

(12) United States Patent

Davis et al.

(54) EXPRESSED LIGAND-VASCULAR INTERCELLULAR SIGNALLING MOLECULE

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- (52) U.S. Cl. 530/350; 435/69.7
- (58) Field of Search 530/350; 435/69.7

(56) **References Cited**

U.S. PATENT DOCUMENTS

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US 6,441,137 B1

Aug. 27, 2002

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WO	WO96/31598	10/1996

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(57) ABSTRACT

The present invention provides for a modified TIE-2 ligand which has been altered by addition, deletion or substitution of one or more amino acids, or by way of tagging, with for example, the Fc portion of human IgG-1, but which retains its ability to bind the TIE-2 receptor. The invention further provides for a modified TIE-2 ligand which is a chimeric TIE-2 ligand comprising at least a portion of a first TIE-2 ligand and a portion of a second TIE-2 ligand which is different from the first. In a specific embodiment, the invention further provides for a chimeric TIE ligand comprising at least a portion of TIE-2 Ligand-1 and a portion of TIE-2 Ligand-2. In addition the present invention provides for isolated nucleic acid molecule encoding the modified TIE-2 ligands described. The invention also provides for therapeutic compositions as well as a method of blocking blood vessel growth, a method of promoting neovascularization, a method of promoting the growth or differentiation of a cell expressing the TIE receptor, a method of blocking the growth or differentiation of a cell expressing the TIE receptor and a method of attenuating or preventing tumor growth in a human.

5 Claims, 47 Drawing Sheets

Fig. 1 A



r EHK-1 ecto/h IgG1 Fc Gelfoam (6ug)

Fig. 1 B



r TIE-2 ecto/h lgG1 Fc Gelfoam (6ug)





AATATC	160 GCTAG	240 •	٥¢	SC SC SC
CTATGCAATA	150 SCTAACAAAT	230 PACCTGAAAAT	310 TACA ATG A	370 5550 430 430 490 550 550 550
CAGCTGACTCAGGCTCATGCTGAACGGTCACACAGAGAGAAACAATAAATCTCAGGCTACTATGCAATA&ATATC	90 100 110 120 130 140 150 160 TCAMGTTTTAACGAAGAAAAACATCATTGCAGTGAAATAAAAATTTTTAAAAATGCAAGCAA	170 180 190 200 210 220 230 240	250 260 270 280 290 300 310 CTAGTITTAGAGGTCAGAAGGAAGGAAGGAAGGAGTGTGGCAGTACA ATG ACA H T>	320 330 340 350 360 370 GTT TTC CTT TTC TTT GCT TTC CTC GCT GCT
GAAACAATI	130 TTTTAAA1	210 STCAAACAA	290 • ACGGAAGGAGTGT	PAC CCC AT FC PAC CCC AT FC PAC CCC AT FC
ACACAGAGAG	120 • AATAAAAA	200 GGGGAAAGA	280 • SCGAGAGGCACG	350 GCC ATT CTG A I L 410 AGA TAT AAC 0 R Y N 470 G N C 530 530
- CTGAACGGTCU	100 110 VAAACATCATTGCAGAGA	180 190 200 TCAAACGCTTTCTTTGAGGGGGGAAAGAGTC	260 270 MGMAGGAGCAAGTTTTGC	TTC CTC GCT F L A 400 MGT GGG AGA S G R 460 E H D 520
AGCTCCATC	100 • GAAAAACATC	180 CTTCAAACGC	260 - Agaagaaagg	330 F A F F A F 390 GAA AAC AG GAA AAC AG 450 CTT CCA GA L P E L P E
IGACTCAGGC	90 • TTTTAACGAU	170 TATGATICITICI	250 ITTAGAGGICAC	320 GTT TTC CTT TCC V F L S 380 380 CGC CGA AGT CCA R R S P 440 440 440 TAC AGT TTC ATT Y T F I S00 S00
CAGC	TCAAG	лттс	CTAGT	320 320 320 320 320 320 320 320

Fig. 4 A

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1620	G TGC AVA	1680 • L N	1740 5 CAC TAC	1800 • L D F	1850 1860 1870 1880 GAMATEEEGAGAMACTEGAGAAACTETTTGAAAACTT	1910 1920 1930 1940 1950 1960 1970 CCCTTCCAGCMTAAGTGGTAGTCACCCAAGGTTCTTGACCGTGAATCTGGAGCCC	2020 2030 2040 2050	2120 JAACTATGGTAGC	
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1580	A D	1640 • L T	1700 ACT CCC 0	1760 • AGT TAC T S Y	1820 CAGAAGCEN	1900 • ATATIGICT	1980 J	2060 2	2140 • ATGGTTAATTTC

80 • ATATC	160 • SCTAG	240 •	ح ۸	50	۶X	AC Y>	
70 TATGCAATAA	150 CTAACAAATGC	230 • ACCTGAAATAA	310 BACA ATG AC	370 C AGC AAT C	430 6 CAA TGT 0 0 C	490 4 GAC CAG T D Q	550
60 • ATCTCAGCTAC	140 TAGAACAAAG	220	300 • GTGCTGGCAGT	360 • ATA GGG TG I G C	420 • • • • • •	480 • AGT ACG ACU S T T	540
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40 ACACAGAGAG	120 •*********	200 2000GAAAGA	280 • GOGAGAGGCI	350 GCC ATT CTG A I L	410 AGA TAT AAC R Y N	470 GGC AAC TGT G N C	530
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Fig. 5 A

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Fig. 5 B see L o ar	620 Y I	680 САС АСС Н Т	740 AGA AAG (сто Сто	860 ATC	$\begin{array}{ccccccc} 920 & 930 & 940 & 950 & 960 \\ \hline \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \$	980	1040	× X
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ACA CGA GG T G G	A TGG TGG TTT W W F	CAT GCT TGT D A C			TA ANT OGA	ACA GGA GGA TGG TGG GCT TGT GGC CCC TCC AAT CTA AAT GGA ATG TTC TAT ACT T G G W W F D A C G P S N L N G M F Y T>
1700	1710	1720	1730	۰.	1740	1750
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1760	1770	1780	0671	٥,	1800	1810
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1820 1830	0 1840	1840 1850 1860 1870	1860	1870	1880 1890	
CCGATTATGA	AGCAACAAAGAA	ATCCCCCACAAGC	TIGCCAGGTGA	GAAACTGT	TTGAMACTIC	ccattatgaaagcaacaagaaagcaagcagcaggaggaggagaactgtttgaaagcaaggaagcaaacaatatt
1900 1910	0 1920	1920 1930 1940 1950 1960 1970	1940	1950	1960	1970
Shericetho	CAGCAATAAGTGG	TAGTTATGTON	VETCACCAAGG	TTCTTGAC	CONCANTERIOS	GICTECETTECAGEAATAMETEGTAGTTATETECAAGECTACETTETECACCETECAGEGCCGTTTECAGTTCAC
1960 1990	0 2000	2010	2020	2030	2030 2040	2050
	ACTTGGGGTGACA	стестсясетес	SCTCGACTATA	GAMACTCI	CACTGACTOT	
2060 2070	0 2080	2090	2100	2110	2120	2130
AGAAACTGCTI	GAGCTTCCTGTGC	TTCANACTACTU	ACTOGACCTTA	TTTCCAN	CTATOGTAGCC	

Fig. 5 C

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1100 1110 1120 1130 1140 1150 AAT CTT TOC ACT AM GAN GTT TTA CTA ANG GGA GGA ANA AGA GAG GAA GAA GAA CAA TTT N L C T K E V L L K G G K R E E E K P F>	I Y I Y Y Y Y Y Y Y Y Y Y Y Y Y Y Y Y Y	ATT MT MT ATG CCA GM CCC MA MG CTG TTT TGC MT ATG CAT GTC MT GGG GGA GGTINNHPEPKKVFCNHDVNGGG	1390 NT GAG TIT ATT TIT V E F I F>	1450 LC TOG GAA GOG AAC	1510 A AAC TAT AGG TTG N Y R L>	1570 c TTA CAC OGT GCT L H G AS
II40 AA AGA GAG K R E	AN AGT GGA	TTTC CAL	1380 6 CTC 005 A	1440 5 TTA ATG G L H	IS00 F GM ALG C	1560 . AGC CTG A
1130 5 ccA ccA A 6 c C	L150 F TTT AAT A F N 1 1250	F C P F C P 1310 MGT CTA GA	1370 GAA TAT TG E Y W	1430 AGA ATT GM R I E	1490 • ATA GGA AN' I G N	1550
1120 T TTA CTA AAG L L K	I CM CCT GC Q A G 1240	K K V K K V 1300 CM GM GGA	1360 CCC TCC GCT P S G	1420 TAC ATG CTA Y H L	1480 AGA TTC CAC R F H	1540 ACA GCA GCA T A G
1110 CT AN GA GT T K E V	A D V Y 1230	TG CCA CM CCC H P E P 1290 I 200 FA CM CMT CCT FA CM CMT CCT	1350 57 TTT CGA MAT	1410 T CAG AGG CAG	1470 A CAG TAT GAC Q Y D	1530 T CAC ACT GGG H T GG
1100 • AAT CTT TGC J N L C	AGA GAC TOT 6 R D C 1220	ATT MAT MAT A I N N N 1280 1280 Toc ACT GTA N W T V	1340 1340 1340 1340 1340 1340 1340 1340	1400 GCC ATT ACC AC A I T S	1460 CGA GCC TAT TC R A Y S	1520 TAT TTA ANA GG Y L K G

80 • SAACAAA	160 • TGACAG	240 *	320 COCTCA	s c>	A AAG K>	G GAC D>	
10 20 30 40 50 60 70 80 GAATTCCTOGGTTGATTTATCTCCTCCAGGCTTGAGGAGGGGAGG	90 100 110 120 130 140 150 160 GGACCGTGAAAGCTGCTCAAGCGTGACCACCCACTGACAGGAGCTGTTCTTCCCACTGCAGGAGC	170 180 190 200 200 210 220 230 240 TITACTGCATGCCTGGAGAAGAGGAAAGAGGAAAGAGGAAAGAGAAGACTTTCATTG	250 260 270 280 290 300 310 320 ACGGACCCATGCCAGCCTAGCGCTTTCAGACGCAGCAGCTCTGGGACGTGTGTTTTGCCCTCA	330 340 350 360 370 380 AGTITIGCTAAGCTGGTTTATTACTGAAGAAAGA ATG TGG CAG ATT GTT TTT ACT CTG AGC TGT M W Q I V F F T L S C>	440 * GAC AGC ATA GGA AAG D S I G K>	500 * 5 CCA GAG ATG P E M	560
60 • IGTAGGATC	140 • •	220 AAAGAGGA	300 * GGACTCTG	0 4 117 TTC TY V F I	ATG M	r r c cuc cue	
50 * RACAACACT	130 1 TGAGCAGGA	210 CTACTGGAA	290 AGCAGCTOG	370 * CAG ATT GTT Q I V	430 ccc AAG AGC R K S	490 * TAC ACT TTC Y T F	550
40 • TGAGGGAGGG		200 * ACCAGGTTTG	280 * ITCAGACGGC	360 * A ATG TGG	420 • TAT AAC AAC TIT CC Y N N F I	480 1962 AGC C S	540
30 * CTCCCAGCCT	110 GCTGACACAG	190 * AGCAGTAAAA	270 AGCCCTGCGT	350 • CTGAAGAAAG	410 GCC TAT AAC A Y N	470 • CAT 666 TCC H 6 S	530
20 IGTTTATCTC	100 TCTGTAAAA	180 • tagagaacac	260 * KAGCGTAGC	340 CGTTTATTA	SCC GCA	98 O	
10 + criecerrieci	90 STGAAAGCTGC	170 ACTECATECCTGC	250 * SACCCAGCCATGG	330 * MGCTAAGCTGCT	0 400 • • • • • • • • • • • • • • • • • • •	0 460 * AAG CAA TAT CAG GTC K Q Y Q V	520
GAATTC	GGACCG	TITACI	ACGGAC	AGTTTIG	390 * GAT CT D L	450 * AAG CA K Q	510 520

6 A Fig.

T ^A CT	GAG E>	ខ្លាំ	AAT N>	E> E>	£ 3	E GA	GAA E>	g A
N C P	CTA G	N AAC C	TTA A L	L G		o & x	CTA G	0
4 U 4 2	ATG G M	T ACA A	GTÀ T V	AAA T K	AGT TTC S F	ATA AAA O I K	E C	1090 1100 CTT CAA AAG CAG CAA CAT GAT L Q K Q Q H D
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Fig.	16	171	1770 + AT	1830 • CG		-	·		

















Fig. 10 D



trkB

BINDING ACTIVITY (RU) 90 80 70 60 50 40 30 20 10 SHEP 1-1+ rTIE2 SHEP 1-1 SHEP 1-1+



Fig. 11 B











Fetal Thymus E17.5 CDR1⁺: Cortical stromal cells A2B5⁺: Medulla stromal cells





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COVALENT MULTIMERIC STRUCTURE OF TL1 AND TL2 AND THEIR INTERCONVERSION BY THE MUTATION OF ONE CYSTEINE







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$\begin{array}{cccccccccccccccccccccccccccccccccccc$	1520 1530 1540 155 * * * * * * * * GGC CCC AGC TAC TCA CTG CAC GGC ACA CGC G P S Y S L H G T R	* CCGTA(* *	1810 1820 1830 1840 1810 1820 1830 1840 * * * * * ATACTCTTTC CCCTGC TGCATGCCCG GGAATCCCTG CCATGAACT

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22
Fig.

80	ASRLHIADWR	af.aai.thi -gcsn.r.sns.r-rynr igha ilh-dg-n cresttdq-y nt.aapve>	s.r-rfnr iqht ilq-dg-n cresttdq-y nt.aapv-e->	<9-V	<dy< th=""><th>ey</th><th></th></dy<>	ey	
70	HILDGLLLLA THAAAQHRGP EAGGHRQIHQ VRRGQCSYTF VVPEPDICQL APTAAPEALG GSNSLQRDLP ASRLHIADWR	nt.aa-	nt.aa-	nt.aa.	ava.	ava.	
60	APTAAPEALG	cresttdq-y	cresttdq-y	cres.t.qy-	-my2222-	-742222-	
50	VVPEPDICQL	ilh-dg-n	ilq-dg-n	-nghli	11t.s.r-	11m.n.r-	
40	VRRGQCSYTF	iqha	iqht	iqha	d.up.	s.up.	·
30	EAGGHRQIHQ	.ns.r-rynr	s.r-rfnr	-nrynr	dst.rY.	dsi.kky.	
20	THAAAQHRGP	-gcsn.r.s.	-gett.r	iigcsn.r.n.	saysnfrksv	aaynnfrksm	
10	MLLDGLLLLA		af.aaahi -gctt.r.	mTL1. mtvflsfaffaailthigcsn.r.nnrynr iqha ilhgn- cres.t.qy- nt.aay-e>	mTL2. mwqiifltfgwd.v saysnfrksv dst.ryqn.p 11t.s.rssssymavady>	mwqivfftlscd.v aaynnfrksm dsi.kkyqh.s 11m.n.rssssyvavaey>	
	mTL3	hTL1.	chTL1.	HILL.	mTL2.	hTL2	

160	EAQVE		, t , v	~ • • • •	~ • • • •
150	AQRAQRAQRV SQLEKILENN TQWLLKLEQS IXVNLRSHLV QAQQDTIQNQ TTTMLALGAN LMADTKAQTH KLTAVEAQVL	ae. r 20. r	ae.r.d.		dsvln.mmny .qd.mkkem. einavav.iei.tlaerd>
140	CAN LAND	ts Is.	ts ls.	.ts .1	.tl
0	IQ TTTMLAI	h aei	h a ei	.av.iei	. aviei
130	QAQQDTIQN		i.nav.	einvv	einav
120	IXVNLRSHLV	ve mk ema ve mk em	.ve.mk.ema	.qd.mkkem.	.qd.mkkem.
110	LOWLEKLEQS	Yn Prop	Au	ΥΥΥ	үпп.
100	SQLEKILENN	ih. hvm. Y	hhvmy		
06	AQRAQRAQRV S	parss.kl qn.nvmyqny .ve.mk.ema .lnavh .aei.ts .lsae.rdt> qdfsf.kl qhhvmvqsv .ve.mk.emlnavh .aei.ts .lsae.rdt	pdfss.kl q	1vsb-	- dsv.l
	mrr3	chTL1.	mTL1.	mTL2.	hTL2.

AQRAQRAQRY SQLEKIIENN TQHLLKLEQS IKVNLRSHLV QAQQDTIQNQ TTTMLALGAN LMXTKAQTH KLTAVEAQVL pdfss.kl qhhvmyqsy .ve.mk.ema .inavh .aei.ts .lsaerd.t> ddf-sf.kl qhhvmyqsy .ve.mk.ema .inavh .aei.ts .lsaerd.t> ddfs-rs.kl qhhvmyqny .ve.mk.ema .inavh .aei.ts .lsaerd.t> -dsv.lln.mny .qd.mkkem. einavav.iei.ts .laerd.t>	170 180 190 200 210 210 240 240 240 220 230 240 240 307 DHMKTQM DENSLSTNKL FRQMLMQSRE LQRLQGRNRA LETRLQALEA QHQAQLNSLQ EKREQLHSLL GHQTGTLANLsrlei.l	320
LMN <u>QTKAQTH</u> .lsaer .lsaer .lsaer .laer	230 EKREQLHSLL .ek.n.qg.v .ek.n.qg.v .ek.n.qg.v .gkde.qv.v	310
TTTMLALGAN .aei.ts .aei.ts .av.iei.ts .av.iei.ts	220 QHQAQLNSLQ K.kee.dt.k r.kee.dt.k K.kee.dt.k K.se.q.mk K.ii.q.ik	300
QAQQDTIQNQ .i.nav.h .l.nav.h .i.nav.h ei.nav	210 LETRLQALEA hkilem.g hkilem.g qkvldm.g kkvl.m.d	290
IKVNLRSHLV .ve.mk.ema .ve.mk.ema .ve.mk.ema .qd.mkkem. .qd.mkkem.	170 180 190 200 200 210 220 230 240 NOTLHNKTOM LENSLSTNKL FROMLMOSRE LORLOGRNRA LETRLOALEA QHQAQLNSLQ EKREQLHSLL GHQTGTLANL srleilyk.l.q.tn. ilkihek.slhkilem.g k.kee.dt.k .ek.n.qg.v tryijqe. srleilyk.l.q.tn. ilkihek.slhkilem.g k.kee.dt.k .ek.n.qg.v srfijqe. srleilyk.l.q.tn. ilkihek.slhkilem.g k.kee.dt.k .ek.n.qg.v srfijqe. trlellk.i.d.ts. inknk.sfqkvldm.g k.seq.mk .qkde.qv.v sk.ssvide.	280
үп	190 ERQMLMQSRE .K.l.q.tn. .K.l.q.tn. .K.i.d.ts. .K.i.d.ts.	270
SQLEKITENN Qh. hvm. Y Qh. hvm. Y Ph. n	180 Thensestruke	260
AQRAQRAQRV pdfss.kl pdfss.kl pdfss.kl -dsvl	170 NOTHHMKTQM srlei.l srlei.l trlel.l	250
мТL3 hTL1. chTL1. мTL1. hTL2. hTL2.	мтыз Мтыз смты мты1. Мты2. Мты2.	

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	TEXITYDS	iin	ni	ifn	iltfp	n.iltfp
310	T NUSSAN	lvyqa.f. X	lvyg.f. k	lvyqa.f. k	.fklt .	vfkht .
300	CPVFQD CAE	ikp.rd	ikp.rd	jkp.rd	[tt.r	lis.r
290	ITX-I SV	gg k-ree	na keee	lkgg k-reed	a ir eec	tv aeec
280	KHNLHALSSN SSSLQQQQQ LTEFVQRLVR IVAQ-DQHPVS L-KTPKPVFQD CAEIKRSGVN TSGVYTIYET NMTKPLKVFC	ekq.nratt. n.vkle .mdt.hnn lctkevllkgg k-reeekp.rdvyqa.f. kiinpe.k>	lcsk-egvllk	ekq.sratn. n.ikle .mdt.hns lctk-egvllkgg k-reeekp.rdvyqa.f. kifnpe.k>	ekk.vtatv. n.lkhd .m.t.ns.lt mmss-pn-skssa ir.eeqtt.rfklti.ltfp .s.eei.ay.>	ekkivtatv. n.vkhd .m.t.nn.lt mmstsns-akdtv aeeqis.rvfkht .n.iltfp .s.eei.ay.>
270	LTEFVORLVR .	mdt.hn.n	.mdt.ht.it	.mdt.hn.s	.m.t.ns.lt 1	.m.t.nn.lt 1
260	SSSLQQQQQ	n.vkle	n.vkle	n.ikle	n.1. k. hd	n.vkhđ
250	KHNLHALSSN	ekq.nratt.	ekq.nkatt.	ekq.sratn.	ekk.vtatv.	ekkivtatv.
	mTL3	hrL1.	chTL1.	mrii.	mTL2.	hTL2.

350 360 360 370 380 390 400 NFQRTWEEYK EGFGNVAREH WLGNEAVHRL TSRTAYLLRV ELHDWEGRQT SIQYENFQLG dg.k mpsg.yfifaiqrq.m.imnra ysdr.hi.> dkg.k mspsg.yfifaiqrq.m.imnra ysdr.hi.> dg.k mpsg.yfifaiqrq.m.imnra ysdr.hi.> dk vplg.yfisqqqhr.v.ki q.knea hsl.dh.y.a>	430440450460470480NSLAPQGTKFSTKDMDNDNCMCKCAQMLSGGWWFDACGLSNLNGTYSVHQHLHKINGIRs.ilh.adal.tpmf.tagnhg.lk>s.ilh.adal.tpmf.tagnhg.lk>s.ilh.adal.tpmf.tagnhg.lk>s.ilh.adal.tpmf.tagnhg.lk>s.isqp.sdspmf.tagnhg.lk>s.isqp.ndgpmf.tagnhg.lk>	
380 390 TSRTAYLLRV ELHDWEGRQT qrq.mimnra qrq.simnra qrq.mimnra .qqhr.v.ki q.knea .nqqr.v.ki h.knea	470 <u>NLNG</u> TYYSVH mf.tag mf.tag mf.tag mf.tag mf.tag	
380 390 TSRTAYLLRV ELHDWEGRQT •.qrq.mimnra •.qrq.simnra •.qrq.mimnra •.qrq.m.imnra •.qrq.m.imnra	460 GWWFDACGLS 	
370 WLGNFAVHRL fifai fifai fifai f.sq.	450 MCKCAQMLSG l.t. l.t. l.t. ist.	
350 360 370 370 370 370 370 370 370 370 370 37	430 440 450 460 470 NSLAPQGTKF STKDMDNDNC MCKCAQMLSG GWWFDACGLS NLNGLYYSVH s.ilh.ad a l.t mf.tag s.ilh.ad a l.t mf.tag s.ilh.ad a l.t mf.tag s.ilh.ad a l.t mf.tag s.istp.sd a l.t mf.tag s.istp.sd a nl.t mf.tag s.istp.sd a nl.t mf.tag s.istp.sd mf.tag mf.tag	
350 NFQRTWEEYK dg.k dg.k dk dk	430 NSLAPQGTKF silh.ad. silh.ad. s.isqp.sd. s.isqp.sd. s.isqp.nd.	MGA• 1df 1df5 adf5 adf5
340 LIQHREDGSV V1 V1 V1	420 NDSSSSAGRK kghtgtkq kghtgtkq kghtgtkq tgltgtki	<u>л</u> нствникв гъ.сі гъ.сі ка.сі ка.сі ка.сі.
330 340 DMETLGGGWT LIQHREDGSV 1 n.dvnv1 n.dvnv1 dvgv1 dvgv1	410 420 SERQRYSLSV NDSSSSAGRK N n.k.n.r.yl kghtgtkq s n.k.n.r.yl kghtgtkq s n.k.n.r.yl kghtgtkq s g.esn.rihl tgltgt.aki s eln.rihl kgltgtki s	490 500 WHYFRGPSYS IHGTRMMLRP 500 k.rrs.t.i 1 k.rrs.t.i 1 .y.wk.sgka.t.i 5 .v.wk.sgka.t.i 5
mTL3 hTL1. chTL1. mTL1. hTL2. hTL2.	mTL3 hTL1. chTL1. mTL1. mTL2. hTL2.	mTL3 hTL1. chTL1. mTL1. hTL2.

Fig. 22 B

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	GCT A	130 HT AGC	AAC N	AAA K	330 ACG	CTA L	460 kg GCT : A	AAG K	С Л С С	
	GTG V	E O	r S	260 GTG V	AAG K	ATG M	46 GAG E	AAC	590 GCG A	
	60 TCT S	CAC H	190 ig GAC	ົ້າຍັດ	ATC	390 CCC P	ATG M	520 CACC	ມີ ອີດເຊັ່	
	ATG M	ပ္ပ ပ	AGG 19	QCAG	320 GCC Å	A GCC	GAC	s TC S	AAC	
	ACC	120 CA C H	ACC S	250 CCACC	ра В С С С С С С С С С С С С С С С С С С	ACG	450 ACC T	сл Р	o caa	
	50 GCC À	Q	GTC V	P C 22	GAG E	380 CAG Q	г. СЦС	TTT F	000 000 000 000 000	
	GTT V	GTC V	180 GAG E	L	L0 CTA L	AAT.	AAG K	510 ACC T	Q	
	GTG V	110 GT A V	СС П Р	AAG K	310 AAG CT K L	Q	440 CGC R	GAG EAG	L CTT	
	40 CTT L	СТТ Г	999 9	240 666 666	AAG K	370 G GCC	ATC	CCA P	570 CAG Q	◄
	стс г	ACA T	170 CCG P	стG г	СТG Г	TA M	Q	500 ATG M	Q	
	CTC	100 30 GAG	сст Р	CAC H	300 TGG W	CAA Q	430 17 GCC	CAG Q	L CTC	
	AGC S	E O	U U U U U	230 CTG L	Q	Q	A A A A A	A GCC	560 AAG K	
	900 990 990	00 00 00	160 G CCC	CCA	ACG	360 CAG Q	ACC T	490 G GAT	Q	
	Q	AGG R	1 GAG E	AAC	290 AAC N	GTC V	Q	AT M	AGG R	
	ст <u>с</u>	90 GAT D	TCT S	220 G GCC	AAC	Q	420 AAC N	AGA R	550 A CAG	
	20 ATG M	GCG Å	AAG K	r US S	Q	350 6AG E	ста 1	r S	cTA CTA	
	A B C C C	GAG E	150 CCC P	TCA	80 CTG	្តីមិ។	сто С	480 ACA	cre L	
	CTA L	80 CAG Q	CTG L	GAA E	A GCA	AAG K	410 AGC S	Q	ст <u></u> г	
	10 C CAG	AGG R	TTG L	210 AGA R	c AG	340 66 TCG K S	ο Υ Ε	AAC N	540 CAG Q	
∢	N CC	ACA	140 TTC F	Q	GAG BAG	3, AGG R	0 99 0	470 C CTG L	AAC N	
n N	10 ATG CTC TCC CAG CTA M L S Q L	70 80 CAA CAG ACA AGG CAG Q Q T R Q	140 TAC ACC TTC TTG CTG Y T F L L	200 210 ACC CTC CAG AGA GAA T L Q R E	210 210 210 210 210 210 210 20 210 20 210 21	340 ATC TTG AGG TCG AAG I L R S K	400 410 GAG CTG GGC ACC AGC E L G T S	470 CAG CTC CTG AAC CAG Q L L N Q	130 540 CTG GAG AAC CAG CTG L E N Q L	
Fig. 23 A	ATG M	CAA	TAC Y	200 ACC T	CAG Q	ATC I	4(GAG E	CÀG Q	530 CTG L	

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AAG K 720 GAG CGC GGC CTG CGC E R G L R 1000 1010 1020 1020 1030 1040 1050 CTC ATC CAG CGT GAG AAT GGC ACT GTC CAG CGG AAC TGG AAG GAT TAC AAA CAG GGC L I Q R R E N G T V N F Q R N W K D Y K Q G $\begin{array}{ccccccccc} 1060 & 1070 & 1080 & 1090 & 1100 & 1110 & 1120 \\ \mbox{TTC GGA GAC CCA GCT GGG GAG CAC TGG CTG GGC AAT GAA GTG GTG CAC CAG CTC ACC AGA AGG GCA \\ \mbox{F G D P A G E H W L G N E V V H Q L T R R A } \end{array}$ CAT H AAG GAA E GAG AAG CGG TTG CAG GCC CTG GAG CAG CAG GAG GAG CTG GCC AGC ATC CTC AGC E K R L Q A L E T K Q Q E E L A S I L S 670680690700710GCG AAG UTG CTG AAC ACG CTG AGC GCC GCC CTC ACC AAC ATCALLNTLNTAKLLNTLSRQSALTNT

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A GCA G	н Срс Тр	AAC CTC N I.	1450 TAC TTC Y F	I		
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TAC TAC	1300 CTT GAC TCA L D S	370 TGT C	ATC	TTG L		
ပို့ ဗ	00 GAC D	A GC L	ပ္ပ ပ္ပ	1500 CGG CCT 1 R P		
GTC V	13 CTT L	GAC	BD DD DD	ь Са Са Са		
1230 GTG V	AGC ACC S T	50 TTT F	14 ATG M	АТА Т		
TCT S	AGC	136 766 W	AAG K	1490 CATGATGATA (MMT		
CTT L	1290 TTT F	р ЦС	TAC Y	14 ATG M		
220 AGG R	AGC	90 00 00	142 AAG K	2GC 79		
1220 1230 TAC AGG CTT TCT GTG GTC Y R L S V V	ACC	L350 GGA G	AAC	N TCT		
СТА L	280 AAC N	1350 3 TCT GGA GGG	GAC	148 GCC A		
10 CAG	1280 1290 CTG CAG AAC ACC AGC TTT L Q N T S F	ATA M	1410 SCT CCC GAC AAC 7 A P D N	1480 FCGT GCC TCT CGC 1 R A S R		
12 AAC N	CTG L	340 GTG V	A GCT J	г. СIG		
E GAG	1270 CTG GTC L V	1340 CAG GTG Q V	1400 TAC TAC CAC GCT Y Y H A	1470 TAC TCA Y S		
s AGT	L CIG	GCC	1400 AC TAC Y Y	TAC 1		
1200 GGC G	s AGC	30 CC	14 TAC Y	S S C		
L CTG	AGC S	1330 AAG TC K C	GTC T V	50 50 P		
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	1260 CGC CAG AGC R Q S	1330 CTC TGC AAG TGT GCC L C K C A	0 9 9 9 9 9	1460 AAG GGC CCC K G P		
1190 TTC F	R CGC	CTC CTC	1390 AAC GGC (N G	AAG K		
06 *	ဗ္ဗိ ပ္ပ ခ်	180 a crg crg D>	270 * ATG TAC M>	360 GTT CAA V>	450 ACC TCG T>	540 676 0>
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	AGT TCA S	ACA TGT	GTG CAC V	A CGT	GAG CTC E	CAA GTT 0
	AAC N N		CAT GTA H	AAT TTA N		CTT GAA L
80	E CTT		E CTTT	0 4 20 6 40 6 40 6 40 7 0	440 6AT CTA D	CTT GAA
	P GG	E CTC	CTG GAC L	o cyc	ACA TGT	5 GTT O
	s RG	ភូទី។	CAT GTA H	ATA TAT I		AAG FTC K
0*	R CGA	A LI + 0	o to	o c vo	430 * AGA AAG (TCT TTC (R K	0400 010 010
~	ပ္ပင္လဲ «	16 MAC N	25 GAA	A 0.00 4	AGA 43	52 CTA SAT
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0 *		n + 10 TTT + 10	0 + TATO	NAC 7	0 * 10 5 40 5 10 5 10	500 * * * AGT 7 * AGT 7
	L DE H	14 VIT (VIT (I	P 20 2	33 FTT 1	410 * TCT CAG AGA GTC S Q	N 25
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• •	CTG I GAC	L L L		TTT C		
40	I ATT C	13(13(716 7	220 * CAC GTG GTG CAC H V	310 PAC 2 VTG 7	400 AGC CTC S L	L 49
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0 * M		N. S.	TAS TAS	2777 (3777 (3777 (390 ATA GGA TAT CCT I G	480 6A6 2 CTC 7 E
	TTC (မီ ဂို ရ	P TCT AGA	TTT C	E CUC	L CTT
	A CO C	GTA CAT	o gra	A CAA	crig GAC 6	R CGA
* 50	AAA	C C C C C C C C C C C C C C C C C C C	CTG GAC	96 + CT - 1 CT - 1 CT - 1	M 480 M 480 M C 4	70 AGA SGA
	NGG N	110 ATT CAA TAA GTT I Q	200 CCT CTG CGA GAC	290 * TGG CTG ACC GAC W L	380 * ACC ATG TGG TAC T M	ACT 4
	CTT GAA	80 00 00 00 00 00 00 00 00 00 00 00 00 0	AAC	o nc	A	GTT
01 *	ပုပ္ရွ		0 + ACH	280 * TAT ACT (ATA TGA (Y T	HCC HCC H	
	CAA V	100 * TAT AAC ATA TTG Y N	190 * AAC ACA TTG TGT N T	28 ATA	370 * CAC ACG GTG TGC H T	460 * CTA AAT GAT TTA L N
≺ 7	H TGP	AGA R AGA	ATG Y	AAT TTA N	AAC 0 NN	GTA (CAT (
J. 74 A	ATG TAC M	AGA	o CAG	GAA EE	o Erc	o CAG
$\overline{\mathbf{n}}$				-	-	-

Fig. 24 A

630	* 22 A	720 GCT A>	- +	900 • ATG Y>	990 • CGT •	1080 GAG CTC EV	1170 GAA CTT EV
	AAC	AGA TCT R	င် နှိုင်	ATC TAG I	R CCA	TTA MTTA	1 ATA Y Y
	CTC CTC	AAC TTG N	CTT E	ပ္က ပ္က ပ္က ပ္	c ag GTC	ច ប្រី ខ្ល	L AAC
620	E CTT	710 * TTTA AAT L	100 AAA TTT K	90 • TTA N	80 * ATT TAA T	6 + CL 5 Y CL 2	s TCA SGT * 60
Ţ	AAG TTC K	CAA CAA	ACT TGA		980 * ATT ATT TAA TAA I I	TGG ACC W	1160 * ATC TCA ATG AGT Y S
	CAC GTG H	AAG TTC K		00 F	ស្តីភ្នំម	TAT ATA Y	A CGA
61.0	GGA AAA CCT TTTT G K	700 * CTG GAA GAC CTT L E	790 * AAT CTT TTA GAA N L	880 6GA CAC A CCT GTG A	970 4 GGG TGG CCC ACC	n rotate Crista	1150 * AAT CAG (TTA CTC (N E
ġ.		7(CTG GAC	15 TTA N	စ်ပို့စွဲ 🖁	် မိုဂ်ရ	106 GGA CCT	115 AAT TTA N
		GAG CTC E	c c c c c c c c c c c c c c c c c c c	TCA AGT S	0000 0000 0000	TCA AGT S	8 U 0
	ATG TAC M	o CAG	L SA L	A TT X	ថ្លីដ៍ច	CCT GGA	CTT E
600	E CTT a	690 870 778 1	780 AAC TTG	870 TTC AAG	0 1 1 1 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	NG NAC	MCG + 00 MCG + 0
	ere Tere	ATA TAT I	CAC GTG H	CAT CAT	A A CCT	្រី ថ្ងៃ ថ្ងៃ ច	D CHC L
	ATC TAG I	TAT ATA Y	GTC CAG	GAA	E SAA	TT F	AAA TTT K
590	AAA TTT K	680 ACA TGT	770 * ACA TGT	> 000 × 00	950 * * M ATG	စ္ + စိုပ်ပ္ စ	E SAN CTT + 30
	A E H	3 E a	ទីទីទីគ	AC AC	CTG 9	CAC LIO	H CAC 11 H GTG H
	CTT	CGT GCA R	ATG TAC M	D GAC	TGT ACA	AAA TTT K	ATA TAT I
80	TTA TTA AAT AAT L L	670 * GTT ACT CAA TGA V T	760 * GAG CTG CTC GAC E L	850 TT AGA GA AA TCT CT P R L	940 + GCC TAC CGG ATG A Y	1030 * GAA TAT A CTT ATA T E Y 1	1120 * CTT AAA 7 GAA TTT 1 L K
ŝ			00	HR	00	103 GAA CTTT E	112 GAA
			CTG GAC L	Р 661 Р	AAG TTC K	AAA TTT K	C C C
_	AAC TTG N	ပ္ပ် ပ္ပံ ဖ	o GTT o		ATC	TGG ACC W	TAT ATA Y
570		660 CTT CAA GAA GTT L Q	750 * AAG CAG TTC GTC K Q	840 676 676 840	930 GAA GAG CTT CTC E E	1020 ACT TGA	1110 * ccc * ccc
	CTTT CTTT	L CTT	AAG	A CTT B	GAA CTT E	ខ្លួបជ្	SE a
	GTA H	AAC	ο E C F C F C	CTC GAG	ACA TGT	S C S C	e S E o
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	L AAC	A GAG CTC E	AGT S	e r GGA	ည် ဦ ရ	c da C	1090 * TCG CAA CTG 7 AGC GTT GAC 7 S 2 L
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	E CAN	A AAC	AAC TTG	AAG TTC	ACA 9	1000 66C AG CCG TCC SCG TCC	10: AGC SC
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- 1 - 	AT D T	ACC TGG	ACC TGG	TTA AAT L	ACG TGC	CTC ETC	TTT AAA F
-							

Fig. 24 B

1260 • GTT O>	GAT GAT CTA D>	1440 * AAA TTT K>	
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н ACA Н TGT	CAA GTT Q	TTC AAG F	
1230 1230 666 CCC	1320 * TCA AGT S	1410 * AAG TTC K	
ACA	ACA	AAT TTA N	ТАА АТТ * >
CTT GAA	AAA TTT K	ACA TGT T	TTC AAG
1220 A GGA T CCT	1310 * TT TGC AA ACG I C	1400 * AG AAC TC TTG O N	1490 * EA GAT ST CTA
AA TT X	A F	00	66.4
CTT 5 GAA	TGT ACA	AGG R	CCA GGT P
1210 • MTT CAC PAA GTG I H	1300 * MC AAA TT TT D K	1390 * CCA CAG 5GT GTC P Q	1480 * ATC CGA PAG GCT I R
R. P.	00	00	~ ~ ~
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CAT TTC CAT TTC GTA AAG H F	A GGA T CCT G	A TGT T ACA C	TCA S AGT
САТ н GTA	CCA GGT P	GCA CGT A	000 000 000

Fig. 24 C

GAA AAC CTT TTG E N ATA TAT I I I CCC GGG GGG AAC TTG N CAT V NCA SCA NCG S CAG GTC Q s ngg er o S S C C 530 CAG GTC Q TAG TAG A GG AGG AGG GAA CTTT E ATG TAC o ng a AAT (TTA (N AGC FCG AGA S AAC TTG N CAC V L L 160 3C CGC 2G GCG 3C GCG 250 * CTG GAG GAC CTC 340 * erc cac o o 430 * ACT GAT TGA CTA T D 520 * G ATT C TAA 70 AAG TTC ACG ACG 200 x of Cho E AAA ATA TAT I TAT AAT L N AAC < GTG CAC AAA FTT R AAC E CAA GAG CTC CTC E D D D AAG K E TAT ATA Y CTC E AGG ACC H ATG M M 00 00 00 00 AAT X AAC TTG TTG AAC L AAG CTG CTG ATG TAC M ACA TGT T CTC GAG 220 * ATG GAT ATG CTA 310 * GAC AAC CTG TTG D N 400 * AAC CAA TTG GTT M Q 490 * CAC TCC GTG AGG H S o cyo CTA CTA E CTT B S AGC TTG AAC L AND R L GAG ATC TAG I ACA ACA ACA ACA ACA ACA ACA ACA сто сус С THG AAC ACT CTG TGA CAT TGA CAC TTA L CAT GGG GTA CCC H G CAC CCC CTG CCC CTG CCC CTG CCC GAG AAT CTC TTA E N ACA TGT P S S O 800 000 000 E SAA ATG AAC TAC TTY M K CAG GTC CAC CAC CAA V ATA TAT I R TCT R 460 + + TGC T 10 * 5 ATT 5 ATT 7 AA 280 * ACC CAT W L 370 * GTG ATG CAC TAC V M org CAG C D L ∢ ACC ACC c AG A CGA SECO ATG TAC M AAG FTTC K K GTG CCAC CCAC V V T TGA 500 F AAT TTA N

<u>-ig. 25</u>

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		76 GAG CTC E	crd Crd D	9 700 ACG 9	1030 * AAA AT TTT TA	1120 * ATT GA TAA CT I E
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Fig. 25 B	00	A H	0.9	ΕÆ	ΦŬ ⁻	F2"
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Aug. 27, 2	002 5.		,
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و دري 6	TTA AAT L	9 CC 9	
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Fig. 25 C			

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Fig. 26 A		κ, [-	U & C	ថបី	320	527

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၂ ပ	F AAA	CAT GTA H		P GGT	GCA CGT A	0000 0000 0000
Fig. 26 C	GAG CTC E	GAA CTT E		CAA GTT Q	GAT CTA D	AAA TTT K
<u>.</u>						
LL_						

0 * 2 4 4	0 + U 0 A	0 * {+ < ^	0 + U Ø A	0 + 4 H A	0 + 4 H A
90 P GGA • GV CCT	• •			450 A CTA T GAT	540 T GAA A CTT
C ATA 5 TAT 1	000 a	A AAT T TTA N	G AAC C TTG N	c car v	A AAT T TTA N
AGC PTCG	AGC TCG S	GAA CTT E	r cag A Grc	o cac o cac	G ACA T TGT
80 80 80 0 0	170 * TCC * AGG	260 * ATG TAC	350 * CAA	440 ACC TGG	530 A CAG r GTC
ATG M	1 AGG	CAC CAC		CTC E	caa caa
AGC TCG S	TCT AGA S	CAT GTA H	AAT TTA N	CAA	CTT GAA
70 * AAG TTC	160 * Acc ccc Acc ccc C R	250 * CTG GAA GAC CTT L E	340 * CAG CAG GTC GTC Q Q	430 * ACA GAT TGT CTA T D	520 VA CTT TT GAA
0 0 K			o cyo "	ACA 4	26~
TTT AAA F		CAT GTA H		CTG GAC L	AAG
AAC TTG		CAA GTT Q		AAG TTC K	GAG CTC E
60 * AAC TTG					510 CTA GAT L
TAT ATA Y	GAG CTC E	AAA TTT K		ACC TGG	AAG TTC K
000 CGG	CCA GGT P	cag GTC Q	GAG CTC E	cag GTC Q	TAC ATG Y
> 50 CGT > 50	140 * CTG # GAC	230 7 AGG	320 * TCG : AGC	410 4 410 1 GAG 1 CTC E	TGG \$
C 60 C 60 C 60	c crc	2 AGA S	3 AAG TTC K	A CGT L	AGG A
TTG AAC L	TTC AAG	TTC AAG F	ATG TAC M	ACT TGA	TTA AAT L
40 GTC V	130 * AC ACT rG TGA	220 * NG GAT NG CTA	N AAC	o to cyc	490 AAT TCA T TTA AGT A N S
GAA L	13 TAC ATG Y	22 TAC ATG Y	310 * GAA AAC CTT TTG E N	400 + TCT CAC AGA GTC	49 AAT TTA N
CTA D	AGC S	GAA CTT E	GTG CAC	CTC GAG L	GAG CTC E
TGT ACA C	ACG ACG	CTC GAG	ATT TAA I	CTC GAG	CTG GAC
AGC A 30 NGC A 30	120 700 * 866	210 210 660 660	300 * ATG Y	390 700 800 800	480 CTG GAC L
crig GAC	8000	900 000	AAT TTA N	ACC TGG	o Cao STC o
ACT TGA		CTG CTG	GAG CTC E	GGA GGA	ATA TAT I
20 * AAA	110 110 GTC CAG CAG GTC V Q	200 * GTC AGG GTC TCC Q R	290 * AAA CTT TTT GAA K L	380 * CTC ATA CTC TAT E I	470 * CTT GAG GAA CTC L E
AAG		cag GTC Q	AAA TTT K	GAG 3	crr 4
GTT CAA	o cyc	GTG CAC	A STT O	CTG BAC	r GGA
10 * ATT TAA	100 A TAT T ATA Y	A GCT + 0	r GAC GAC	370 * CC ATG GG TAC	AGA SGA
90	100 LOCAA TAT GTT ATA Q Y	190 * AAT GCT TTA CGA	280 * ACC GAC O	A P P	460 * ACT TCT TGA AGA T S
T TGG	TTC :	AGG AGG	c AG GTC	A GCT	CAA GTT Q
TAC N	AAG TTCC	GTG CAC	ACT TGA	ACG	AAT TTA N
Fig. 27 A					
L ealans					

630 *	AAG K	720 AAC TTG N>	810 • AAG TTC K>	900 • • • • •	990 400 660 400 700 700 700	1080 + TTT AAA F>
	TTA AAT L	ACC ACC	4 5	£2	\$£0	1080 * ATT TTT TAA AAA I F>
	ACC TGG	ACC	TTA (AAT (ACT TGA T	GAA CTTT E	TTT AAA F
02 *		710 GCT CGA	800 * * CAA	90 47 77 77 77 77 77 77 77	80 80 80 80 80	170 * GAG CTC E
6	TTG AAC	7 AGA C TCT C	665T 67A 67A	ATC 8 IAG	980 * CAT CGT GTA GCA H R	1070 * G AAT GAG C TTA CTC N E
	GAG 7 CTC 7	AAC A TTG 7 N	GAA C E E	e CCA	CAA GTT Q	ဗ္ဗပ္ဂ ဗ
• •	E TT	700 • CAA TTA 7 GTT AAT 7 Q L	ANA ATT X	880 890 * AAA AGT GGA ATT TAC ACT AN TTT TCA CCT TAG ATG TGA TN K S G I Y T 3	970 * GTA ATA (CAT TAT (V I	L CTG
610	AAG GAA TTC CTT K E	T TO	790 * ACT AAA (TGA TTT (T K	88 MAA FTT	97 ATE TAT	1060 * ACG CTG ACC GAC W L
	H C SH	MAG C	TGC A ACG 7 C	N N	TGA	
	AAA TTTT (CTT 1 GAA 7	TTT AAA F	စ ပ အ	E
•	6 L 0		780 * AAT (TTA (N	870 GGT +	0 4 100 100 4 0	1050 1050
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	ATG MC MC MC MC MC MC MC MC MC MC MC MC MC		T AA J	ATO	9 0 0 8 0 0 8 0 0	50 50 50 50 4
o *	E CTT 7	TAG 0	v v v RTG C	850 860 * Constants Consta	950 * AAT TTA 0	1040 * GGA AAT CCT TTA G N
55	TTA G AAT C L	68 MTA 7 TT 7 T	770 * CAC AAC GTG TTG H N	8(STA 2 V	GTC 9	
	ATC 7 TAG 7 I	ТАТ <i>1</i> АТА 1 Ү	< GTC CAG C	D D	D CTA C	TTT (AAA (F
.	KAA I VTT 7 K	TGT 1	ACA TGT	A LGC	A C C C C C C C C C C C C C C C C C C C	F 4
580	CAT AAA I GTA TTT 7 H K	670 * GTT ACA C GTT TGT J Q T	760 * CTC ACA CTG TGT D T	BCA (185	940 * AAT ATG (TTA TAC (N M	1030 ATG GG MC CC
		a CG CG CG CG CG CG CG CG CG CG CG CG CG	ATG (TAC (M	D CTG	Acc Acc	AAA TTTT K
		TGA C	L C C L	RCT RGA	T AA T	TA TA
570	LAT 1	660 GTT 2 CAA 3	750 6AG (CTC (840 * AAA	930 930 5 676 1 7 CAC A	1020 GAA
-,	S AGT S	L	, end Bach	P S C	ŽÉ×	AAG TTC
	AAC 7 TTG 1 N	5 0 0 0 0 0	CAA (GTT (AAA TTT K	AAA TTT X	ο Ο Ο Ο Χ
560	AN I	650 CAA C	740 CAG CAG CAG	ы ста ста зо в ста зо	0 * U 0 A	ရ * ဖွပ္က စ
ũ	GAA AAA CTT TTT E K	650 * CTT CAA GAA GTT L Q	740 • TTC CAG TTC GTC	830 * GAA GAG CTT CTC E E	920 • CTT GGG TTT • CTT GGG TTT • C	1010 * AGA GGC TCT CCG 1 R G
	CAT C GTA C H	AAC C TTG C	CAG 2 GTC 2	GAG CTC	P GGT	CLAN
• •		E C C C C C C C C C C C C C C C C C C C		AGA RCT	M + 0 M HAC	TTC AAG
550 *	AAG ATC TTC TAG K I	640 * AAA GAG TTT CTC K E	730 * GTC CTT CAG GAA V L	820 * AAA AGA TTT TCT K R	910 * AAT ATG TTA TAC	1000 * GAT TTC CTA AAG D F
ב	TTG 2 AAC	GAG 2 E	AGT S	e CCI 9	AAT TTA N	CTA GAT
	ATC TAG	E CTT C		GGA GCT G	ATT TAA I	AGT TCA S

Fig. 27 B

• • • •		1.08				
	1170	TTC CAC AAG GTG F H>	1260 6GT GCT CCA CGA 6 A>	1350 + TGT 66C ACA CC6 C 6>	1440 * CCC AGT GGG TCA P S>	
		AGA TCT 8	GTG H	GCT CGA	ဖ ပ ဖ ဖွ ပ ဖ	
	1160	TAT GAC ATA CTG Y D	1250 * ATC TTA TAG AAT I L	1340 * TTT GAT AAA CTA F D	1430 * TTC AAA AAG TTT F K	
	•	TCA CAG AGT GTC S Q	to * AGC CTG TCG GAC S L	M MCC ACC	20 * CAC TAC GTG ATG H Y	
	1150 *	TAT ATA Y	12, AGC TCG S	1330 • • GGA TGG • CCT ACC	142 ACC ACC	
		CGA GCC GCT CGG R A	AAA CAG TTT GTC K Q	ACA GGA TGT CCT T G	ATA AAG TAT TTC I K	
	1140	GGG AAC CCC TTG G N	1230 A GCA GGA CGT CGT CGT CCT	1320 * ATG TTA 7 TAC AAT	1410 * AAT GOG TTA CCC N G	
		CTT CTT	ACA TGT	CTC GAG	CTG GAC L	
	1130	BAC TGG CTG ACC D W	1220 * ACT GGG TGA CCC	1310 * TGT GCC C ACA CGG G	1400 * GGA AAA CCT TTT G K	тда АСТ * >
	•	TTA ATG AAT TAC L M	agr cac cca gre g H	C FGC AAA ACG TTT C K	AAC CAT FTG GTA N H	D SAT TTT SAT AAA D F
	1120	ATT GAG TAA CTC I E	1210 TTA AAA GGT AAT TTT CCA L K G	1300 TGT ATG TGC ACA TAC ACG C M C	1390 GGA CAA AAC CCT GTT TTG G Q N	1480 * TTTA GAT NAT CTA
	-	AGA TCT R	TAT ATA Y	AAC TTTG N	900 CGC	CGA CCT CCT GGA R P
	1110	ATG CTA TAC GAT M L	1200 * AGG TTG TTCC AAC R L	1290 * AAT GAC TTA CTG N D	1380 * TAT ACT ATA TGA Y T	1470 * ATG ATT TAC TAA M I
	~ •	TAC ATG Y	TAT ATA Y	c TA C TA	TTC AAG F	M
	1100	AGG CAG TCC GTC R Q	1190 * CAA AAC GTT TTG Q N	1280 * GAT GCT CTA CGA D A		1460 * ACA ACT ? TGT TGA T T T
	• *	AGT CAG TCA GTC S Q	30 * GAA AAG CTT TTC E K) ACT AAA GA TTT T K	60 CTA AAT GAT TTA L N	50 * CGT TCC GCA AGG R S
	1090	ACC TGG	1180 GGA AAT GAJ CCT TTA CT7 G N E	1270 * C AGC ACT G TCG TGA	13 AAT TTA	145 TTA AAT L
Fig. 27 C		GCC ATT CGG TAA A I	ATA GG TAT CC I G	GAT TTC CTA AAG D F	CCC TCC GGG AGG P S	TAC TCC ATG AGG Y S
Fig.)					

U.S. Patent

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EXPRESSED LIGAND-VASCULAR INTERCELLULAR SIGNALLING MOLECULE

This application is a divisional application of U.S. Ser. 5 No. 08/740, 223 Oct. 25, 1996; now issued as U.S. Pat. No. 6,265,564, which claims the priority of U.S. Provisional application No. 60/022,999 filed Aug. 2, 1996. Throughout this application various publications are referenced. The disclosures of these publications in their entireties are 10 hereby incorporated by reference into this application.

INTRODUCTION

The present invention relates generally to the field of 15 genetic engineering and more particularly to genes for receptor tyrosine kinases and their cognate ligands, their insertion into recombinant DNA vectors, and the production of the encoded proteins in recipient strains of microorganisms and recipient eukaryotic cells. More specifically, the 20 present invention is directed to a novel modified TIE-2 ligand that binds the TIE-2 receptor, as well as to methods of making and using the modified ligand. The invention further provides a nucleic acid sequence encoding the modified ligand, and methods for the generation of nucleic acid encoding the modified ligand and the gene product. The modified TIE-2 ligand, as well as nucleic acid encoding it, may be useful in the diagnosis and treatment of certain diseases involving endothelial cells and associated TIE receptors, such as neoplastic diseases involving tumor angiogenesis, wound healing, thromboembolic diseases, atherosclerosis and inflammatory diseases. In addition, the modified ligand may be used to promote the proliferation and/or differentiation of hematopoietic stem cells.

ligands described herein may be used to promote the growth, survival, migration, and/or differentiation and/or stabilization or destabilization of cells expressing TIE receptor. Biologically active modified TIE-2 ligand may be used for the in vitro maintenance of TIE receptor expressing cells in $_{40}$ culture. Cells and tissues expressing TIE receptor include, for example, cardiac and vascular endothelial cells, lens epithelium and heart epicardium and early hematopoietic cells. Alternatively, such human ligand may be used to support cells which are engineered to express TIE receptor. Further, modified TIE-2 ligand and its cognate receptor may be used in assay systems to identify further agonists or antagonists of the receptor.

BACKGROUND OF THE INVENTION

The cellular behavior responsible for the development, maintenance, and repair of differentiated cells and tissues is regulated, in large part, by intercellular signals conveyed via growth factors and similar ligands and their receptors. The receptors are located on the cell surface of responding cells and they bind peptides or polypeptides known as growth factors as well as other hormone-like ligands. The results of this interaction are rapid biochemical changes in the responding cells, as well as a rapid and a long-term readjustment of cellular gene expression. Several receptors associated with various cell surfaces may bind specific growth factors.

The phosphorylation of tyrosine residues in proteins by tyrosine kinases is one of the key modes by which signals are transduced across the plasma membrane. Several cur- 65 rently known protein tyrosine kinase genes encode transmembrane receptors for polypeptide growth factors and

hormones such as epidermal growth factor (EGF), insulin, insulin-like growth factor-I (IGF-I), platelet derived growth factors (PDGF-A and -B), and fibroblast growth factors (FGFs). (Heldin et al., Cell Regulation, 1: 555–566 (1990); Ullrich, et al., Cell, 61: 243-54 (1990)). In each instance, these growth factors exert their action by binding to the extracellular portion of their cognate receptors, which leads to activation of the intrinsic tyrosine kinase present on the cytoplasmic portion of the receptor. Growth factor receptors of endothelial cells are of particular interest due to the possible involvement of growth factors in several important physiological and pathological processes, such as vasculogenesis, angiogenesis, atherosclerosis, and inflammatory diseases. (Folkman, et al. Science, 235: 442-447 (1987)). Also, the receptors of several hematopoietic growth factors are tyrosine kinases; these include c-fms, which is the colony stimulating factor 1 receptor, Sherr, et al., Cell, 41: 665-676 (1985), and c-kit, a primitive hematopoietic growth factor receptor reported in Huang, et al., Cell, 63: 225-33 (1990).

The receptor tyrosine kinases have been divided into evolutionary subfamilies based on the characteristic structure of their ectodomains. (Ullrich, et al. Cell, 61: 243-54 (1990)). Such subfamilies include, EGF receptor-like kinase (subclass I) and insulin receptor-like kinase (subclass II), each of which contains repeated homologous cysteine-rich sequences in their extracellular domains. A single cysteinerich region is also found in the extracellular domains of the eph-like kinases. Hirai, et al., Science, 238: 1717-1720 (1987); Lindberg, et al. Mol. Cell. Biol., 10: 6316-24 (1990); Lhotak, et al., Mol. Cell. Biol. 11: 2496-2502 (1991). PDGF receptors as well as c-fms and c-kit receptor tyrosine kinases may be grouped into subclass III; while the FGF receptors form subclass IV. Typical for the members of More generally, the receptor activating modified TIE-2 35 both of these subclasses are extracellular folding units stabilized by intrachain disulfide bonds. These so-called immunoglobulin (Ig)-like folds are found in the proteins of the immunoglobulin superfamily which contains a wide variety of other cell surface receptors having either cellbound or soluble ligands. Williams, et al., Ann. Rev. Immunol., 6: 381–405 (1988).

> Receptor tyrosine kinases differ in their specificity and affinity. In general, receptor tyrosine kinases are glycoproteins which consist of (1) an extracellular domain capable of 45 binding the specific growth factor(s); (2) a transmembrane domain which usually is an alpha-helical portion of the protein; (3) a juxtamembrane domain where the receptor may be regulated by, e.g., protein phosphorylation; (4) a tyrosine kinase domain which is the enzymatic component $_{50}$ of the receptor; and (5) a carboxyterminal tail which in many receptors is involved in recognition and binding of the substrates for the tyrosine kinase.

> Processes such as alternative exon splicing and alternative choice of gene promoter or polyadenylation sites have been 55 reported to be capable of producing several distinct polypeptides from the same gene. These polypeptides may or may not contain the various domains listed above. As a consequence, some extracellular domains may be expressed as separate, secreted proteins and some forms of the receptors may lack the tyrosine kinase domain and contain only the extracellular domain inserted in the plasma membrane via the transmembrane domain plus a short carboxyl terminal tail.

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A gene encoding an endothelial cell transmembrane tyrosine kinase, originally identified by RT-PCR as an unknown tyrosine kinase-homologous cDNA fragment from human leukemia cells, was described by Partanen, et al., Proc. Natl. Acad. Sci. USA, 87: 8913–8917 (1990). This gene and its encoded protein are called "TIE" which is an abbreviation for "tyrosine kinase with Ig and EGF homology domains." Partanen, et al. Mol. Cell. Biol. 12: 1698–1707 (1992).

It has been reported that tie mRNA is present in all human fetal and mouse embryonic tissues. Upon inspection, tie message has been localized to the cardiac and vascular endothelial cells. Specifically, tie mRNA has been localized to the endothelia of blood vessels and endocardium of 9.5 to ¹⁰ 18.5 day old mouse embryos. Enhanced tie expression was shown during neovascularization associated with developing ovarian follicles and granulation tissue in skin wounds. Korhonen, et al. Blood 80: 2548–2555 (1992). Thus the TIEs have been suggested to play a role in angiogenesis, ¹⁵ which is important for developing treatments for solid tumors and several other angiogenesis-dependent diseases such as diabetic retinopathy, psoriasis, atherosclerosis and arthritis.

Two structurally related rat TIE receptor proteins have ²⁰ been reported to be encoded by distinct genes with related profiles of expression. One gene, termed tie-1, is the rat homolog of human tie. Maisonpierre, et al., Oncogene 8: 1631–1637 (1993). The other gene, tie-2, may be the rat homolog of the murine tek gene, which, like tie, has been ²⁵ reported to be expressed in the mouse exclusively in endothelial cells and their presumptive progenitors. Dumont, et al. Oncogene 8: 1293–1301 (1993). The human homolog of tie-2 is described in Ziegler, U.S. Pat. No. 5,447,860 which issued on Sep. 5, 1995 (wherein it is referred to as "ork"), ³⁰ which is incorporated in its entirety herein.

Both genes were found to be widely expressed in endothelial cells of embryonic and postnatal tissues. Significant levels of tie-2 transcripts were also present in other embryonic cell populations, including lens epithelium, heart epicardium and regions of mesenchyme. Maisonpierre, et al., Oncogene 8: 1631–1637 (1993).

The predominant expression of the TIE receptor in vascular endothelia suggests that TIE plays a role in the development and maintenance of the vascular system. This could include roles in endothelial cell determination, proliferation, differentiation and cell migration and patterning into vascular elements. Analyses of mouse embryos deficient in TIE-2 illustrate its importance in angiogenesis, particularly for vascular network formation in endothelial cells. Sato, T. N., et al., Nature 376:70–74 (1995). In the mature vascular system, the TIEs could function in endothelial cell survival, maintenance and response to pathogenic influences. 50

The TIE receptors are also expressed in primitive hematopoietic stem cells, B cells and a subset of megakaryocytic cells, thus suggesting the role of ligands which bind these receptors in early hematopoiesis, in the differentiation and/ or proliferation of B cells, and in the megakaryocytic differentiation pathway. Iwama, et al. Biochem. Biophys. Research Communications 195:301–309 (1993); Hashiyama, et al. Blood 87:93–101 (1996), Batard, et al. Blood 87:2212–2220 (1996). ing cells of the host-vector system under conditions permitting production of the polypeptide and recovering the polypeptide so produced. The invention herein described of an isolated nucleic acid molecule encoding a modified TIE-2 ligand further provides for the development of the ligand as a therapeutic for the treatment of patients suffering from disorders involving cells, tissues or organs which express the TIE-2 receptor. The present invention also provides for an antibody which

SUMMARY OF THE INVENTION

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The present invention provides for a composition comprising a modified TIE-2 ligand substantially free of other proteins. As used herein, modified TIE-2 ligand refers to a ligand of the TIE family of ligands, whose representatives comprise ligands TL1, TL2, TL3 and TL4 as described herein, which has been altered by addition, deletion or

substitution of one or more amino acids, or by way of tagging, with for example, the Fc portion of human IgG-1, but which retains its ability to bind the TIE-2 receptor. Modified TIE-2 ligand also includes a chimeric TIE-2 ligand comprising at least a portion of a first TIE-2 ligand and a portion of a second TIE-2 ligand which is different from the first. By way of non-limiting example, the first TIE-2 ligand is TL1 and the second TIE-2 ligand is TL2. The invention envisions other combinations using additional TIE-2 ligand family members. For example, other combinations for creating a chimeric TIE-2 ligand are possible, including but not limited to those combinations wherein the first ligand is selected from the group consisting of TL1, TL2, TL3 and TL4, and the second ligand, different from the first ligand, is selected from the group consisting of TL1, TL2, TL3 and TL4.

The invention also provides for an isolated nucleic acid molecule encoding a modified TIE-2 ligand. In one embodiment, the isolated nucleic acid molecule encodes a TIE-2 ligand of the TIE family of ligands, whose representatives comprise ligands TL1, TL2, TL3 and TL4 as described herein, which has been altered by addition, deletion or substitution of one or more amino acids, or by way of tagging, with for example, the Fc portion of human IgG-1, but which retains its ability to bind the TIE-2 receptor. In another embodiment, the isolated nucleic acid molecule encodes a modified TIE-2 ligand which is a chimeric TIE-2 ligand comprising at least a portion of a first TIE-2 ligand and a portion of a second TIE-2 ligand which is different from the first. By way of non-limiting example, the first TIE-2 ligand is TL1 and the second TIE-2 ligand is TL2. The invention envisions other combinations using additional TIE-2 ligand family members. For example, other combinations are possible, including but not limited to those combinations wherein the isolated nucleic acid molecule encodes a modified TIE-2 ligand which is a chimeric TIE-2 ligand comprising a portion of a first ligand selected from the group consisting of TL1, TL2, TL3 and TL4, and a portion of a second ligand, different from the first ligand, selected from the group consisting of TL1, TL2, TL3 and TL4

The isolated nucleic acid may be DNA, cDNA or RNA. The invention also provides for a vector comprising an isolated nucleic acid molecule encoding a modified TIE-2 ligand. The invention further provides for a host-vector 45 system for the production in a suitable host cell of a polypeptide having the biological activity of a modified TIE-2 ligand. The suitable host cell may be bacterial, yeast, insect or mammalian. The invention also provides for a method of producing a polypeptide having the biological 50 activity of a modified TIE-2 ligand which comprises growing cells of the host-vector system under conditions permitting production of the polypeptide and recovering the polypeptide so produced.

The invention herein described of an isolated nucleic acid molecule encoding a modified TIE-2 ligand further provides for the development of the ligand as a therapeutic for the treatment of patients suffering from disorders involving cells, tissues or organs which express the TIE-2 receptor. The present invention also provides for an antibody which specifically binds such a therapeutic molecule. The antibody may be monoclonal or polyclonal. The invention also provides for a method of using such a monoclonal or polyclonal antibody to measure the amount of the therapeutic molecule in a sample taken from a patient for purposes of monitoring the course of therapy.

The present invention also provides for an antibody which specifically binds a modified TIE-2 ligand as described

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herein. The antibody may be monoclonal or polyclonal. Thus the invention further provides for therapeutic compositions comprising an antibody which specifically binds a modified TIE-2 ligand, in a pharmaceutically acceptable vehicle. The invention also provides for a method of blocking blood vessel growth in a mammal by administering an effective amount of a therapeutic composition comprising an antibody which specifically binds a receptor activating modified TIE-2 ligand as described herein, in a pharmaceutically acceptable vehicle.

The invention further provides for therapeutic compositions comprising a modified TIE-2 ligand as described herein, in a pharmaceutically acceptable vehicle. The invention also provides for a method of promoting neovascularization in a patient by administering an effective amount of a therapeutic composition comprising a receptor activating modified TIE-2 ligand as described herein, in a pharmaceutically acceptable vehicle. In one embodiment, the method may be used to promote wound healing. In another embodiment, the method may be used to treat ischemia. In 20 vet another embodiment, a receptor activating modified TIE-2 ligand as described herein is used, alone or in combination with other hematopoietic factors, to promote the proliferation or differentiation of hematopoietic stem cells, B cells or megakaryocytic cells.

Alternatively, the invention provides that a modified TIE-2 ligand may be conjugated to a cytotoxic agent and a therapeutic composition prepared therefrom. The invention further provides for a receptorbody which specifically binds a modified TIE-2 ligand. The invention further provides for therapeutic compositions comprising a receptorbody which specifically binds a modified TIE-2 ligand in a pharmaceutically acceptable vehicle. The invention also provides for a method of blocking blood vessel growth in a mammal by 35 administering an effective amount of a therapeutic composition comprising a receptorbody which specifically binds a modified TIE-2 ligand in a pharmaceutically acceptable vehicle.

The invention also provides for a TIE-2 receptor antagonist as well as a method of inhibiting TIE-2 biological activity in a mammal comprising administering to the mammal an effective amount of a TIE-2 antagonist. According to the invention, the antagonist may be a modified TIE-2 ligand as described herein which binds to, but does not activate, the TIE-2 receptor.

BRIEF DESCRIPTION OF THE FIGURES

FIGS. 1A and 1B-TIE-2 receptorbody (TIE-2 RB) inhibits the development of blood vessels in the embryonic chicken chorioallantoic membrane (CAM). A single piece of resorbable gelatin foam (Gelfoam) soaked with 6 µg of RB was inserted immediately under the CAM of 1-day chick embryos. After 3 further days of incubation, 4 day old embryos and surrounding CAM were removed and examined. FIG. 1A: embryos treated with EHK-1 RB (rEHK-1 ecto/hIgG1 Fc) were viable and possessed normally developed blood vessels in their surrounding CAM. FIG. 1B: all embryos treated with TIE-2 RB (r TIE-2 ecto/h IgG1 Fc) were dead, diminished in size and were almost completely 60 devoid of surrounding blood vessels.

FIG. 2—Vector pJFE14.

FIG. **3**—Restriction map of λ gt10.

FIGS. 4A-4D-Nucleic acid (SEQ ID NO: 1) and deduced amino acid (single letter code) sequences (SEQ ID 65 3: early corpus luteum; and Column 4: atretic follicle; Row NO: 2) of human TIE-2 ligand 1 from clone Igt10 encoding htie-2 ligand 1.

FIGS. 5A-5D-Nucleic acid (SEQ ID NO: 3) and deduced amino acid (single letter code) sequences (SEQ ID NO: 4) of human TIE-2 ligand 1 from T98G clone.

FIGS. 6A-6D-Nucleic acid (SEQ ID NO: 5) and deduced amino acid (single letter code) sequences (SEQ ID NO: 6) of human TIE-2 ligand 2 from clone pBluescript KS encoding human TIE 2 ligand 2.

FIG. 7-Western blot showing activation of TIE-2 receptor by TIE-2 ligand 1 (Lane L1) but not by TIE-2 ligand 2 (Lane L2) or control (Mock).

FIG. 8—Western blot showing that prior treatment of HAEC cells with excess TIE-2 ligand 2 (Lane 2) antagonizes the subsequent ability of dilute TIE-2 ligand 1 to activate the TIE-2 receptor (TIE2-R) as compared with prior treatment of HAEC cells with MOCK medium (Lane 1).

FIG. 9—Western blot demonstrating the ability of TL2 to competitively inhibit TL1 activation of the TIE-2 receptor using the human cell hybrid line, EA.hy926.

FIGS. 10A-10D-Histogram representation of binding to rat TIE-2 IgG immobilized surface by TIE-2 ligand in C2C12 ras (FIG. 10A), Rat2 ras (FIG. 10B), SHEP (FIG. 10C), and T98G (FIG. 10D) concentrated (10x) conditioned medium. Rat TIE-2 (rTIE2) specific binding is demonstrated 25 by the significant reduction in the binding activity in the presence of 25 μ g/ml soluble rat TIE-2 RB as compared to a minor reduction in the presence of soluble trkB RB.

FIGS. 11A-11B-Binding of recombinant human TIE-2 ligand 1 (hTL1) (FIG. 11A) and human TIE-2 ligand 2 (hTL2) (FIG. 11B), in COS cell supernatants, to a human TIE-2 receptorbody (RB) immobilized surface. Human TIE-2-specific binding was determined by incubating the samples with 25 μ g/ml of either soluble human TIE-2 RB or trkB RB; significant reduction in the binding activity is observed only for the samples incubated with human TIE-2 RB

FIG. 12—Western blot showing that TIE-2 receptorbody (denoted TIE-2 RB or, as here, TIE2-Fc) blocks the activation of TIE-2 receptors by TIE-2 ligand 1 (TL1) in HUVEC cells, whereas an unrelated receptorbody (TRKB-Fc) does not block this activation.

FIG. 13—Agarose gels showing serial dilutions [undiluted (1) to 10_{-4}] of the TL1 and TL2 RT-PCR products obtained from E14.5 mouse fetal liver (Lanes 1-total, Lanes 3-stromal enriched, and Lanes 4- c-kit+TER119 hematopoietic precursor cells) and E14.5 mouse fetal thymus (Lanes 2-total).

FIG. 14—Agarose gels showing serial dilutions $_{50}$ [undiluted (1) to 10^{-4}] of the TL1 and TL2 RT-PCR products obtained from E17.5 mouse fetal thymus cortical stromal cells (Lanes 1-CDR1+/A2B5-) and medullary stromal cells (Lane CDR1-/A2B5+).

FIG. 15—A schematic representation of the hypothesized 55 role of the TIE-2/TIE ligands in angiogenesis. TL1 is represented by (\bullet) , TL2 is represented by (*), TIE-2 is represented by (T), VEGF is represented by ([]), and flk-1 (a VEGF receptor) is represented by (Y).

FIG. 16-In situ hybridization slides showing the temporal expression pattern of TIE-2, TL1, TL2, and VEGF during angiogenesis associated with follicular development and corpus luteum formation in the ovary of a rat that was treated with pregnant mare serum. Column 1: Early preovulatory follicle; Column 2: pre-ovulatory follicle; Column A: bright field; Row B: VEGF; Row C: TL2; Row D: TL1 and Row E: TIE-2 receptor.

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FIG. 17-Comparison of amino acid sequences of mature TL1 protein (SEQ ID NO: 7) and mature TL2 protein (SEQ ID NO: 8). The TL1 sequence is the same as that set forth in FIGS. 4A-4D (SEQ ID NO: 2), except that the putative leader sequence has been removed. Similarly, the TL2 sequence is the same as that set forth in FIGS. 6A-6D (SEQ ID NO: 6), except that the putative leader sequence has been removed. Arrows indicate residues Arg49, Cys245 and Arg264 of TL1, which correspond to the residues at amino acid positions 69, 265 and 284, respectively, of TL1 as set 10 forth in FIGS. 4A-4D (SEQ ID NO: 2).

FIG. 18—Western blot of the covalent multimeric structure of TL1 and TL2 (Panel A) and the interconversion of TL1 and TL2 by the mutation of one cysteine (Panel B).

FIG. 19—A typical curve of TIE-2-IgG binding to immobilized TL1 in a quantitative cell-free binding assay.

FIG. 20-A typical curve showing TIE-2 ligand 1 ligandbody comprising the fibrinogen-like domain of the ligand bound to the Fc domain of IgG (TL1-fFc) binding to immobilized TIE-2 ectodomain in a quantitative cell-free binding assay.

FIGS. 21A-21C-Nucleotide (SEQ ID NO: 9) and deduced amino acid (single letter code) sequences (SEQ ID NO: 10) of TIE ligand-3. The coding sequence starts at 25 tatives comprise ligands TL1, TL2, TL3 and TL4 as position 47. The fibrinogen-like domain starts at position 929.

FIGS. 22A-22B-Comparison of Amino Acid Sequences of TIE Ligand Family Members. mTL3 =mouse TIE ligand-3 (SEQ ID NO: 11); hTL1=human TIE-2 ligand1 30 (SEQ ID NO: 12); chTL1=chicken TIE-2 ligand1 (SEQ ID NO: 13); mTL1=mouse TIE-2 ligand 1 (SEQ ID NO: 14); mTL2=mouse TIE-2 ligand 2 (SEQ ID NO: 15); hTL2= human TIE-2 ligand 2 (SEQ ID NO: 16). The boxed regions indicate conserved regions of homology among the family 35 TIE-2 ligand is TL1 and the second TIE-2 ligand is TL2. The members.

FIGS. 23A-23C-Nucleotide (SEQ ID NO: 17) and deduced amino acid (single letter code) sequences (SEQ ID NO: 18) of TIE ligand-4. Arrow indicates nucleotide position 569.

FIGS. 24A-24C-Nucleotide (SEQ ID NO: 19) and deduced amino acid (single letter code) sequences (SEQ ID NO: 20) of chimeric TIE ligand designated 1N1C2F (chimera 1). The putative leader sequence is encoded by nucleotides 1-60.

FIGS. 25A-25C-Nucleotide (SEQ ID NO: 21) and deduced amino acid (single letter code) sequences (SEQ ID NO: 22) of chimeric TIE ligand designated 2N2C1F (chimera 2). The putative leader sequence is encoded by $_{50}$ nucleotides 1-48.

FIGS. 26A-26C-Nucleotide (SEQ ID NO: 23) and deduced amino acid (single letter code) sequences (SEQ ID NO: 24) of chimeric TIE ligand designated 1N2C2F (chimera 3). The putative leader sequence is encoded by 55 within the sequence can be substituted by another amino nucleotides 1-60.

FIGS. 27A-27C-Nucleotide (SEQ ID NO: 25) and deduced amino acid (single letter code) sequences (SEQ ID NO: 26) of chimeric TIE ligand designated 2N1C1F (chimera 4). The putative leader sequence is encoded by $_{60}$ nucleotides 1-48.

DETAILED DESCRIPTION OF THE **INVENTION**

As described in greater detail below, applicants have 65 created novel modified TIE-2 ligands that bind the TIE-2 receptor. The present invention provides for a composition

comprising a modified TIE-2 ligand substantially free of other proteins. As used herein, modified TIE-2 ligand refers to a ligand of the TIE family of ligands, whose representatives comprise ligands TL1, TL2, TL3 and TL4 as described herein, which has been altered by addition, deletion or substitution of one or more amino acids, or by way of tagging, with for example, the Fc portion of human IgG-1, but which retains its ability to bind the TIE-2 receptor. Modified TIE-2 ligand also includes a chimeric TIE-2 ligand comprising at least a portion of a first TIE-2 ligand and a portion of a second TIE-2 ligand which is different from the first. By way of none limiting example, the first TIE-2 ligand is TL1 and the second TIE-2 ligand is TL2. The invention envisions other combinations using additional TIE-2 ligand family members. For example, other combinations for creating a chimeric TIE-2 ligand are possible, including but not limited to those combinations wherein the first ligand is selected from the group consisting of TL1, TL2, TL3 and TL4, and the second ligand, different from the first ligand, is selected from the group consisting of TL1, TL2, TL3 and 20 TL4.

The invention also provides for an isolated nucleic acid molecule encoding a modified TIE-2 ligand. In one embodiment, the isolated nucleic acid molecule encodes a TIE-2 ligand of the TIE family of ligands, whose represendescribed herein, which has been altered by addition, deletion or substitution of one or more amino acids, or by way of tagging, with for example, the Fc portion of human IgG-1, but which retains its ability to bind the TIE-2 receptor. In another embodiment, the isolated nucleic acid molecule encodes a modified TIE-2 ligand which is a chimeric TIE-2 ligand comprising at least a portion of a first TIE-2 ligand and a portion of a second TIE-2 ligand which is different from the first. By way of non-limiting example, the first invention envisions other combinations using additional TIE-2 ligand family members. For example, other combinations are possible, including but not limited to those combinations wherein the isolated nucleic acid molecule encodes a modified TIE-2 ligand which is a chimeric TIE-2 ligand comprising a portion of a first ligand selected from the group consisting of TL1, TL2, TL3 and TL4, and a portion of a second ligand, different from the first ligand, selected from the group consisting of TL1, TL2, TL3 and 45 TL4.

The present invention comprises the modified TIE-2 ligands and their amino acid sequences, as well as functionally equivalent variants thereof, as well as proteins or peptides comprising substitutions, deletions or insertional mutants of the described sequences, which bind TIE-2 receptor and act as agonists or antagonists thereof. Such variants include those in which amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues acid(s) of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the class of nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

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Also included within the scope of the invention are proteins or fragments or derivatives thereof which exhibit the same or similar biological activity as the modified TIE-2 ligands described herein, and derivatives which are differentially modified during or after translation, e.g., by glycosylation, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Functionally equivalent molecules also include molecules that contain modifications, including N-terminal modifications, which result from expression in a particular recombinant host, such as, for example, N-terminal methylation which occurs in certain bacterial (e.g. E. coli) expression systems.

The present invention also encompasses the nucleotide sequences that encode the proteins described herein as modified TIE-2 ligands, as well as host cells, including 15 yeast, bacteria, viruses, and mammalian cells, which are genetically engineered to produce the proteins, by e.g. transfection, transduction, infection, electroporation, or microinjection of nucleic acid encoding the modified TIE-2 ligands described herein in a suitable expression vector. The 20 present invention also encompasses introduction of the nucleic acid encoding modified TIE-2 ligands through gene therapy techniques such as is described, for example, in Finkel and Epstein FASEB J. 9:843-851 (1995); Guzman, et al. PNAS (USA) 91:10732-10736 (1994). 25

One skilled in the art will also recognize that the present invention encompasses DNA and RNA sequences that hybridize to a modified TIE-2 ligand encoding nucleotide sequence, under conditions of moderate stringency, as defined in, for example, Sambrook, et al. Molecular Cloning: A Laboratory Manual, 2 ed. Vol. 1, pp. 101-104, Cold Spring Harbor Laboratory Press (1989). Thus, a nucleic acid molecule contemplated by the invention includes one having a nucleotide sequence deduced from an amino acid sequence of a modified TIE-2 ligand prepared as described herein, as 35 well as a molecule having a sequence of nucleotides that hybridizes to such a nucleotide sequence, and also a nucleotide sequence which is degenerate of the above sequences as a result of the genetic code, but which encodes a ligand that binds TIE-2 receptor and which has an amino acid 40 sequence and other primary, secondary and tertiary characteristics that are sufficiently duplicative of a modified TIE-2 ligand described herein so as to confer on the molecule the same biological activity as the modified TIE-2 ligand described herein.

The present invention provides for an isolated nucleic acid molecule encoding a modified TIE-2 ligand that binds and activates TIE-2 receptor comprising a nucleotide sequence encoding TIE-2 ligand 1 wherein the portion of the nucleotide sequence that encodes the N-terminal domain of 50 TIE-2 ligand 1 is replaced by a nucleotide sequence that encodes the N-terminal domain of TIE-2 ligand 2. The invention also provides for such a nucleic acid molecule, with a further modification such that the portion of the nucleotide sequence that encodes the coiled-coil domain of 55 ligand comprising at least a portion of a first TIE-2 ligand TIE-2 ligand 1 is replaced by a nucleotide sequence that encodes the coiled-coil domain of TIE-2 ligand 2.

The present invention also provides for an isolated nucleic acid molecule encoding a modified TIE-2 ligand that binds and activates TIE-2 receptor comprising a nucleotide 60 sequence encoding TIE-2 ligand 1 wherein the portion of the nucleotide sequence that encodes the N-terminal domain of TIE-2 ligand 1 is replaced by a nucleotide sequence that encodes the N-terminal domain of TIE-2 ligand 2 and which is further modified to encode a different amino acid instead 65 21), FIGS. 26A-26C (SEQ ID NO: 23), or FIGS. 27A-C of the cysteine residue encoded by nucleotides 784-786 as set forth in FIGS. 27A-27C (SEQ ID NO: 25). A serine

residue is preferably substituted for the cysteine residue. In another embodiment, the nucleic acid molecule is further modified to encode a different amino acid instead of the arginine residue encoded by nucleotides 199-201 as set forth in FIGS. 27A-27C (SEQ ID NO: 25). A serine residue is preferably substituted for the arginine residue.

The present invention also provides for an isolated nucleic acid molecule encoding a modified TIE-2 ligand that binds and activates TIE-2 receptor comprising a nucleotide sequence encoding TIE-2 ligand 1 which is modified to encode a different amino acid instead of the cysteine residue at amino acid position 245. A serine residue is preferably substituted for the cysteine residue.

The invention further provides for an isolated nucleic acid molecule encoding a modified TIE-2 ligand that binds but does not activate TIE-2 receptor comprising a nucleotide sequence encoding TIE-2 ligand 1 wherein the portion of the nucleotide sequence that encodes the N-terminal domain of TIE-2 ligand 1 is deleted. The invention also provides for such a nucleic acid molecule further modified so that the portion of the nucleotide sequence that encodes the coiledcoil domain of TIE-2 ligand 1 is deleted and the portion encoding the fibrinogen-like domain is fused in-frame to a nucleotide sequence encoding a human immunoglobulin gamma-1 constant region (IgG1 Fc).

The invention further provides for an isolated nucleic acid molecule encoding a modified TIE-2 ligand that binds but does not activate TIE-2 receptor comprising a nucleotide sequence encoding TIE-2 ligand 2 rein the portion of the nucleotide sequence that encodes the N-terminal domain of TIE-2 ligand 2 is deleted. The invention also provides such a nucleic acid molecule further modified so that the portion of the nucleotide sequence that encodes the coiled-coil domain of TIE-2 ligand 2 is deleted and the portion encoding the fibrinogen-like domain is used in-frame to a nucleotide sequence encoding a human immunoglobulin gamma-1 constant region (IgG1 Fc).

The invention further provides for an isolated nucleic acid molecule encoding a modified TIE-2 ligand that binds but does not activate TIE-2 receptor comprising a nucleotide sequence encoding TIE-2 ligand 1 wherein the portion of the nucleotide sequence that encodes the fibrinogen-like domain of TIE-2 ligand 1 is replaced by a nucleotide sequence that encodes the fibrinogen-like domain of TIE-2 ligand 2. The invention also provides for such a nucleic acid molecule further modified so that the portion of the nucleotide sequence that encodes the coiled-coil domain of TIE-2 ligand 1 is replaced by a nucleotide sequence that encodes the coiled-coil domain of TIE-2 ligand 2.

The invention further provides for a modified TIE-2 ligand encoded by any of nucleic acid molecules of the invention.

The present invention also provides for a chimeric TIE-2 and a portion of a second TIE-2 ligand which is different from the first, wherein the first second TIE-2 ligands are selected from the group consisting of TIE-2 and-1, TIE-2 Ligand-2, TIE Ligand-3 and TIE Ligand-4. Preferably, the chimeric TIE ligand comprises at least a portion of TIE-2 Ligand-1 and a ion of TIE-2 Ligand-2.

The invention also provides a nucleic acid molecule that encodes a chimeric TIE ligand as set forth in FIGS. 24A-24C (SEQ ID NO: 19), FIGS. 25A-25C (SEQ ID NO: (SEQ ID NO: 25). The invention also provides a chimeric TIE ligand as set forth in FIGS. 24A-24C (SEQ ID NOS: 19

and 20), FIGS. 25A-25C (SEQ ID NOS: 21 and 22), FIGS. 26A-26C (SEQ ID NOS: 23 and 24), or FIGS. 27A-C (SEQ ID NOS: 25 and 26). The invention further provides a chimeric TIE ligand as set forth in FIGS. 27A-27C (SEQ ID NO: 26), modified to have a different amino acid instead of 5 the cysteine residue encoded by nucleotides 784-786.

Any of the methods known to one skilled in the art for the insertion of DNA fragments into a vector may be used to construct expression vectors encoding a modified TIE-2 ligand using appropriate transcriptional/translational control 10 signals and the protein coding sequences. These methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinations (genetic recombination). Expression of a nucleic acid sequence encoding a modified TIE-2 ligand or peptide fragments thereof may be regulated 15 TIE-2 ligand, which may then be recovered in a biologically by a second nucleic acid sequence which is operably linked to the a modified TIE-2 ligand encoding sequence such that the modified TIE-2 ligand protein or peptide is expressed in a host transformed with the recombinant DNA molecule. For example, expression of a modified TIE-2 ligand described 20 receptor. Such biologically active forms could, for example, herein may be controlled by any promoter/enhancer element known in the art. Promoters which may be used to control expression of the ligand include, but are not limited to the long terminal repeat as described in Squinto et al., (Cell 65:1-20 (1991)); the SV40 early promoter region (Bernoist 25 and Chambon, Nature 290:304-310), the CMV promoter, the M-MuLV 5' terminal repeat, promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., Cell 22:787-797 (1980)), the herpes thymidine kinase promoter (Wagner et al., Proc. Natl. Acad. Sci. U.S.A. 30 78:144-1445 (1981)), the adenovirus promoter, the regulatory sequences of the metallothionein gene (Brinster et al., Nature 296:39-42 (1982)); prokaryotic expression vectors such as the β -lactamase promoter (Villa-Kamaroff, et al., Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731 (1978)), or the 35 gene inserted in an expression vector can be detected by tac promoter (DeBoer, et al., Proc. Natl. Acad. Sci. U.S.A. 80:21-25 (1983)), see also "Useful proteins from recombinant bacteria" in Scientific American, 242:74-94 (1980); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADH (alcohol dehydrogenase) promoter, 40 PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals; elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 45 Cell 38:639-646 (1984); Ornitz et al., Cold Spring Harbor Symp. Quant. Biol. 50:399-409 (1986); MacDonald, Hepatology 7:425-515 (1987); insulin gene control region which is active in pancreatic beta cells [Hanahan, Nature 315:115-122 (1985)]; immunoglobulin gene control region 50 gene product expressed by the recombinant. Such assays can which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al. 55 body or portion thereof or by binding to antibodies produced 1986, Cell 45:485–495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58); 60 alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al, 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94); myelin basic protein gene control 65 region which is active in oligodendrocytes in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light

chain-2 gene control region which is active in skeletal muscle (Shani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372–1378). The invention further encompasses the production of antisense compounds which are capable of specifically hybridizing with a sequence of RNA encoding a modified TIE-2 ligand to modulate its expression. Ecker, U.S. Pat. No. 5,166,195, issued Nov. 24, 1992.

Thus, according to the invention, expression vectors capable of being replicated in a bacterial or eukaryotic host comprising a nucleic acid encoding a modified TIE-2 ligand as described herein, are used to transfect a host and thereby direct expression of such nucleic acid to produce a modified active form. As used herein, a biologically active form includes a form capable of binding to TIE receptor and causing a biological response such as a differentiated function or influencing the phenotype of the cell expressing the induce phosphorylation of the tyrosine kinase domain of TIE receptor. Alternatively, the biological activity may be an effect as an antagonist to the TIE receptor. In alternative embodiments, the active form of a modified TIE-2 ligand is one that can recognize TIE receptor and thereby act as a targeting agent for the receptor for use in both diagnostics and therapeutics. In accordance with such embodiments, the active form need not confer upon any TIE expressing cell any change in phenotype.

Expression vectors containing the gene inserts can be identified by four general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, (c) expression of inserted sequences and (d) PCR detection. In the first approach, the presence of a foreign DNA—DNA hybridization using probes comprising sequences that are homologous to an inserted modified TIE-2 ligand encoding gene. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. For example, if a nucleic acid encoding a modified TIE-2 ligand is inserted within the marker gene sequence of the vector, recombinants containing the insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign based, for example, on the physical or functional properties of a modified TIE-2 ligand gene product, for example, by binding of the ligand to TIE receptor or a portion thereof which may be tagged with, for example, a detectable antiagainst the modified TIE-2 ligand protein or a portion thereof. Cells of the present invention may transiently or, preferably, constitutively and permanently express a modified TIE-2 ligand as described herein. In the fourth approach, DNA nucleotide primers can be prepared corresponding to a tie specific DNA sequence. These primers could then be used to PCR a tie gene fragment. (PCR Protocols: A Guide To Methods and Applications, Edited by Michael A. Innis et al., Academic Press (1990)).

The recombinant ligand may be purified by any technique which allows for the subsequent formation of a stable, biologically active protein. Preferably, the ligand is secreted

into the culture medium from which it is recovered. Alternatively, the ligand may be recovered from cells either as soluble proteins or as inclusion bodies, from which it may be extracted quantitatively by 8M guanidinium hydrochloride and dialysis in accordance with well known methodology. In order to further purify the ligand, affinity chromatography, conventional ion exchange chromatography, hydrophobic interaction chromatography, reverse phase chromatography or gel filtration may be used.

In additional embodiments of the invention, as described in greater detail in the Examples, a modified TIE-2 ligand encoding gene may be used to inactivate or "knock out" an endogenous gene by homologous recombination, and thereby create a TIE ligand deficient cell, tissue, or animal. For example, and not by way of limitation, the recombinant 15 TIE ligand-4 encoding gene may be engineered to contain an insertional mutation, for example the neo gene, which would inactivate the native TIE ligand-4 encoding gene. Such a construct, under the control of a suitable promoter, may be introduced into a cell, such as an embryonic stem cell, by a 20 technique such as transfection, transduction, or injection. Cells containing the construct may then be selected by G418 resistance. Cells which lack an intact TIE ligand-4 encoding gene may then be identified, e.g., by Southern blotting, PCR detection, Northern blotting or assay of expression. Cells 2.5 lacking an intact TIE ligand-4 encoding gene may then be fused to early embryo cells to generate transgenic animals deficient in such ligand. Such an animal may be used to define specific in vivo processes, normally dependent upon the ligand. 30

The present invention also provides for antibodies to a modified TIE-2 ligand described herein which are useful for detection of the ligand in, for example, diagnostic applications. For preparation of monoclonal antibodies directed toward a modified TIE-2 ligand, any technique which pro-35 vides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor 40 et al., 1983, Immunology Today 4:72), and the EBVhybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in "Monoclonal Antibodies and Cancer Therapy," Alan R. Liss, Inc. pp. 77-96) and the like are within the scope of the present invention. 45

The monoclonal antibodies may be human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies. Human monoclonal antibodies may be made by any of numerous techniques known in the art (e.g., Teng et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 50 80:7308-7312; Kozbor et al., 1983, Immunology Today 4:72-79; Olsson et al., 1982, Meth. Enzymol. 92:3-16). Chimeric antibody molecules may be prepared containing a mouse antigen-binding domain with human constant regions (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 55 nists of the TIE-2 receptor. Such assay systems would be 81:6851, Takeda et al., 1985, Nature 314:452).

Various procedures known in the art may be used for the production of polyclonal antibodies to epitopes of a modified TIE-2 ligand described herein. For the production of antibody, various host animals, including but not limited to 60 rabbits, mice and rats can be immunized by injection with a modified TIE-2 ligand, or a fragment or derivative thereof. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), 65 Pharmacia Biosensor, Piscataway, N.J.): or 2) block the mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols,

polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and Corynebacterium parvum.

A molecular clone of an antibody to a selected a modified TIE-2 ligand epitope can be prepared by known techniques. Recombinant DNA methodology (see e.g., Maniatis et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) may be used to construct nucleic acid sequences which encode a monoclonal antibody molecule, or antigen binding region thereof.

The present invention provides for antibody molecules as well as fragments of such antibody molecules. Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')₂ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the F(ab'), fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent. Antibody molecules may be purified by known techniques, e.g., immunoabsorption or immunoaffinity chromatography, chromatographic methods such as HPLC (high performance liquid chromatography), or a combination thereof.

The present invention further encompasses an immunoassay for measuring the amount of a modified TIE-2 ligand in a biological sample by

- a) contacting the biological sample with at least one antibody which specifically binds a modified TIE-2 ligand so that the antibody forms a complex with any modified TIE-2 ligand present in the sample; and
- b) measuring the amount of the complex and thereby measuring the amount of the modified TIE-2 ligand in the biological sample.

The invention further encompasses an assay for measuring the amount of TIE receptor in a biological sample by

- a) contacting the biological sample with at least one ligand of the invention so that the ligand forms a complex with the TIE receptor; and
- b) measuring the amount of the complex and thereby measuring the amount of the TIE receptor in the biological sample.

The present invention also provides for the utilization of a modified TIE-2 ligand which activates the TIE-2 receptor as described herein, to support the survival and/or growth and/or migration and/or differentiation of TIE-2 receptor expressing cells. Thus, the ligand may be used as a supplement to support, for example, endothelial cells in culture.

Further, the creation by applicants of a modified TIE-2 ligand for the TIE-2 receptor enables the utilization of assav systems useful for the identification of agonists or antagouseful in identifying molecules capable of promoting or inhibiting angiogenesis. For example, in one embodiment, antagonists of the TIE-2 receptor may be identified as test molecules that are capable of interfering with the interaction of the TIE-2 receptor with a modified TIE-2 ligand that binds the TIE-2 receptor. Such antagonists are identified by their ability to 1) block the binding of a biologically active modified TIE-2 ligand to the receptor as measured, for example, using BIAcore biosensor technology (BIAcore; ability of a biologically active modified TIE-2 ligand to cause a biological response. Such biological responses

include, but are not limited to, phosphorylation of the TIE receptor or downstream components of the TIE signal transduction pathway, or survival, growth or differentiation of TIE receptor bearing cells.

In one embodiment, cells engineered to express the TIE 5 receptor may be dependent for growth on the addition of a modified TIE-2 ligand. Such cells provide useful assay systems for identifying additional agonists of the TIE receptor, or antagonists capable of interfering with the activity of the modified TIE-2 ligand on such cells. 10 Alternatively, autocrine cells, engineered to be capable of co-expressing both a modified TIE-2 ligand and receptor, may provide useful systems for assaying potential agonists or antagonists.

Therefore, the present invention provides for introduction 15 of a TIE-2 receptor into cells that do not normally express this receptor, thus allowing these cells to exhibit profound and easily distinguishable responses to a ligand which binds this receptor. The type of response elicited depends on the cell utilized, and not the specific receptor introduced into the 20 to the controls and in each of the dilutions, and then cell. Appropriate cell lines can be chosen to yield a response of the greatest utility for assaying, as well as discovering, molecules that can act on tyrosine kinase receptors. The molecules may be any type of molecule, including but not limited to peptide and non-peptide molecules, that will act in 25 measuring the amount of radioactivity per cell, or by crosssystems to be described in a receptor specific manner.

One of the more useful systems to be exploited involves the introduction of a TIE receptor (or a chimeric receptor comprising the extracellular domain of another receptor tyrosine kinase such as, for example, trkC and the intracel- 30 lular domain of a TIE receptor) into a fibroblast cell line (e.g., NIH3T3 cells) thus such a receptor which does not normally mediate proliferative or other responses can, following introduction into fibroblasts, nonetheless be assayed by a variety of well established methods to quantitate effects 35 of fibroblast growth factors (e.g., thymidine incorporation or other types of proliferation assays; see in Zoelen, 1990, "The Use of Biological Assays For Detection Of polypeptide Growth Factors" in Progress Factor Research, Vol. 2, pp. 131–152; Zhan and M. Goldfarb, 1986, Mol. Cell. Biol., Vol. 40 6, pp. 3541-3544). These assays have the added advantage that any preparation can be assayed both on the cell line having the introduced receptor as. well as the parental cell line lacking the receptor; only specific effects on the cell line with the receptor would be judged as being mediated 45 through the introduced receptor. Such cells may be further engineered to express a modified TIE-2 ligand, thus creating an autocrine system useful for assaying for molecules that act as antagonists/agonists of this interaction. Thus, the present invention provides for host cells comprising nucleic 50 acid encoding a modified TIE-2 ligand and nucleic acid encoding TIE receptor.

The TIE receptormodified TIE-2 ligand interaction also provides a useful system for identifying small molecule agonists or antagonists of the TIE receptor. For example, 55 including, but not limited to, cell growth and/or differentiafragments, mutants or derivatives of a modified TIE-2 ligand may be identified that bind TIE receptor but do not induce any other biological activity. Alternatively, the characterization of a modified TIE-2 ligand enables the further characterization of active portions of the molecule. Further, the 60 identification of a ligand enables the determination of the X-ray crystal structure of the receptor/ligand complex, thus enabling identification of the binding site on the receptor. Knowledge of the binding site will provide useful insight into the rational resign of novel agonists and antagonists. 65

The specific binding of a test molecule to TIE receptor may be measured in a number of ways. For example, the actual binding of test molecule to cells expressing TIE may be detected or measured, by detecting or measuring (i) test molecule bound to the surface of intact cells; (ii) test molecule cross-linked to TIE protein in cell lysates; or (iii) test molecule bound to TIE in vitro. The specific interaction between test molecule and TIE may be evaluated by using reagents that demonstrate the unique properties of that interaction.

As a specific, nonlimiting example, the methods of the invention may be used as follows. Consider a case in which a modified TIE-2 ligand in a sample is to be measured. Varying dilutions of the sample (the test molecule), in parallel with a negative control (NC) containing no modified TIE-2 ligand activity, and a positive control (PC) containing a known amount of a modified TIE-2 ligand, may be exposed to cells that express TIE in the presence of a detectably labeled modified TIE-2 ligand (in this example, radioiodinated ligand). The amount of modified TIE-2 ligand in the test sample may be evaluated by determining the amount of ¹²⁵I-labeled modified TIE-2 ligand that binds comparing the sample values to a standard curve. The more modified TIE-2 ligand in the sample, the less ¹²⁵I-ligand that will bind to TIE.

The amount of ¹²⁵I-ligand bound may be determined by linking a modified TIE-2ligand to cell surface proteins using DSS, as described in Meakin and Shooter, 1991, Neuron 6:153-163, and detecting the amount of labeled protein in cell extracts using, for example, SDS polyacrylamide gel electrophoresis, which may reveal a labeled protein having a size corresponding to TIE receptor/modified TIE-2 ligand. The specific test molecule/TIE interaction may further be tested by adding to the assays various dilutions of an unlabeled control ligand that does not bind the TIE receptor and therefore should have no substantial effect on the competition between labeled modified TIE-2 ligand and test molecule for TIE binding. Alternatively, a molecule known to be able to disrupt TIE receptor/modified TIE-2 ligand binding, such as, but not limited to, anti-TIE antibody, or TIE receptorbody as described herein, may be expected to interfere with the competition between ¹²⁵I-modified TIE-2 ligand and test molecule for TIE receptor binding.

Detectably labeled modified TIE-2 ligand includes, but is not limited to, a modified TIE-2 ligand linked covalently or noncovalently to a radioactive substance, a fluorescent substance, a substance that has enzymatic activity, a substance that may serve as a substrate for an enzyme (enzymes and substrates associated with calorimetrically detectable reactions are preferred) or to a substance that can be recognized by an antibody molecule that is preferably a detectably labeled antibody molecule.

Alternatively, the specific binding of test molecule to TIE may be measured by evaluating the secondary biological effects of a modified TIE-2 ligand/TIE receptor binding, tion or immediate early gene expression or phosphorylation of TIE. For example, the ability of the test molecule to induce differentiation can be tested in cells that lack tie and in comparable cells that express tie; differentiation in tieexpressing cells but not in comparable cells that lack tie would be indicative of a specific test molecule/TIE interaction. A similar analysis could be performed by detecting immediate early gene (e.g. fos and jun) induction in tieminus and tie-plus cells, or by detecting phosphorylation of TIE using standard phosphorylation assays known in the art. Such analysis might be useful in identifying agonists or antagonists that do not competitively bind to TIE.

Similarly, the present invention provides for a method of identifying a molecule that has the biological activity of a modified TIE-2 ligand comprising (i) exposing a cell that expresses tie to a test molecule and (ii) detecting the specific binding of the test molecule to TIE receptor, in which specific binding to TIE positively correlates with TIE-like activity. Specific binding may be detected by either assaying for direct binding or the secondary biological effects of binding, as digcussed supra. Such a method may be particularly useful in identifying new members of the TIE ligand 10 family or, in the pharmaceutical industry, in screening a large array of peptide and non-peptide molecules (e.g., peptidomimetics) for TIE associated biological activity. In a preferred, specific, nonlimiting embodiment of the invention, a large grid of culture wells may be prepared that 15 contain, in alternate rows, PC12 (or fibroblasts, see infra) cells that are either tie-minus or engineered to be tie-plus. A variety of test molecules may then be added such that each column of the grid, or a portion thereof, contains a different test molecule. Each well could then be scored for the 20 able to determine if the blocker molecule is binding spepresence or absence of growth and/or differentiation. An extremely large number of test molecules could be screened for such activity in this manner.

In additional embodiments, the invention provides for methods of detecting or measuring TIE ligand-like activity 25 or identifying a molecule as having such activity comprising (i) exposing a test molecule to a TIE receptor protein in vitro under conditions that permit binding to occur and (ii) detecting binding of the test molecule to the TIE receptor protein, in which binding of test molecule to TIE receptor 30 assay for identifying additional agonists of correlates with TIE ligand-like activity. According to such methods; the TIE receptor may or may not be substantially purified, may be affixed to a solid support (e.g. as an affinity column or as an ELISA assay), or may be incorporated into an artificial membrane. Binding of test molecule to TIE 35 end and extending to the nucleotide at about position 1160 receptor may be evaluated by any method known in the art. In preferred embodiments, the binding of test molecule may be detected or measured by evaluating its ability to compete with detectably labeled known TIE ligands for TIE receptor binding.

The present invention also provides for a method of detecting the ability of a test molecule to function as an antagonist of TIE ligand-like activity comprising detecting the ability of the molecule to inhibit an effect of TIE ligand binding to TIE receptor on a cell that expresses the receptor. 45 Such an antagonist may or may not interfere with TIE receptor/modified TIE-2 ligand binding. Effects of a modified TIE-2 ligand binding to TIE receptor are preferably biological or biochemical effects, including, but not limited to, cell survival or proliferation, cell transformation, imme- 50 diate early gene induction, or TIE phosphorylation.

The invention further provides for both a method of identifying antibodies or other molecules capable of neutralizing the ligand or blocking binding to the receptor, as well as the molecules identified by the method. By way of 55 fibrinogen-like domain, which has been "clustered" using nonlimiting example, the method may be performed via an assay which is conceptually similar to an ELISA assay. For example, TIE receptorbody may be bound to a solid support, such as a plastic multiwell plate. As a control, a known amount of a modified TIE-2 ligand which has been Myc-60 tagged may then be introduced to the well and any tagged modified TIE-2 ligand which binds the receptorbody may then be identified by means of a reporter antibody directed against the Myc-tag. This assay system may then be used to screen test samples for molecules which are capable of i) 65 binding to the tagged ligand or ii) binding to the receptorbody and thereby blocking binding to the receptorbody by

the tagged ligand. For example, a test sample containing a putative molecule of interest together with a known amount of tagged ligand may be introduced to the well and the amount of tagged ligand which binds to the receptorbody may be measured. By comparing the amount of bound tagged ligand in the test sample to the amount in the control, samples containing molecules which are capable of blocking ligand binding to the receptor may be identified. The molecules of interest thus identified may be isolated using methods well known to one of skill in the art.

Once a blocker of ligand binding is found, one of skill in the art would know to perform secondary assays to determine whether the blocker is binding to the receptor or to the ligand, as well as assays to determine if the blocker molecule can neutralize the biological activity of the ligand. For example, by using a binding assay which employs BlAcore biosensor technology (or the equivalent), in which either TIE receptorbody or a modified TIE-2 ligand or ligandbody is covalently attached to a solid support (e.g. carboxymethyl dextran on a gold surface), one of skill in the art would be cifically to the ligand, ligandbody or to the receptorbody. To determine if the blocker molecule can neutralize the biological activity of the ligand, one of skill in the art could perform a phosphorylation assay (see Example 5) or alternatively, a functional bioassay, such as a survival assay, by using primary cultures of, for example, endothelial cells. Alternatively, a blocker molecule which binds to the receptorbody could be an agonist and one of skill in the art would know to how to determine this by performing an appropriate

In addition, the invention further contemplates compositions wherein the TIE ligand is the receptor binding domain of a TIE-2 ligand described herein. For example, TIE-2 ligand 1 contains a "coiled coil" domain (beginning at the 5' of FIGS. 4A-4D (SEQ ID NO: 1) and about position 1157 of FIGS. 5A-5D (SEQ ID NO: 3) and a fibrinogen-like domain (which is encoded by the nucleotide sequence of FIGS. 4A-4D [SEQ ID NO: 1] beginning at about position 40 1161 and about position 1158 of FIGS. **5A–5D** [SEQ ID NO: 3]). The fibrinogen-like domain of TIE-2 ligand 2 is believed to begin on or around the same amino acid sequence as in ligand 1 (FRDCA) which is encoded by nucleotides beginning around 1197 of FIGS. 6A-6D (SEQ ID NO: 5). The fibrinogen-like domain of TIE ligand-3 is believed to begin on or around the amino acid sequence which is encoded by nucleotides beginning around position 929 as set forth in FIGS. 21A-21C (SEQ ID NO: 10). Multimerization of the coiled coil domains during production of the ligand hampers purification. As described in Example 19, Applicants have discovered, however, that the fibrinogen-like domain comprises the TIE-2 receptor binding domain. The monomeric forms of the fibringen-like domain do not, however, appear to bind the receptor. Studies utilizing myc-tagged anti-myc antibodies, do bind the TIE-2 receptor. [Methods of production of "clustered ligands and ligandbodies are described in Davis, et al. Science 266:816-819 (1994)]. Based on these finding, applicants produced "ligandbodies' which comprise the fibrinogen-like domain of the TIE-2 ligands coupled to the Fc domain of IgG ("Fc's"). These ligandbodies, which form dimers, efficiently bind the TIE-2 receptor. Accordingly, the present invention contemplates the production of modified TIE ligandbodies which may be used as targeting agents, in diagnostics or in therapeutic applications, such as targeting agents for tumors and/or associated vasculature wherein a TIE antagonist is indicated.

The invention herein further provides for the development of the ligand, a fragment or derivative thereof, or another molecule which is a receptor agonist or antagonist, as a therapeutic for the treatment of patients suffering from disorders involving cells, tissues or organs which express the 5 TIE receptor. Such molecules may be used in a method of treatment of the human or animal body, or in a method of diagnosis.

Because TIE receptor has been identified in association with endothelial cells and, as demonstrated herein, blocking 10 of TIE-2 ligand 1 appears to prevent vascularization, applicants expect that a modified TIE-2 ligand described herein may be useful for the induction of vascularization in diseases or disorders where such vascularization is indicated. Such diseases or disorders would include wound healing, 15 examined in in vitro and in vivo[] biological systems and ischaemia and diabetes. The ligands may be tested in animal models and used therapeutically as described for other agents, such as vascular endothelial growth factor (VEGF), another endothelial cell-specific factor that is angiogenic. The Ferrara reference, as well as other studies, describe in vitro and in vivo studies that may be used to demonstrate the effect of an angiogenic factor in enhancing blood flow to ischemic myocardium, enhancing wound healing, and in other therapeutic settings wherein neoangiogenesis is 25 desired. [see Sudo, et al. European Patent Application 0 550 296 A2 published Jul. 7, 1993; Banai, et al. Circulation 89:2183-2189 (1994); Unger, et al. Am. J. Physiol. 286:H1588-H1595 (1994); Lazarous, et al. Circulation 91:145–153 (1995)]. According to the invention, a modified 30 TIE-2 ligand may be used alone or in combination with one or more additional pharmaceutically active compounds such as, for example, VEGF or basic fibroblast growth factor (bFGF), as well as cytokines, neurotrophins, etc.

modified TIE-2 ligands which bind but do not activate the receptor as described herein, receptorbodies as described herein in Examples 2 and 3, and TIE-2 ligand 2 as described in Example 9, would be useful to prevent or attenuate vascularization, thus preventing or attenuating, for example, 40 tumor growth. These agents may be used alone or in combination with other compositions, such as anti-VEGF antibodies, that have been shown to be useful in treating conditions in which the therapeutic intent is to block angiogenesis. Applicants expect that a modified TIE-2 ligand described herein may also be used in combination with agents, such as cytokine antagonists such as IL-6 antagonists, that are known to block inflammation.

For example, applicants have determined that TIE ligands are expressed in cells within, or closely associated with, 50 are used to diagnose or treat patients in which the desired tumors. For example, TIE-2 ligand 2 appears to be tightly associated with tumor endothelial cells. Accordingly, it and other TIE antagonists may also be useful in preventing or attenuating, for example, tumor growth. In addition, TIE ligands or ligandbodies may be useful for the delivery of 55 embodiments, treatment may comprise use of a therapeutitoxins to a receptor bearing cell. Alternatively, other molecules, such as growth factors, cytokines or nutrients, may be delivered to a TIE receptor bearing cell via TIE ligands or ligandbodies. TIE ligands or ligandbodies such as modified TIE-2 ligand described herein may also be used as 60 compositions comprising a modified TIE-2 ligand or ligandiagnostic reagents for TIE receptor, to detect the receptor in vivo or in vitro. Where the TIE receptor is associated with a disease state, TIE ligands or ligandbodies such as a modified TIE-2 ligand may be useful as diagnostic reagents for detecting the disease by, for example, tissue staining or 65 whole body imaging. Such reagents include radioisotopes, flurochromes, dyes, enzymes and biotin. Such diagnostics or

targeting agents may be prepared as described in Alitalo, et al. WO 95/26364 published Oct. 5, 1995 and Burrows, F. and P. Thorpe, PNAS (USA) 90:8996-9000 (1993) which is incorporated herein in its entirety.

In other embodiments, the TIE ligands, a receptor activating modified TIE-2 ligand described herein are used as hematopoietic factors. A variety of hematopoietic factors and their receptors are involved in the proliferation and/or differentiation and/or migration of the various cells types contained within blood. Because the TIE receptors are expressed in early hematopoietic cells, the TIE ligands are expected to play a comparable role in the proliferation or differentiation or migration of these cells. Thus, for example, TIE containing compositions may be prepared, assayed,

used therapeutically as described in any of the following: Sousa, U.S. Pat. No. 4,810,643, Lee, et al., Proc. Natl. Acad. Sci. USA 82:4360-4364 (1995) Wong, et al. Science, 228:810-814 (1985); Yokota, et al. Proc. Natl. Acad. Sci Ferrara, et al. U.S. Pat. No. 5,332,671 issued Jul. 26, 1994. 20 (USA) 81:1070 (1984); Bosselman, et al. WO 9105795 published May 2, 1991 entitled "Stem Cell Factor" and Kirkness, et al. WO 95/19985 published Jul. 27, 1995 entitled "Haemopoietic Maturation Factor". Accordingly, receptor activating modified TIE-2 ligand may be used to diagnose or treat conditions in which normal hematopoiesis is suppressed, including, but not limited to anemia, thrombocytopenia, leukopenia and granulocytopenia. In a preferred embodiment, receptor activating modified TIE-2 Iigand may be used to stimulate differentiation of blood cell precursors in situations where a patient has a disease, such as acquired immune deficiency syndrome (AIDS) which has caused a reduction in normal blood cell levels, or in clinical settings in which enhancement of hematopoietic populations is desired, such as in conjunction with bone marrow Conversely, antagonists of the TIE receptor, such as 35 transplant, or in the treatment of aplasia or myelosuppression caused by radiation, chemical treatment or chemotherapy.

> The receptor activating modified TIE-2 ligands of the present invention may be used alone, or in combination with another pharmaceutically active agent such as, for example, ctyokines, neurotrophins, interleukins, etc. In a preferred embodiment, the ligands lay be used in conjunction with any of a number of the above referenced actors which are known to induce stem cell or other hematopoietic precursor proliferation, or factors acting on later cells in the hematopoietic pathway, including, but not limited to, hemopoietic maturation factor, thrombopoietin, stem cell factor, erythropoietin, G-CSF, GM-CSF, etc.

> In an alternative embodiment, TIE receptor antagonists result is inhibition of a hematopoietic pathway, such as for the treatment of myeloproliferative or other proliferative disorders of blood forming organs such as thrombocythemias, polycythemias and leukemias. In such cally effective amount of the a modified TIE-2 ligand, TIE antibody, TIE receptorbody, a conjugate of a modified TIE-2 ligand, or a ligandbody or fFC as described herein.

> The present invention also provides for pharmaceutical dbodies described herein, peptide fragments thereof, or derivatives in a pharmacologically acceptable vehicle. The modified TIE-2 ligand proteins, peptide fragments, or derivatives may be administered systemically or locally. Any appropriate mode of administration known in the art may be used, including, but not limited to, intravenous, intrathecal, intraarterial, intranasal, oral, subcutaneous,

intraperitoneal, or by local injection or surgical implant. Sustained release formulations are also provided for.

The present invention also provides for an antibody which specifically binds such a therapeutic molecule. The antibody may be monoclonal or polyclonal. The invention also pro-5 vides for a method of using such a monoclonal or polyclonal antibody to measure the amount of the therapeutic molecule in a sample taken from a patient for purposes of monitoring the course of therapy.

The invention further provides for a therapeutic composition comprising a modified TIE-2 ligand or ligandbody and a cytotoxic agent conjugated thereto. In one embodiment, the cytotoxic agent may be a radioisotope or toxin.

The invention also provides for an antibody which specifically binds a modified TIE-2 ligand. The antibody may 15 be monoclonal or polyclonal.

The invention further provides for a method of purifying a modified TIE-2 ligand comprising:

- a) coupling at least one TIE binding substrate to a solid matrix;
- b) incubating the substrate of a) with a cell lysate so that the substrate forms a complex with any modified TIE-2 ligand in the cell lysate;
- c) washing the solid matrix; and
- d) eluting the modified TIE-2 ligand from the coupled 25 substrate.

The substrate may be any substance that specifically binds the modified TIE-2 ligand. In one embodiment, the substrate is selected from the group consisting of anti-modified TIE-2 ligand antibody, TIE receptor and TIE receptorbody. The 30 invention further provides for a receptorbody which specifically binds a modified TIE-2 ligand, as well as a therapeutic composition comprising the receptorbody in a pharmaceutically acceptable vehicle, and a method of blocking blood vessel growth in a human comprising administering an 35 effective amount of the therapeutic composition.

The invention also provides for a therapeutic composition comprising a receptor activating modified TIE-2. ligand or ligandbody in a pharmaceutically acceptable vehicle, as well as a method of promoting neovascularization in a patient 40 comprising administering to the patient an effective amount of the therapeutic composition.

In addition, the present invention provides for a method for identifying a cell which expresses TIE receptor which comprises contacting a cell with a detectably labeled modi- 45 fied TIE-2 ligand or ligandbody, under conditions permitting binding of the detectably labeled ligand to the TIE receptor and determining whether the detectably labeled ligand is bound to the TIE receptor, thereby identifying the cell as one provides for a therapeutic composition comprising a modified TIE-2 ligand or ligandbody and a cytotoxic agent conjugated thereto. The cytotoxic agent may be a radioisotone or toxin.

The invention also provides a method of detecting expres- 55 sion of a modified TIE-2 ligand by a cell which comprises obtaining mRNA from the cell, contacting the mRNA so obtained with a labeled nucleic acid molecule encoding a modified TIE-2 ligand, under hybridizing conditions, determining the presence of mRNA hybridized to the labeled 60 molecule, and thereby detecting the expression of a modified TIE-2 ligand in the cell.

The invention further provides a method of detecting expression of a modified TIE-2 ligand in tissue sections which comprises contacting the issue sections with a labeled 65 of the rat TIE-2 receptor fused to the human immunoglonucleic acid molecule encoding a modified TIE-2 ligand, under hybridizing conditions, determining the presence of

mRNA hybridized to the labelled molecule, and thereby detecting the expression of a modified TIE-2 ligand in tissue sections.

EXAMPLE 1

IDENTIFICATION OF THE ABAE CELL LINE AS REPORTER CELLS FOR THE TIE-2 RECEPTOR

Adult BAE cells are registered in the European Cell Culture Repository, under ECACC#92010601. (See PNAS 75:2621 (1978)). Northern (RNA) analyses revealed moderate levels of tie-2 transcripts in the ABAE (Adult Bovine Arterial Endothelial) cell line, consistent with in situ hybridization results that demonstrated almost exclusive localization of tie-2 RNAs to vascular endothelial cells. We therefore examined ABAE cell lysates for the presence of TIE-2 protein, as well as the extent to which this TIE-2 protein is tyrosine-phosphorylated under normal versus serumdeprived growth conditions. ABAE cell lysates were harvested and subjected to immunoprecipitation, followed by Western blot analyses of immunoprecipitated proteins with TIE-2 specific and phosphotyrosine specific antisera. Omission or inclusion of TIE-2 peptides as specific blocking molecules during TIE-2 immunoprecipitation allowed unambiguous identification of TIE-2 as a moderately detectable protein of ~150 kD whose steady-state phosphotyrosine levels diminish to near undetectable levels by prior serumstarvation of the cells.

Culture of ABAE cells and harvest of cell lysates was done as Follows. Low-passage-number ABAE cells were plated as a monolayer at a density of 2×10^6 cells/150 mm plastic petri plate (Falcon) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% bovine calf drum (10% BCS), 2 mM L-glutamine (Q) and 1% each of penicillin and streptomycin (P-S) in an atmosphere of 5% CO2. Prior to harvest of cell lysates, cells were serumstarved for 24 hours in DMEM/Q/P-S, followed aspiration of the medium and rinsing of the plates with ice-cold phosphate buffered saline (PBS) supplemented with sodium orthovanadate, sodium fluoride and sodium benzamidine. Cells were lysed in a small volume of this rinse buffer that had been supplemented with 1% NP40 detergent and the protease inhibitors PMSF and aprotinin. Insoluble debris was removed from the cell lysates by centrifugation at 14,000×G for 10 minutes, at 4° C. and the supernatants were subjected to immunoprecipitation with antisera specific for TIE-2 receptor, with or without the presence of blocking which expresses TIE receptor. The present invention also 50 peptides added to $\sim 20 \ \mu g/ml$ lysate. immunoprecipitated proteins were resolved by PAGE (7.5% Laemmli gel), and then electro-transferred to PVDF membrane and incubated either with various TIE-2- or phosphotyrosine-specific antisera. TIE-2 protein was visualized by incubation of the membrane with HRP-linked secondary antisera followed by treatment with ECL reagent (Amersham).

EXAMPLE 2

CLONING AND EXPRESSION OF TIE-2 RECEPTORBODY FOR AFFINITY-BASED STUDY OF TIE-2 LIGAND INTERACTIONS

An expression construct was created that would yield a secreted protein consisting of the entire extracellular portion bulin gamma-1 constant region IgG1 Fc). This fusion protein is called a TIE-2 "ureceptorbody" (RB), and would be

normally expected to exist as a dimer in solution based on formation of disulfide linkages between individual IgG1 Fc tails. The Fc portion of the TIE-2 RB was prepared as follows. A DNA fragment encoding the Fc portion of human IgG1 that spans from the hinge region to the carboxyterminus of the protein, was amplified from human placental cDNA by PCR with oligonucleotides corresponding to the published sequence of human IgG1; the resulting DNA fragment was cloned in a plasmid vector. Appropriate DNA restriction fragments from a plasmid encoding the fulllength TIE-2 receptor and from the human IgG1 Fc plasmid were ligated on either side of a short PCR-derived fragment that was designed so as to fuse, in-frame, the TIE-2 and human IgG1 Fc protein-coding sequences. Thus, the resulting TIE-2 ectodomain-Fc fusion protein precisely substi-15 tuted the IgG1 Fc in place of the region spanning the TIE-2 transmembrane and cytoplasmic domains. An alternative method of preparing RBs is described in Goodwin, et. al. Cell 73:447-456 (1993).

Milligram quantities of TIE-2 RB were obtained by 20 cloning the TIE-2 RB DNA fragment into the pVL1393 baculovirus vector and subsequently infecting the Spodoptera frugiperda SF-21AE insect cell line. Alternatively, the cell line SF-9 (ATCC Accession No. CRL-1711) or the cell line BTI-TN-5b1-4 may be used. 25 DNA encoding the TIE-2 RB was cloned as an Eco RI-NotI fragment into the baculovirus transfer plasmid pVL1393. Plasmid DNA purified by cesium chloride density gradient centrifugation was recombined into viral DNA by mixing 3 μg of plasmid DNA with 0.5 μg of Baculo-Gold DNA ₃₀ (Pharminigen), followed by introduction into liposomes using 30 µg Lipofectin (GIBCO-BRL). DNA-iposome mixtures were added to SF-21AE cells (2×10^6 cells/60 mm dish) in TMN-FH medium (Modified Grace's Insect Cell Medium (GIBCO-BRL) for 5 ours at 27° C., followed by incubation 35 at 27° C. for 5 days in TMN-FH medium supplemented with 5% fetal calf serum. Tissue culture medium was harvested for plaque purification of recombinant viruses, which was carried out using methods previously described (O'Reilly, D. R., L. K. Miller, and V. A. Luckow, Baculovirus Expres- 40 sion Vectors-A Laboratory Manual. 1992, New York: W. H. Freeman) except that the agarose overlay contained 125 μg/mL X-gal (5-bromo-4-chloro-3-indolyl-β-Dgalactopyranoside; GIBCO-BRL). After 5 days of incubation at 27° C., non-recombinant plaques were scored by 45 helial cells, which provide the major source of endothelial positive chromogenic reaction to the X-gal substrate, and their positions marked. Recombinant plaques were then visualized by addition of a second overlay containing 100 μ g/mL MTT (3-[4,5-dimethylthiazol-2-yl]2,5, diphenyltetrazolium bromide; Sigma). Putative recombinant 50 endothelial and hematopoietic cell lineages, termed the virus plaques were picked by plug aspiration, and purified by multiple rounds of plaque isolation to assure homogeneity. Virus stocks were generated by serial, low-multiplicity passage of plaque-purified virus. Low passage stocks of one virus clone (vTIE-2 receptorbody) were produced.

SF-21AE cells were cultured in serum free medium (SF-900 II, Gibco BRL) containing 1×antibioticlantimycotic solution (Gibco BRL) and 25 mg/L Gentamycin (Gibco BRL). Pluronic F-68 was added as a surfactant to a final concentration of 1 g/L. Cultures (4 L) were raised in a 60 invade the embryo to join with limited, intra-embryonicallybioreactor (Artisan Cell Station System) for at least three days prior to infection. Cells were grown at 27° C., with gassing to 50% dissolved oxygen, at a gas flow rate of 80 mL/min (aeration at a sparge ring). Agitation was by means of a marine impeller at a rate of 100 rpm. Cells were 65 harvested in mid-logarithmic growth phase ($\sim 2 \times 10^6$ cells/ mL), concentrated by centrifugation, and infected with 5

plaque forming units of vTIE-2 receptorbody per cell. Cells and inoculum were brought to 400 mL with fresh medium, and virus was adsorbed for 2 hours at 27° C. in a spinner flask. The culture was then resuspended in a final volume of 8 L with fresh serum-free medium, and the cells incubated in the bioreactor using the previously described conditions.

Culture medium from vTIE-2 receptorbody-infected SF21AE cells were collected by centrifugation (500 \times g, 10 minutes) at 72 hours post-infection. Cell supernatants were brought to pH 8 with NaOH. EDTA was added to a final concentration of 10 mM and the supernatant pH was readjusted to 8. Supernatants were filtered (0.45 μ m, Millipore) and loaded on a protein A column (protein A sepharose 4 fast flow or HiTrap protein A, both from Pharmacia). The column was washed with PBS containing 0.5 M NaCl until the absorbance at 280 nm decreased to baseline. The column was washed in PBS and eluted with 0.5 M acetic acid. Column fractions were immediately neutralized by eluting into tubes containing 1 M Tris pH 9. The peak fractions containing the TIE-2 receptorbody were pooled and dialyzed versus PBS.

EXAMPLE 3

DEMONSTRATION THAT TIE-2 HAS A CRITICAL ROLE IN DEVELOPMENT OF THE VASCULATURE

Insight into the function of TIE-2 was gained by introduction of "excess" soluble TIE-2 receptorbody (TIE-2 RB) into a developing system. The potential ability of TIE-2 RB to bind, and thereby neutralize, available TIE-2 ligand could result in an observable disruption of normal vascular development and characterization of the ligand. To examine whether TIE-2 RB could be used to disrupt vascular development in early chick embryos, small pieces of a biologically resorbable foam were soaked with E-2 RB and inserted immediately beneath the chorioallantoic membrane at positions just lateral to the primitive embryo.

Early chicken embryos develop atop the yolk from a small disk of cells that is covered by the chorioallantoic membrane (CAM). The endothelial cells that will come to line the vasculature in the embryo arise from both extra- and intraembryonic cell sources. Extra-embryonically-derived endotcells in the embryo, originate from accretions of mesenchyme that are situated laterally around the embryo-proper, just underneath the CAM. As these mesenchyme cells mature, they give rise to a common progenitor of both the hemangioblast. In turn, the hemangioblast gives rise to a mixed population of angioblasts (the endothelial cell progenitor) and hematoblasts (the pluripotential hematopoietic precursor). Formation of rudiments of the circulatory 55 system begins when endothelial cell progeny segregate to form a one-cell-thick vesicle that surrounds the primitive blood cells. Proliferation and migration of these cellular components eventually produces a vast network of bloodfilled microvessels under the CAM that will ultimately derived vascular elements.

Newly fertilized chicken eggs obtained from Spafas, Inc. (Boston, Mass.) were incubated at 99.5° F., 55% relative humidity. At about 24 hrs. of development, the egg shell was wiped down with 70% ethanol and a dentist's drill was used to make a 1.5 cm. hole in the blunt apex of each egg. The shell membrane was removed to reveal an air space directly

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above the embryo. Small rectangular pieces of sterile Gelfoam (Upjohn) were cut with a scalpel and soaked in equal concentrations of either TIE-2- or EHK-1 receptorbody. EHK-1 receptorbody was made as set forth in Example 2 using the EHK-1 extracellular domain instead of the TIE-2 extracellular domain (Maisonpierre et al., Oncogene 8:3277–3288 (1993). Each Gelfoam piece absorbed approximately 6 μ g of protein in 30 μ l. Sterile watchmakers forceps were used to make a small tear in the CAM at a position several millimeters lateral to the primitive embryo. The majority of the piece of RB-soaked Gelfoam was inserted under the CAM and the egg shell was sealed over with a piece of adhesive tape. Other similarly-staged eggs were treated in parallel with RB of the unrelated, neuronally expressed receptor tyrosine kinase, EHK-1 (Maisonpierre et al., Oncogene 8:3277-3288 (1993). Development was allowed to proceed for 4 days and then the embryos were examined by visual inspection. Embryos were removed by carefully breaking the shells in dishes of warmed PBS and carefully. cutting away the embryo with surrounding CAM. 20 N-hydroxysuccinimide (NHS) and N-ethyl-N'-(3-Of 12 eggs treated with each RB, 6 TIE-2 RB and 5 EHK-1 RB treated embryos had developed beyond the stage observed at the start of the experiment. A dramatic difference was seen between these developed embryos, as shown in FIGS. 1A and 1B. Those treated with EHK-1 RB appeared 25 to have developed relatively normally. Four out of five EHK-1 embryos were viable as judged by the presence of a beating heart. Furthermore, the extra-embryonic vasculature, which is visually obvious due to the presence of red blood cells, was profuse and extended several centime- 30 ters laterally under the CAM. By contrast, those treated with TIE-2 RB were severely stunted, ranging from 2-5 mm. in diameter, as compared with more than 10 mm in diameter for the EHK-1 RB embryos. All of the TIE-2 RB treated embryos were dead and their CAMs were devoid of blood 35 vessels. The ability of TIE-2 RB to block vascular development in the chicken demonstrates that TIE-2 ligand is necessary for development of the vasculature.

EXAMPLE 4

IDENTIFICATION OF A TIE-2-SPECIFIC BINDING ACTIVITY IN CONDITIONED MEDIUM FROM THE ras ONCOGENE-TRANSFORMED C2C12 MOUSE MYOBLAST CELL LINE

Screening of ten-fold-concentrated cell-conditioned media (10×CCM) from various cell lines for the presence of soluble, TIE-2-specific binding activity (BIAcore; Pharmacia Biosensor, Piscataway, N.J.) revealed binding activity in 50 serum-free medium from oncogenic-ras-transformed C2C12 cells (C2C12-ras), RAT 2-ras (which is a ras transformed fibroblast cell line), human glioblastoma T98G and the human neuroblastoma cell line known as SHEP-1.

The C2C12-ras 10×CCM originated from a stably trans- 55 fected line of C2C12 myoblasts that was oncogenically transformed by transfection with the T-24 mutant of H-ras by standard calcium phosphate-based methods. An SV40 based neomycin-resistance expression plasmid was physically linked with the ras expression plasmid in order to 60 permit selection of transfected clones. Resulting G418resistant ras-C2C12 cells were routinely maintained as a monolayer on plastic dishes in DMEM/glutamine/penicillinstreptomycin supplemented with 10% fetal calf serum (FCS). Serum-free C2C12-ras 10×CCM was made by plat- 65 ing cells at 60% confluence in a serum free defined media for 12 hours. [Zhan and Goldfarb, Mol. Cell. Biol. 6:

3541-3544 (1986)); Zhan, et al. Oncogene 1: 369-376 (1987)]. The medium was discarded and replaced with fresh DMEM/Q/P-S for 24 hours. This medium was harvested and cells were re-fed fresh DMEMIQ/P-S, which was also harvested after a further 24 hours. These CCM were supplemented with the protease inhibitors PMSF (1 mM) and aprotinin (10 μ g/ml), and ten-fold concentrated on sterile size-exclusion membranes (Amicon). TIE-2-binding activity could be neutralized by incubation of the medium with an excess of TIE-2 RB, but not by incubation with EHK-1 RB, prior to BIAcore analysis.

Binding activity of the 10×CCM was measured using biosensor technology (BIAcore; Pharmacia Biosensor, Piscataway, N.J.) which monitors biomolecular interactions in real-time via surface plasmon resonance. Purified TIE-2 RB was covalently coupled through primary amines to the carboxymethyl dextran layer of a CM5 research grade sensor chip (Pharmacia Biosensor; Piscataway, N.J.). The sensor chip surface was activated using a mixture of dimethylaminopropyl)carbodiimide (EDC), followed by immobilization of TIE-2 RB (25 µg/mL, pH 4.5) and deactivation of unreacted sites with 1.0 M ethanolamine (pH 8.5). A negative control surface of the EHK-1 receptorbody was prepared in a similar manner.

The running buffer used in the system was HBS (10 mM Hepes, 3.4 mM EDTA, 150 mM NaCl, 0.005% P20 surfactant, pH 7.4). The 10×CCM samples were centrifuged for 15 min at 4° C. and further clarified using a rile, low protein-binding 0.45 µm filter (Millipore; Bedford, Mass.). Dextran (2 mg/ml) and P20 surfactant (0.005%) were added to each CCM sample. Aliquots of $40 \,\mu$ L were injected across the immobilized surface (either TIE-2 or EHK-1) at a flow rate of 5 μ L/min and the receptor binding was monitored for 8 min. The binding activity (resonance units, RU) was measured as the difference between a baseline value determined 30 s prior to the sample injection and a measurement taken at 30 s post-injection. Regeneration of the surface was accomplished with one 12-µL pulse of 3 M MgCl₂.

The instrument noise level is 20 RU; therefore, any binding activity with a signal above 20 RU may be interpreted as a real interaction with the receptor. For C2C12-ras conditioned media, the binding activities were in the range 45 60-90 RU for the TIE-2 RB immobilized surface. For the same samples assayed on a EHK-1 RB immobilized surface, the measured activities were less than 35 RU. Specific binding to the TIE-2 receptorbody was evaluated by incubating the samples with an excess of either soluble TIE-2 or EHK-1 RB prior to assaying the binding activity. The addition of soluble EHK-1 RB had no effect on the TIE-2 binding activity of any of the samples, while in the presence of soluble TIE-2 binding to the surface is two-thirds less than that measured in the absence of TIE-2. A repeat assay using >50× concentrated C2C12-ras CCM resulted in a four-fold enhancement over background of the TIE-2 specific binding signal.

EXAMPLE 5

C2C12-ras CCM CONTAINS AN ACTIVITY THAT INDUCES TYROSINE PHOSPHORYLATION OF TIE-2 RECEPTOR

C2C12-ras 10×CCM was examined for its ability to induce tyrosine phosphorylation of TIE-2 in ABAE cells. Serum-starved ABAE cells were briefly incubated with C2C12-ras CCM, lysed and subjected to immunoprecipitation and Western analyses as described above. Stimulation of serum-starved ABAE cells with serum-free C2C12-ras 10× CCM was done as follows. The medium of ABAE cells starved as described above was removed and replaced with either defined medium or 10×CCM that had been pre-5 warmed to 37° C. After 10 minutes, the media were removed and the cells were twice rinsed on ice with an excess of chilled PBS supplemented with orthovanadate/NaF/ benzamidine. Cell lysis and TIE-2-specific immunoprecipitation was done as described above. 10

ABAE cells incubated for 10 minutes with defined medium showed no induction of TIE-2 tyrosine phosphorylation, whereas incubation with C2C12-ras CCM stimulated at least a 100×increase in TIE-2 phosphorylation. This activity was almost totally depleted by pre-incubation ¹⁵ of the C2C12-ras 10×CCM for 90 minutes at room temperature with 13 μ g of TIE-2 RB coupled to protein G-Sepharose beads. Medium incubated with protein G Sepharose alone was not depleted of this phosphorylating activity.

EXAMPLE 6

EXPRESSION CLONING OF TIE-2 LIGAND

COS-7 cells were cultured in Dulbecco's modified ²⁵ Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% each of penicillin and streptomycin (P/S) and 2 mM glutamine in an atmosphere of 5% CO₂. The mouse myoblast C2C12 ras cell line was cultured in Eagle's 30 minimal essential medium (EMEM) with 10% FBS, (P/S) and 2 mM glutamine. Full length mouse TIE-2 ligand cDNA clones were obtained by screening a C2C12 ras cDNA library in the pJFE14 vector expressed in COS cells. This vector, as shown in FIG. 2, is a modified version of the vector pSR_a (Takebe, et al. 1988, Mol. Cell. Biol. 8:466–472). The library was created using the two BSTX1 restriction sites in the pJFE14 vector.

COS-7 cells were transiently transfected with either the pJFE14 library or control vector by the DEAE-dextran 40 transfection protocol. Briefly, COS-7 cells were plated at a density of 1.0×10⁶ cells/100 mm plate 24 hours prior to transfection. For transfection, the cells were cultured in serum-free DMEM containing 400 μ g/ml of DEAE-dextran, 1 μ M chloroquine, and 2 mM glutamine, and 1 μ g of the 45 appropriate DNA for 3-4 hours at 37° C. in an atmosphere of 5% CO₂. The transfection media was aspirated and replaced with PBS with 10% DMSO for 2-3 min. Following this DMSO "shock", the COS-7 cells were placed into DMEM with 10% FBS, 1% each of penicillin and 50 ing htie-2 ligand 1 (ATCC Accession No. 75928). Phage streptomycin, and 2 mM glutamine for 48 hours.

Because the TIE-2 ligand is secreted it was necessary to permeabilize the cells to detect binding of the receptorbody probe to the ligand. Two days after transfection the cells were rinsed with PBS and then incubated with PBS con- 55 malian expression vector may be accomplished as follows. taining 1.8% formaldehyde for 15-30 min. at room temperature. Cells were then washed with PBS and incubated for 15 min. with PBS containing 0.1% Triton X-100 and 10% Bovine Calf Serum to permeabilize the cells and block non-specific binding sites.

The screening was conducted by direct localization of staining using a TIE-2 receptorbody (RB), which consisted of the extracellular domain of TIE-2 fused to the IgG1 constant region. This receptorbody was prepared set forth in Example 2. A 100 mm dish of transfected, fixed and per- 65 meabilized COS cells was probed by incubating them for 30 min with TIE-2 RB. The cells were then washed twice with

PBS and incubated for an additional 30 min with PBS/10% Bovine Calf Serum/anti-human IgG-alkaline phosphatase conjugate. After three PBS washes, cells were incubated in alkaline-phosphatase substrate for 30-60 min. The dish was then inspected microscopically for the presence of stained cells. For each stained cell, a small area of cells including the stained cell was scraped from the dish using a plastic pipette tip and plasmid DNA was then rescued and used to electroporate bacterial cells. Single bacterial colonies resulting from the electroporation were picked and plasmid DNA prepared from these colonies was used to transfect COS-7 cells which were probed for TIE-2 ligand expression as evidenced by binding to TIE-2 receptorbodies. This allowed identification of single clones coding for TIE-2 ligand. Confirmation of TIE-2 ligand expression was obtained by phosphorylation of the TIE-2 receptor using the method set forth in Example 5. A plasmid clone encoding the TIE-2 ligand was deposited with the ATCC on Oct. 7, 1994 and designated as "pJFE14 encoding TIE-2 ligand" under ATCC 20 Accession No. 75910.

EXAMPLE 7

ISOLATION AND SEQUENCING OF FULL LENGTH cDNA CLONE ENCODING HUMAN TIE-2 LIGAND

A human fetal lung cDNA library in lambda gt-10 (see FIG. 3) was obtained from Clontech Laboratories, Inc. (Palo Alto, Calif.). Plaques were plated at a density of 1.25×10^6 / 20×20 cm plate, and replica filters taken following standard procedures (Sambrook, et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., page 8.46, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

Isolation of human tie-2 ligand clones was carried out as 35 follows. A 2.2 kb XhoI fragment from the deposited tie-2 ligand clone (ATCC NO. 75910-see Example 6 above) was labeled by random priming to a specific activity of approximately 5×10^8 cpm/ng. Hybridization was carried out at 65° C. in hybridization solution containing 0.5 mg/ml salmon sperm DNA. The filters were washed at 65° C. in 2×SSC, 0.1% SDS and exposed to Kodak XAR-5 film overnight at -70° C. Positive phage were plaque purified. High titre phage lysates of pure phage were used for isolation of DNA via a Qiagen column using standard techniques (Qiagen, Inc., Chatsworth, Calif., 1995 catalog, page 36). Phage DNA was digested with EcoRI to release the cloned cDNA fragment for subsequent suboloning. A lambda phage vector containing human tie-2 ligand DNA was deposited with the ATCC on Oct. 26, 1994 under the designation λ gt10 encod-DNA may be subjected directly to DNA sequence analysis by the dideoxy chain termination method (Sanger, et al., 1977, Proc. Natl. Acad. Sci. U.S.A. 74: 5463-5467).

Subcdoning of the human tie-2 ligand DNA into a mam-The clone λ gt10 encoding htie-2 ligand 1 contains an EcoRI site located 490 base pairs downstream from the start of the coding sequence for the human TIE-2 ligand. The coding region may be excised using unique restriction sites stream and downstream of the initiator and stop codons respectively. For example, an SpeI site, located 70 bp 5' to the initiator codon, and a Bpu1102i (also known as BIpI) site, located 265 bp 3' to the stop codon, may be used to excise the complete coding region. This may then be subcloned into the pJFE14 cloning vector, using the XbaI (compatible to the SpeI overhang) and the PstI sites (the PstI and Bpu1102i sites are both made blunt ended).

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The coding region from the clone λ gt10 encoding htie-2 ligand 1 was sequenced using the ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, Calif.). The nucleotide and deduced amino acid sequence of human TIE-2 ligand from 5 the clone λ gt10 encoding htie-2 ligand 1 is shown in FIGS. 4A-4D (SEQ ID NOS: 1 and 2).

In addition, full length human tie-2 ligand cDNA clones were obtained by screening a human glioblastoma T98G TIE-2 ligand were identified by DNA hybridization using a 2.2 kb XhoI fragment from the deposited tie-2 ligand clone (ATCC NO. 75910) as a probe (see Example 6 above). The coding region was sequenced using the ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing 15 Kit (Applied Biosystems, Inc., Foster City, Calif.). This sequence was nearly identical to that of clone λ gt10 encoding htie-2 ligand 1. As shown in FIGS. 4A-4D (SEQ ID NOS: 1 and 2), the clone λ gt10 encoding htie-2 ligand 1 contains an additional glycine residue which is encoded by 20 nucleotides 1114-1116. The coding sequence of the T98G clone does not contain this glycine residue but otherwise is identical to the coding sequence of the clone λ gt10 encoding htie-2 ligand 1. FIGS. 5A-5D (SEQ ID NOS: 3 and 4) sets forth the nucleotide and deduced amino acid sequence of 25 human TIE-2 ligand from the T98G clone.

EXAMPLE 8

ISOLATION AND SEQUENCING OF SECOND FULL LENGTH cDNA CLONE A ENCODING HUMAN TIE-2 LIGAND

A human fetal lung cDNA library in lambda gt-10 (see FIG. 3) was obtained from Clontech Laboratories, Inc. (Palo Alto, Calif.). Plaques were plated at a density of 1.25×10^6 / 20×20 cm plate, and replica filters taken following standard 35 procedures (Sambrook, et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., page 8.46, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). Duplicate filters were screened at low stringency (2×SSC, 55° C.) with probes made to the human TIE-2 ligand 1 sequence. One of 40 the duplicate filters was probed with a 5' probe, encoding amino acids 25-265 of human TIE-2 ligand 1 as set forth in FIGS. 4A-4D (SEQ ID NO: 2). The second duplicate filter was probed with a 3' probe, encoding amino acids 282-498 of human TIE-2 ligand 1 sequence (see FIGS. 4A-4D [SEQ 45 ID NO: 2]). Both probes were hybridized at 55° C. in hybridization solution containing 0.5 mg/ml salmon sperm DNA. Filters were washed in 2×SSC at 55° C. and exposed overnight to X-ray film. In addition, duplicate filters were also hybridized at normal stringency (2×SSC, 65° C.) to the 50 full length coding probe of mouse TIE-2 ligand 1 (F3-15, XhoI insert). Three positive clones were picked that fulfilled the following criteria: i. hybridization had not been seen to the full length (mouse) probe at normal stringency, and ii. hybridization was seen at low stringency to both 5' and 3' 55 probes. EcoRI digestion of phage DNA obtained from these clones indicated two independent clones with insert sizes of approximately 2.2 kb and approximately 1.8 kb. The 2.2 kb EcoRI insert was subcloned into the EcoRI sites of both pBluescript KS (Stratagene) and a mammalian expression 60 vector suitable for use in COS cells. Two orientations were identified for the mammalian expression vector. The 2.2 kb insert in pBluescript KS was deposited with the ATCC on Dec. 9, 1994 and designated as pBluescript KS encoding human TIE 2 ligand 2. The start site of the TIE-2 ligand 2 65 coding sequence is approximately 355 base pairs downstream of the pBluescript EcoRI site.

COS-7 cells were transiently transfected with either the expression vector or control vector by the DEAE-dextran transfection protocol. Briefly, COS-7 cells were plated at a density of 1.0×10⁶ cells/100 mm plate 24 hours prior to transfection. For transfection, the cells were cultured in serum-free DMEM containing 400 μ g/ml of DEAE-dextran, 1 μ M chloroquine, and 2 mM glutamine, and 1 μ g of the appropriate DNA for 3-4 hours at 37° C. in an atmosphere of 5% CO2. The transfection media was aspirated and cDNA library in the pJFE14 vector. Clones encoding human 10 replaced with phosphate-buffered saline with 10% DMSO for 2-3 min. Following this DMSO "shock", the COS-7 cells were placed into DMEM with 10% FBS, 1% each of penicillin and streptomycin, and 2 mM glutamine for 48 hours

> Because the TIE-2 ligand is secreted it was necessary to permeabilize the cells to detect binding of the receptorbody probe to the ligand. Transfected COS-7 cells were plated at a density of 1.0×10^6 cells/100 mm plate. The cells were rinsed with PBS and then incubated with PBS containing 1.8% formaldehyde for 15-30 min. at room temperature. Cells were then washed with PBS and incubated for 15 min. with PBS containing 0.1% Triton X-100 and 10% Bovine Calf Serum to permeabilize the cells and block non-specific binding sites. The screening was conducted by direct localization of staining using a TIE-2 receptorbody, which consisted of the extracellular domain of TIE-2 fused to the IgG1 constant region. This receptorbody was prepared as set forth in Example 2. Transfected COS cells were probed by incubating them for 30 min with TIE-2 receptorbody. The cells were then washed twice with PBS, fixed with methanol, and then incubated for an additional 30 min with PBS/10% Bovine Calf Serum/anti-human IgG-alkaline phosphatase conjugate. After three PBS washes, cells were incubated in alkaline-phosphatase substrate for 30-60 min. The dish was then inspected microscopically for the presence of stained cells. Cells expressing one orientation of the clone, but not the other orientation, were seen to bind the TIE-2 receptorbody.

> One of skill in the art will readily see that the described methods may be used to further identify other related members of the TIE ligand family.

> The coding region from the clone pBluescript KS encoding human TIE-2 ligand 2 was sequenced using the ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, Calif.). The nucleotide and deduced amino acid sequence of human TIE-2 ligand from the clone pBluescript KS encoding human TIE-2 ligand 2 is shown in FIGS. 6A-6D (SEQ ID NOS: 5 and 6).

EXAMPLE 9

TIE-2 LIGAND 2 IS A RECEPTOR ANTAGONIST

Conditioned media from COS cells expressing either TIE-2 ligand 2 (TL2) or TIE-2 ligand 1 (TL1) were compared for their ability to activate TIE-2 receptors naturally present in human endothelial cell lines.

Lipofectamine reagent (GIBCO-BRL, Inc.) and recommended protocols were used to transfect COS-7 cells with either the pJFE14 expression vector alone, pJFE14 vector containing the human TIE-2 ligand 1 cDNA, or with a pMT21 expression vector (Kaufman, R. J., 1985, Proc. Natl. Acad. Sci. USA 82: 689-693) containing the human TIE-2 ligand 2 cDNA. COS media containing secreted ligands were harvested after three days and concentrated 20-fold by

diafiltration (DIAFLO ultrafiltration membranes, Amicon, Inc.). The quantity of active TIE-2 ligand 1 and TIE-2 ligand 2 present in these media was determined and expressed as the amount (in resonance units, R.U.) of TIE-2 receptor specific binding activity measured by a BIAcore binding ₅ assay.

Northern (RNA) analyses revealed significant levels of TIE-2 transcripts in HAEC (Human Aortic Endothelial Cell) human primary endothelial cells (Clonetics, Inc.). Therefore, these cells were used to examine whether TIE-2 receptor is tyrosine-phosphorylated when exposed to COS media containing the TIE-2 ligands. HAEC cells were maintained in a complete endothelial cell growth medium (Clonetics, Inc.) that contained 5% fetal bovine serum, soluble bovine brain extract, 10 ng/ml human EGF, 1 mg/ml hydrocortisone, 50 mg/ml gentamicin and 50 ng/ml amphotericin-B. Assessment of whether TL1 and TL2 could activate TIE-2 receptor in the HAEC cells was done as follows. Semi-confluent HAEC cells were serum-starved for two hours in highglucose Dulbecco's MEM with added L-glutamine and penicillin-streptomycin at 37° C. followed by replacement of the starvation medium with ligand-containing conditioned COS media for 7 minutes at 37° C. in a 5% CO₂ incubator. The cells were subsequently lysed and TIE-2 receptor protein was recovered by immunoprecipitation of 25 the lysates with TIE-2 peptide antiserum, followed by Western blotting with antiphosphotyrosine antiserum, exactly as described in example 1. The results are shown in FIG. 7. Phosphotyrosine levels on the TIE-2 receptor (TIE-2-R) were induced by treatment of HEAC cells with TIE-2 ligand 30 1 (Lane L1) but not by TIE-2 ligand 2 (Lane L2) conditioned COS media. MOCK is conditioned media from COS transfected with JFE14 empty vector.

Evidence that both TL1 and TL2 specifically bind to the TIE-2 receptor was demonstrated by using a BlAcore to ₃₅ assay the TIE-2 receptor specific binding activities in transfected COS media and by immunostaining of TL1- and TL2-expressing COS cells with TIE-2 receptorbodies.

Because TL2 did not activate the TIE-2 receptor, applicants set out to determine whether TL2 might be capable of 40 serving as an antagonist of TL1 activity. HAEC phosphorylation assays were performed in which cells were first incubated with an "excess" of TL2, followed by addition of dilute TL1. It was reascned that prior occupancy of TIE-2 receptor due to high levels of TL2 might prevent subsequent 45 stimulation of the receptor allowing exposure to TL1 present at a limiting concentration.

Semi-confluent HAEC cells were serum-starved as described above and then incubated for 3 min., at 37° C. with 1-2 ml. of 20×COS/JFE14-TL2 conditioned medium. 50 Control plates were treated with 20×COS/JFE14-only medium (MOCK). The plates were removed from the incubator and various dilutions of COS/JFE14-TL1 medium were then added, followed by further incubation of the plates for 5–7 min. at 37° C. Cells were subsequently rinsed, lysed 55 and TIE-2-specific tyrosine phosphorylation in the lysates was examined by receptor immunoprecipitation and Western blotting, as described above. TL1 dilutions were made using 20×COS/JFE14-TL1 medium diluted to 2×, 0.5×, 0.1×, or 0.02× by addition of 20×COS/JFE14-alone medium. An 60 assay of the initial 20x TL1 and 20xTL2 COS media using BlAcore biosensor technology indicated that they contained similar amounts of TIE-2-specific binding activities, i.e., 445 R.U. and 511 R.U. for TL1 and TL2, respectively. The results of the antiphosphotyrosine Western blot, shown in 65 FIG. 8, indicate that when compared to prior treatment of HAEC cells with MOCK medium (lane 1), prior treatment

of HAEC cells with excess TIE-2 ligand 2 (lane 2) antagonizes the subsequent ability of dilute TIE-2 ligand 1 to activate the TIE-2 receptor (TIE-2-R).

The ability of TL2 to competitively inhibit TL1 activation of the TIE-2-R was further demonstrated using the human cell hybrid line, EA.hy926 (see Example 21 for detailed description of this cell line and its maintenance). Experiments were performed in which unconcentrated COS cell media containing TL1 were mixed at varying dilutions with either MOCK- or TL2-conditioned media and placed on serum-starved EA.hy926 cell monolayers for 5 minutes at 37° C. The media were then removed, the cells were harvested by lysis and TIE-2-specific tyrosine phosphorylation was examined by Western blots, as described above. 15 FIG. 9 shows an experiment which contains three groups of treatments, as viewed from left to right. As shown in the four lanes at the left, treatment of the EA.hy926 cells with 1×COS-TL1 alone robustly activated the endogenous TIE-2-R in these cells, whereas 1×TL2 COS medium was 20 inactive. However, mixture of TL1 with either MOCK or TL2 demonstrated that TL2 can block the activity of TL1 in a dose-dependent fashion. In the central three pairs of lanes the ratio of TL2 (or MOCK) was decreased while the amount of TL1 in the mixture was correspondingly increased from $0.1 \times$ to $0.3 \times$. At any of these mixture ratios the TL1:TL2 lanes showed a reduced level of TIE-2-R phosphorylation compared to that of the corresponding TL1:MOCK lanes. When the amount TL1 was held steady and the amount of TL2 (or MOCK) was decreased, however (shown in the three pairs of lanes at the right), a point was reached at which the TL2 in the sample was too dilute to effectively inhibit TL1 activity. The relative amount of each ligand present in these conditioned COS media could be estimated from their binding units as measured by the BIAcore assay and from Western blots of the COS media with ligand-specific antibodies. Consequently, we can infer that only a few-fold molar excess of TL2 is required to effectively block the activity of TL1 in vitro. This is significant because we have observed distinct examples in vivo (see Example 17 and FIG. 16) where TL2 mRNAs achieve considerable abundance relative to those of TL1. Thus, TL2 may be serving an important physiological role in effectively blocking signaling by the TIE-2-R at these sites.

Taken together these data confirm that, unlike TL1, TL2 is unable to stimulate endogenously expressed TIE-2-R on endothelial cells. Furthermore, at a few fold molar excess TL2 can block TL1 stimulation of the TIE-2 receptor, indicating that TL2 is a naturally occurring TIE-2 receptor antagonist.

EXAMPLE 10

IDENTIFICATION OF TIE-2-SPECIFIC BINDING ACTIVITY IN CONDITIONED MEDIUM AND COS CELL SUPERNATANTS

Binding activity of 10×CCM from the cell lines C2C12ras, Rat2 ras, SHEP, and T98G, or COS cell supernatants after transfection with either human TIE-2 ligand 1 (hTL1) or human TIE-2 ligand 2 (hTL2) was measured using biosensor technology (BIAcore; Pharmacia Biosensor, Piscataway, N.J.) which monitors biomolecular interactions in real-time via surface plasmon resonance (SPR). Purified rat or human TIE-2 RB was covalently coupled through primary amines to the carboxymethyl dextran layer of a CM5 research grade sensor chip (Pharmacia Biosensor; Piscataway, N.J.). The sensor chip surface was activated using a mixture of N-hydroxysuccinimide (NHS) and

N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide (EDC), followed by immobilization of TIE-2 RB (25 µg/mL, pH 4.5) and deactivation of unreacted sites with 1.0 M ethanolamine (pH 8.5). In general, 9000-10000 RU of each receptorbody was coupled to the sensor chip.

The running buffer used in the system was HBS (10 mM Hepes, 150 mM NaCl, 0.005% P20 surfactant, pH 7.4). The samples were centrifuged for 15 min at 4° C. and further clarified using a sterile, low protein binding 0.45 μ m filter (Millipore; Bedford, Mass.). Dextran (2 mg/ml and P20 surfactant (0.005%) were added to each sample. Aliquots of $40 \,\mu\text{L}$ were injected across the immobilized surface (either rat or human TIE-2) at a flow rate of 5 μ L/min and the receptor binding was monitored for 8 min. The binding activity (resonance units, RU) was measured as the difference between a baseline value determined 30 s prior to the sample injection and a measurement taken at 30 s postinjection. Regeneration of the surface was accomplished with one $15-\mu L$ pulse of 3 M MgCl₂.

were tested on the rat TIE-2 RB immobilized surface, while the recombinant hTL1 and hTL2 were tested on the human TIE-2 RB immobilized surface. In each case, specific binding to the TIE-2 receptorbody was evaluated by incubating 25 the samples with 25 μ g/ml of either soluble TIE-2 (rat or human) RB or trkB RB prior to assaying the binding activity. As shown in FIGS. 10A-10D and FIGS. 11A-11B, the addition of soluble trkB RB causes a slight decrease in the TIE-2 binding activity, while the addition of soluble TIE-2 30 RB significantly reduces the binding activity as compared to that measured in the absence of TIE-2 RB.

EXAMPLE 11

TIE-2 RB SPECIFICALLY BLOCKS ACTIVATION OF THE TIE-2 RECEPTOR BY TIE-2 LIGAND 1

The applicants sought to determine whether soluble TIE-2 of TIE-2 receptor by TIE-2 ligand 1 (TL1). To do this, TL1-containing COS media were preincubated with either TIE-2- or TrkB-RB and then compared for their ability to activate TIE-2 receptors naturally present in a human endothelial cell line.

Conditioned COS media were generated from COS-7 cells transfected with either the pJFE14 expression vector alone (MOCK), or pJFE14 vector containing the human TIE-2 ligand 1 cDNA (TL1) and harvested as described in Example 9 hereinabove, with the exception that the media 50 5b1-4 may be used. DNA enoding the TL2-Fc was cloned as were sterile filtered but not concentrated. The quantity of TL1 was determined and expressed as the amount (in resonance units, R.U.) of TIE-2 receptor-specific binding activity measured by BIAcore binding assay.

tie-2 transcripts in HUVEC (Human Umbilical Vein Endothelial Cell) human primary endothelial cells (Clonetics, Inc.). Therefore, these cells were used to examine whether TIE-2 receptor can be tyrosine-phosphorylated when exposed in the presence of TIE-2- or TrkB-RBs to COS 60 media containing TL1. HUVEC cells were maintained at 37° C., 5% CO₂ in a complete endothelial cell growth medium (Clonetics, Inc.) that contained 5% fetal bovine serum, soluble bovine brain extract with 10 µg/ml heparin, 10 ng/ml human EGF, 1 ug/ml hydrocortisone, 50 µg/ml gentamicin 65 and 50 ng/ml amphotericin-B. Assessment of whether TL1 could activate TIE-2 receptor in the HUVEC cells was done

as follows. Confluent dishes of HUVEC cells were serumstarved for two-to-four hours in low-glucose Dulbecco's MEM at 37° C., 5% CO_2 , followed by 10 minute incubation in starvation medium that included 0.1 mM sodium 5 orthovanadate, a potent inhibitor of phosphotyrosine phosphatases. Meanwhile, conditioned COS media were preincubated 30 min. at room temperature with either TIE-2- or TrkB-RB added to 50 μ g/ml. The starvation medium was then removed from the HUVEC dishes and incubated with the RB-containing COS media for 7 minutes at 37° C. 10 HUVEC cells were subsequently lysed and TIE-2 receptor protein was recovered by immunoprecipitation with TIE-2 peptide antiserum, followed by Western blotting with an anti-phosphotyrosine antibody, as described in Example 1. The results are shown in FIG. 12. Phosphotyrosine levels on the TIE-2 receptor were induced by treatment of HUVEC cells with TIE-2 ligand 1 (TL1) relative to that seen with control medium (MOCK) and this induction is specifically blocked by prior incubation with TIE-2-RB (TIE-2-Fc) but The CCM samples (C2C12-ras, Rat2-ras, SHEP, T98G)²⁰ not by incubation with TrkB-RB (TrkB-Fc). These data indicate that soluble TIE-2 RB can serve as a selective inhibitor to block activation of TIE-2 receptor by TIE-2 ligand 1.

EXAMPLE 12

CONSTRUCTION OF TIE-2 LIGANDBODIES

An expression construct was created that would yield a secreted protein consisting of the entire coding sequence of human TIE-2 ligand 1 (TL1) or TIE-2 ligand 2 (TL2) fused to the human immunoglobulin gamma-1 constant region (IgG1 Fc). These fusion proteins are called TIE-2 "ligandbodies" (TL1-Fc or TL2-Fc). The Fc portion of TL1-Fc and TL2-Fc was prepared as follows. A DNA fragment encoding the Fc portion of human IgG1 that spans from the hinge region to the carboxy-terminus of the protein, was amplified from human placental cDNA by PCR with oligonucleotides corresponding to the published sequence of human IgG1; the resulting DNA fragment was cloned in a plasmid vector. RB can serve as a competitive inhibitor to block activation 40 Appropriate DNA restriction fragments from a plasmid encoding full-length TL1 or TL2 and from the human IgG1 Fc plasmid were ligated on either side of a short PCRderived fragment that was designed so as to fuse, in-frame, TL1 or TL2 with human IgG1 Fc protein-coding sequences.

Milligram quantities of TL2-Fc were obtained by cloning the TL2-Fc DNA fragment into the pVL1393 baculovirus vector and subsequently infecting the Spodoptera frugiperda SF-21AE insect cell line. Alternatively, the cell line SF9 (ATCC Accessuion No. CRL-1711) or the cell line BTI-TNan Eco RI-NotI fragment into the baculovirus transfer plasmid pVL1393. Plasmid DNA was recombined into viral DNA by mixing 3 μ g of plasmid DNA with 0.5 μ g of baculo-Gold DNA (Pharminigen), followed by introduction Northern (RNA) analyses revealed significant levels of 55 into liposomes using 30 µg Lipofectin (GIBCO-BRL). DNA-liposome mixtures were added to SF-21AE cells 92×106 cells/60 mm dish) in TMN-FH medim (Modified Grace's Insect Cell Nedium (GIBCO-BRL) for 5 hours at 27° C., followed by incubation at 27° C. for 5 days in TMN-FH meduim supplemented with 5% fatal calf serum. Tissue culture medium was harvested for plaque purification of recombinant viruses, which was carried out using methods previously described (O'Reilly, D. R., L. K. Miller, and V. A. Luckow, Baculovirus Expression Vectors-A Laboratory Manual. 1992, New York: W. H. Freeman) except that the agarose overlay contained 125 mg/mL X-gal (5-bromo-4-chloro-3-indolyl-b- D-galactopyranoside; GIBCO-BRL).

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After 5 days of incubation at 27° C., non-recombinant plaques were scored by positive chromogenic reacton to the X-gal substrate, and their position marked. Recombinant plaques were then visualized by addition of a second overlay containing 100 mg/mL MTT (3-[4,5-dimethylthiazol-2yl]2, 5, diphenyltetrazolium bromide; Sigma). Putative recombinant virus plaques were picked by plug aspiration, and purified by multiple rounds of plaque isolation to assure homoeneity. Virus stocks were generated by serial, low-miltiplicity passage of plaque-purified virus. Low passage stocks of one virus clone (vTL2-Fc Clone #7) were produced.

SF-21AE cells were cultured in serum-free medium (SF-900 II, Gibco BRL) containing 1×antibiotic/antimycotic solution (Gibco BRL) and 25 mg/L Gentamycin (Gibco 15 BRL). Pluronic F-68 was added as a surfactant to a final concentration of 1 g/L. Cultures (4 L) were raised in a bioreactor (Artisan Cell Station System) for at least three days prior to infection. Cells were grown at 27° C., with gassing to 50% dissolved oxygen, at a gas flow rate of 80 $\,^{20}$ mL/min (aeration at a sparge ring). Agitation was by means of a marine impeller at a rate of 100 rpm. Cells were harvested in mid-logarithmic growth phase (~2×10 6 cells/ mL), concentrated by centrifugation, and infected with 5 plaque forming units of vTL2-Fc per cell. Cells and inocu- 25 stromal cells (FIG. 14). lum were brought to 400 mL with fresh medium, and virus was adsorbed for 2 hours at 27° C. in a spinner flask. The culture was then resuspended in a final volume of 8 L with fresh serum-free medium, and the cells incubated in the 30 bioreactor using the previously described conditions.

Culture medium from vTL2-Fc-infected SF21AE cells were collected by centrifugation (500×g, 10 minutes) at 72 hours post-infection. Cell supernatants were brought to pH 8 with NaOH. EDTA was added to a final concentration of 35 10 mM and the supernatant pH was readjusted to 8. Supernatants were filtered (0.45 μ m, Millipore) and loaded on a protein A column (protein A sepharose 4 fast flow or HiTrap protein A, both from Pharmacia). The column was washed with PBS containing 0.5 M NaCl until the absorbance at 280 40 nm decreased to baseline. The column was washed in PBS and eluted with 0.5 M acetic acid. Column fractions were immediately neutralized by eluting into tubes containing 1 M Tris pH 9. The peak fractions containing the TL2-Fc were pooled and dialyzed versus

EXAMPLE 13

EXPRESSION OF TIE-1, TIE-2, TL1, AND TL2 IN RENAL CELL CARCINOMA

In situ hybridization experiments were performed on human renal cell carcinoma tumor tissue using TIE-1, TIE-2, TL1, and TL2 cDNA probes. TIE-2, TIE-1, TL1, and TL2 expression were all up-regulated in the tumor vasculature. Ligand expression appeared to be localized to either the 55 vascular endothelial cells (TL2) or very near the vascular endothelial cells in the mesenchyme (TL1). VEGF has been shown to be dramatically up-regulated in this tumor tissue. Brown, et al. Am. J. Pathol. 143:1255–1262 (1993).

EXAMPLE 14

EXPRESSION OF TIE-1, TIE-2, TL1, AND TL2 IN WOUND HEALING

In situ hybridization experiments were performed on 65 cross-sectional tissue slices obtained from a rat cutaneous wound model using TIE-1, TIE-2, TL1, and TL2 cDNA

probes. The wound healing model involves pressing a small cork bore against the skin of a rat and removing a small, cylindrical plug of skin. As healing begins at the base of the wound, a vertical slice of tissue is taken and used for in situ hybridization. In the tested tissue sample, TL1 and TL2 appeared to be slightly up-regulated by four days postinjury. In contrast to the slightly up-regulated expression of TL1 and TL2 in this tissue, VEGF expression, which may precede TL1 and TL2 expression, is dramatically up-regulated.

EXAMPLE 15

EXPRESSION OF TIE LIGANDS IN FETAL LIVER AND THYMUS

Reverse transcription-PCR (RT-PCR) was performed on mouse E14.5 fetal liver and mouse E17.5 fetal thymus. Agarose gel electrophoresis of the RT-PCR products revealed that in the mouse fetal liver, TIE-2 ligand 1 (TL1) RNA is enriched in the stromal region, but is absent in c-kit⁺TER119 hematopoietic precursor cells. In this same tissue, TIE-2 ligand 2 (TL2) RNA is enriched in the stromal cells, but absent in the hematopoietic precursor cells (FIG. **13**). In the mouse fetal thymus, TL2 is enriched in the stromal cells (FIG. **14**).

EXAMPLE 16

THE TIE RECEPTOR/LIGAND SYSTEM IN ANGIOGENESIS

Although the TIE-2/TIE ligand system appears to play an important role in endothelial cell biology, it has not been shown to play a significant, active role in the early to intermediate stages of vascularization (e.g. angioblast or endothelial cell proliferation and migration, tubule formation, and other early stage events in vascular modeling). In contrast to the receptors and factors known to mediate these aspects of vascular development, the temporally late pattern of expression of TIE-2 and TL1 in the course of vascularization suggests that this system plays a distinct role in the latter stages vascular development, including the structural and functional differentiation and stabilization of new blood vessels. The pattern of expression of TIE-2/TL1 also is consistent with a continuing role in the maintenance of the structural integrity and/or physiological ⁴⁵ characteristics of an established vasculature.

TIE Ligand 2 (TL2) appears to be a competitive inhibitor of TL1. The spatiotemporal characteristics of TL2 expression suggest that this single inhibitory molecule may play multiple, context-dependent roles essential to appropriate vascular development or remodeling (e.g. de-stabilization/ de-differentiation of mature endothelial cells allowing the formation of new vessels from existing vasculature, inhibition of inappropriate blood vessel formation, and regression/ involution of mature blood vessels). FIG. **15** is a schematic representation of the hypothesized role of the TIE-2/TIE ligands in angiogenesis. In this figure TL1 is represented by (\bigcirc), TL2 is represented by (*), TIE-2 is represented by (T), VEGF is represented by ([]), and flk-1 (a VEGF receptor) is represented by (Y).

EXAMPLE 17

EXPRESSION OF TIE LIGANDS IN THE FEMALE REPRODUCTIVE SYSTEM: EXPRESSION IN THE OVARY

Preliminary observations made in experiments examining the expression of the TIE receptors and ligands in the female

reproductive system are consistent with the hypothesis the TL1 plays a role in neovascularization which temporally follows that of VEGF. The pattern of TL2 expression is also consistent with an antagonism of the action of TL1, and a specific role in vascular regression. To verify this, expression of relevant mRNAs can be examined following experimental induction of follicular and luteal development so that their temporal relation to various aspects of neovascularization/vascular regression can be more clearly defined (e.g. in conjunction with endothelial cell staining, vascular fills). Angiogenesis associated with follicular development and corpus luteum formation in staged ovaries of mature, female rats or following induced ovulation in pre-pubertal animals was followed using in situ hybridization. FIG. 16 contains photographs of in situ hybridization slides showing the temporal expression pattern of TIE-2, 15 TL1, TL2, and VEGF during the ovarian cycle [Column 1: Early pre-ovulatory follicle; Column 2: pre-ovulatory follicle; Column 3: early corpus luteum; and Column 4: atretic follicle; Row A:bright field; Row B:VEGF; Row C: TL2; revealed that VEGF, TL1 and TL2 are expressed in a temporally and spatially coordinate fashion with respect to the development and regression of vasculature in the ovary, specifically with respect to the establishment of the vascular an ovarian follicle to a corpus luteum (CL).

Briefly, VEGF expression increases in the follicular granule layer prior to its vascularization during the process of luteinization. During the process of CL formation, highest levels of VEGF expression are apparent in the center of the developing CL in the vicinity of luteinizing cells which are not yet vascularized. VEGF levels remain moderately high and are diffusely distributed in the developed CL. In contrast, noticeably enhanced expression of TIE-2 ligand 1 occurs only late in process of CL formation, after a primary vascular plexus has been established. Later, TL1 expression 35 is apparent throughout the CL at which time the definitive capillary network of the CL has been established.

TL2 exhibits a more complex pattern of expression than either VEGF or TL1. In the developing CL, TL2 is expressed at highest levels at the front of the developing capillary 40 plexus-between the central avascular region of the CL where VEGF expression is highest, and the most peripheral portion of the CL where TL1 expression is dominant and where the luteinization process is complete and the vascular system is most mature. TL2 also appears to be expressed at high levels 45 in the follicular layer of large follicles which are undergoing atresia. While TL1 is also apparent in atretic follicles, VEGF is not expressed.

The pattern of expression described above is most consistent with a role for VEGF in the initiation of angiogenesis, $_{50}$ with TL1 acting late in this process-for example in modeling and/or stabilization of the definitive vascular network. In contrast, TL2 is present both in areas of active expansion of a newly forming vascular network (during CL formation), and in regions which fail to establish a new vasculature and 55 vascular regression is in progress (atretic follicles). This suggests a more dynamic and complex role for TL2, possibly involving destabilization of existing vasculature (necessary for regression) or developing vasculature (necessary for the dynamic modeling of newly forming 60 vessels).

EXAMPLE 18

A RECEPTORBODY BINDING ASSAY AND A LIGAND BINDING AND COMPETITION ASSAY

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A quantitative cell-free binding assay with two alternate formats has been developed for detecting either TIE-2 38

receptorbody binding or ligand binding and competition. In the receptorbody binding version of the assay, TIE-2 ligands (purified or partially purified; either TL1 or TL2) are coated onto an ELISA plate. Receptorbody at varying concentrations is then added, which binds to the immobilized ligand in a dose-dependent manner. At the end of 2 hours, excess receptorbody is washed away, then the amount bound to the plate is reported using a specific anti-human Fc antibody which is alkaline phosphatase tagged. Excess reporter antibody is washed away, then the AP reaction is developed using a colored substrate. The assay is quantitated using a spectrophotometer. FIG. 19 shows a typical TIE-2-IgG binding curve. This assay has been used to evaluate the integrity of TIE-2-IgG after injection into rats and mice. The assay can also be used in this format as a ligand competition assay, in which purified or partially-purified TIE ligands compete with immobilized ligand for receptorbody. In the ligand binding and competition version of the binding assay, TIE-2 ectodomain is coated onto the ELISA plate. The Row D: TL1 and Row E: TIE-2 receptor]. These studies 20 Fc-tagged fibrinogen-like domain fragments of the TIE ligands (TL1-fFc and TL2-fFc) then bind to the ectodomain, and can be detected using the same anti-human Fc antibody as described above. FIG. 20 shows an example of TL1-fFc binding to TIE-2 ectodomain. This version of the assay can system which is generated in the course of the conversion of 25 also be used to quantitate levels of TL1-fFc in serum or other samples. If untagged ligand (again, either purified or unpurified) is added at the same time as the TL1-fFc, then a competition is set up between tagged ligand fragment and full-length ligand. The full-length ligand can displace the 30 Fc-tagged fragment, and a competition curve is generated.

EXAMPLE 19

EA.hv926 CELL LINE CAN BE USED AS A REPORTER CELL LINE FOR TIE LIGAND ACTIVITY

EA.hy926 is a cell hybrid line that was established by fusion of HUVEC with the human lung carcinoma-derived line, A549 [Edgell, et al. Proc. Natl. Acad. Sci. (USA) 80, 3734-3737 (1983). EA.hy926 cells have been found to express significant levels of TIE-2 receptor protein with low basal phosphotyrosine levels. The density at which EA.hy926 cells are passaged prior to their use for receptor assays, as well as their degree of confluency at the time of assay, can affect TIE-2 receptor abundance and relative inducibility in response to treatment with ligand. By adopting the following regimen for growing these cells the EA.hy926 cell line can be used as a dependable system for assay of TIE-2 ligand activities.

EA.hy926 cells are seeded at 1.5×10^6 cells in T-75 flasks (Falconware) and re-fed every other day with high-glucose Dulbecco's MEM, 10% fetal bovine serum, L-glutamine, penicillin-streptomycin, and 1×hypoxanthine-aminopterinthymidine (HAT, Gibco/BRL). After three to four days of growth, the cells are passaged once again at 1.5×10^6 cells per T-75 flask and cultured an additional three to four days. For phosphorylation assays, cells prepared as described above were serum-starved by replacement of the culture medium with high-glucose DMEM and incubation for 2-3 hours at 37° C. This medium was aspirated from the flask and samples of conditioned media or purified ligand were added to the flask in a total volume of 1.5 ml followed by incubation at 37° C. for 5 minutes. Flasks were removed from the incubator and placed on a bed of ice. The medium was removed and replaced with 1.25 ml Lysis Buffer containing 1% nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS in 20 mM Tris, pH 7.6, 150 mM NaCl, 50 mM NaF, 1

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mM sodium orthovanadate, 5 mM benzamidine, and 1 mM EDTA containing the protease inhibitors PMSF, aprotinin, and leupeptin. After 10 minutes on ice to allow membrane solubilization, plates were scraped and cell lysates were clarified by microcentrifugation at top speed for 10 minutes at 4° C. TIE-2 receptor was immunoprecipitated from the clarified supernatant by incubation in the cold with an anti-TIE-2 polyclonal antiserum and Protein G-conjugated Sepharose beads. The beads were washed three times with cold cell lysis buffer and boiled 5 minutes in Laemmli sample buffer, which was then loaded on 7.5% SDSpolyacrylamide gels. Resolved proteins were electrotransferred to PVDF (Lamblia-P) membrane and then subjected to Western blot analysis using anti-phosphotyrosine antibody and the ECL reagent. Subsequent comparison of total TIE-2 protein levels on the same blots was done by stripping the anti-phosphotyrosine antibody and reincubating with a polyclonal antiserum specific to the ectodomain of TIE-2.

EXAMPLE 20

ISOLATION AND SEQUENCING OF FULL LENGTH cDNA CLONE ENCODING MAMMALIAN TIE LIGAND-3

TIE ligand-3 (TL3) was cloned from a mouse BAC genomic library (Research Genetics) by hybridizing library duplicates, with either mouse TL1 or mouse TL2 probes corresponding to the entire coding sequence of those genes. Each copy of the library was hybridized using phosphate buffer at 55° C. overnight. After hybridization, the filters were washed using 2×SSC, 0.1% SDS at 60° C., followed by $_{30}$ exposure of X ray film to the filters. Strong hybridization signals were identified corresponding to mouse TL1 and mouse TL2. In addition, signals were identified which weakly hybridized to both mouse TL1 and mouse TL2. DNA corresponding to these clones was purified, then digested with restriction enzymes, and two fragments which hybridized to the original probes were subcloned into a bacterial plasmid and sequenced. The sequence of the fragments contained two exons with homology to both mouse TL1 and mouse TL2. Primers specific for these sequences were used as PCR primers to identify tissues containing transcripts corresponding to TL3. A PCR band corresponding to TL3 was identified in a mouse uterus cDNA library in lambda gt-11. (Clontech Laboratories, Inc., Palo Alto, Calif.).

Plaques were plated at a density of $1.25 \times 10^6 / 20 \times 20$ cm $_{45}$ plate and replica filters taken following standard procedures (Sambrook, et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., page 8.46, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). Duplicate filters were screened at radioactive probe made to the mouse TL3 sequence. Hybridization was at 65° C. in a solution containing 0.5 mg/ml salmon sperm DNA. Filters were washed in 2×SSC at 65° C. and exposed for 6 hours to X-ray film. Two positive clones that hybridized in duplicate were picked. EcoRI digestion of 55 phage DNA obtained from these clones indicated two independent clones with insert sizes of approximately 1.2 kb and approximately 2.2 kb. The 2.2 kb EcoRI insert was subcloned into the EcoRI site of pBluescript KS (Stratagene). Sequence analysis showed that the longer clone was lacking 60 an initiator methionine and signal peptide but otherwise encoded a probe homologous to both mouse TL1 and mouse TL2.

Two TL3-specific PCR primers were then synthesised as follows:

US2: cctctgggctcgccagtttgttagg (SEQ ID NO: 29)

US1: ccagctggcagatatcagg (SEQ ID NO: 30)

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The following PCR reactions were performed using expression libraries derived from the mouse cell lines C2C12ras and MG87. In the primary PCR reaction, the specific primer US2 was used in conjunction with vectorspecific oligos to allow amplification in either orientation. PCR was in a total volume of 100 ml using 35 cycles of 94° C., 1 min; 42° C. or 48° C. for 1 min; 72° C., 1 min. The secondary PCR reaction included the second specific primer, US1, which is contained within the primary PCR product, in conjunction with the same vector oligos. The secondary reactions were for 30 cycles, using the same temperatures and times as previous. PCR products were gel isolated and submitted for sequence analysis. On the basis of sequences obtained from a total of four independent PCR reactions using two different cDNA libraries, the 5' end of the TL3 sequence was deduced. Northern analysis revealed moderate to low levels of mouse TL3 transcript in mouse placenta. The expression of mouse TL3 consisted of a transcript of approximately 3 kb. The full length TL3 coding sequence is 20 set forth in FIGS. 21A-21C (SEQ ID NOS: 9 and 10).

The mouse TL3 sequence may then be used to obtain a human clone containing the coding sequence of human TL3 by hybridizing either a human genomic or cDNA library with a probe corresponding to mouse TL3 as has been 25 described previously, for example, in Example 8 supra.

EXAMPLE 21

ISOLATION OF FULL LENGTH GENOMIC **CLONE ENCODING HUMAN TIE LIGAND-4**

TIE ligand-4 (TL4) was cloned from a mouse BAC genomic library (BAC HUMAN (II), Genome Systems Inc.) by hybridizing library duplicates, with either a human TL1 35 radioactive probe corresponding to the entire fibrinogen coding sequence of TL1 (nucleotides 1153 to 1806 of FIGS. 4A-4D [SEQ ID NO: 1]) or a mouse TL3 radioactive probe corresponding to a segment of 186 nucleotides from the fibrinogen region of mouse TL3 (nucleotides 1307 to 1492 40 of FIGS. 21A-21C [SEQ ID NO: 9]). Each probe was labeled by PCR using exact oligonucleotides and standard PCR conditions, except that dCTP was replaced by P³²dCTP. The PCR mixture was then passed through a gel filtration column to separate the probe from free P^{32} dCTP. Each copy of the library was hybridized using phosphate buffer, and radioactive probe at 55° C. overnight using standard hybridization conditions. After hybridization, the filters were washed using 2×SSC, 0.1% SDS at 55° C., followed by exposure of X ray film. Strong hybridization "normal" stringency (2×SSC, 65° C.) with a 200 bp PCR 50 signals were observed corresponding to human TL1. In addition, signals were identified which weakly hybridized to both human TL1 and mouse TL3. DNA corresponding to these clones was purified using standard procedures, then digested with restriction enzymes, and one fragment which hybridized to the original probes was subcloned into a bacterial plasmid and sequenced. The sequence of the fragments contained one exon with homology to both human TL1 and mouse TL3 and other members of the TIE ligand family. Primers specific for these sequences may be used as PCR primers to identify tissues containing transcripts corresponding to TL4.

> The complete sequence of human TL4 may be obtained by sequencing the full BAC clone contained in the deposited bacterial cells. Exons may be identified by homology to known members of the TIE-ligand family such as TL1, TL2 and TL3. The full coding sequence of TL4 may then be determined by splicing together the exons from the TL4

genomic clone which, in turn, may be used to produce the TL4 protein. Alternatively, the exons may be used as probes to obtain a full length cDNA clone, which may then be used to produce the TL4 protein. Exons may also be identified from the BAC clone sequence by homology to protein 5 domains such as fibrinogen domains, coiled coil domains, or protein signals such as signal peptide sequences. Missing exons from the BAC clone may be obtained by identification of contiguous BAC clones, for example, by using the ends of the deposited BAG clone as probes to screen a human 10 genomic library such as the one used herein, by using the exon sequence contained in the BAG clone to screen a cDNA library, or by performing either 5' or 3' RACE procedure using oligonucleotide primers based on the TL4 exon sequences.

Identification of Additional TIE Ligand Family Members

The novel TIE ligand-4 sequence may be used in a 20 rational search for additional members of the TIE ligand family using an approach that takes advantage of the existence of conserved segments of strong homology between the known family members. For example, an alignment of the amino acid sequences of the TIE ligands shows several 25 regions of conserved sequence (see boxed regions of FIGS. 22A-22B [SEQ ID NOS: 11, 12, 13, 14, 15, and 16]). Degenerate oligonucleotides essentially based on these boxes in combination with either previously known or novel TIE ligand homology segments may be used to identify new 30 TIE ligands.

The highly conserved regions among TL1, TL2 and TL3 may be used in designing degenerate oligonucleotide primers with which to prime PCR reactions using cDNAs. cDNA templates may be generated by reverse transcription of tissue RNAs using oligo d(T) or other appropriate primers. Aliquots of the PCR reactions may then be subjected to electrophoresis on an agarose gel. Resulting amplified DNA fragments may be cloned by insertion into plasmids, sequenced and the DNA sequences compared with those of all known TIE ligands.

Size-selected amplified DNA fragments from these PCR reactions may be cloned into plasmids, introduced into E. coli by electroporation, and transformants plated on selective agar. Bacterial colonies from PCR transformation may 45 to produce in large quantities. Finally, production and puribe analyzed by sequencing of plasmid DNAs that are purified by standard plasmid procedures.

Cloned fragments containing a segment of a novel TIE ligand may be used as hybridization probes to obtain full length cDNA clones from a cDNA library. For example, the 50 constructed as follows. human TL4 genomic sequence may be used to obtain a human cDNA clone containing the complete coding sequence of human TL4 by hybridizing a human cDNA library with a probe corresponding to human TL4 as has been described previously.

EXAMPLE 22

CLONING OF THE FULL CODING SEQUENCE OF hTL4

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Both 5' and 3' coding sequence from the genomic human TL-4 clone encoding human TIE ligand-4 (hTL-4 ATCC Accession No. 98095) was obtained by restriction enzyme digestion, Southern blotting and hybridization of the hTL-4 clone to coding sequences from mouse TL2, followed by 65 subcloning and sequencing the hybridizing fragments. Coding sequences corresponding to the N-terminal and

C-terminal amino acids of hTL4 were used to design PCR primers (shown below), which in turn were used for PCR amplification of TL4 from human ovary cDNA. A PCR band was identified as corresponding to human TL4 by DNA sequencing using the ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, Calif.). The PCR band was then subcloned into vector pCR-script and several plasmid clones were analyzed by sequencing. The complete human TL4 coding sequence was then compiled and is shown in FIGS. 23A-23C (SEQ ID NOS: 17 and 18). In another embodiment of the invention, the nucleotide at position 569 is changed from A to G, resulting in an amino acid change from Q to R.

15 The PCR primers used as described above were designed as follows:

- hTL4atg 5'-gcatgctatctcgagccaccATGCTCTCCCAG CTAGCCATGCTGCAG-3' (SEQ ID NO: 27)
- hTL4not 5'-gtgtcgacgcggccgctctagatcagacTTAGA TGTCCAAAG GCCGTATCATCAT-3' (SEQ ID NO: 28)

Lowercase letters indicate "tail" sequences added to the PCR primers to facilitate cloning of the amplified PCR fragments.

EXAMPLE 23

CONSTRUCTION AND CHARACTERIZATION OF MODIFIED TIE LIGANDS

A genetic analysis of TIE-2 ligand-1 and TIE-2 ligand-2 (TL1 and TL2) was undertaken to gain insight into a number of their observed properties. Although TL1 and TL2 share similar structural homology, they exhibit different physical 35 and biological properties. The most prominent feature that distinguishes the two ligands is that although they both bind to the TIE-2 receptor, TL1 is an agonist while TL2 is an antagonist. Under non-reducing electrophoretic conditions both proteins exhibit covalent, multimeric structures. TL1 is 40 produced as a mixture of disulfide cross-linked multimers, primarily trimers and higher order species, without any dimeric species. But TL2 is produced almost exclusively as a dimeric species. Also, while TL2 is produced well in most expression systems, TL1 is expressed poorly and is difficult fication conditions also appear to predispose TL1 to inactivation by proteolytic cleavage at a site near the amino terminus.

To study these differences, several modified ligands were

23.1. Cysteine substitution-Investigations into what factors might be contributing to the different physical and biological properties of the two molecules revealed the presence in TL1 of a cysteine residue (CYS 265 in FIGS. 4A-4D (SEQ ID 55 NO: 2); CYS 245 in FIG. 17 (SEQ ID NOS: 7 and 8) preceding the fibrinogen-like domain in TL1 but absent in TL2-i.e., there was no corresponding cysteine residue in TL2. The CYS265 residue in TL1 is encoded by TGC and is located at about nucleotides 1102-1104 (see FIGS. 4A-4D [SEQ ID NO: 1]) at the approximate junction between the coiled-coil and fibrinogen-like domains. Because cysteine residues are generally involved in disulfide bond formation, the presence of which can contribute to both the tertiary structure and biological properties of a molecule, it was thought that perhaps the presence of the CYS265 residue in TL1 might be at least partially responsible for the different properties of the two molecules.

To test this hypothesis, an expression plasmid was constructed which contained a mutation in TL1 in which the CYS (residue 265 in FIGS. 4A-4D [SEQ ID NOS: 1 and 2]; residue 245 in FIG. 17 [SEQ ID NOS: 7 and 8]) was replaced with an amino acid (serine) which does not form 5 disulfide bonds. In addition to this TL1/CYS mutant, a second expression plasmid was constructed which mutated the approximately corresponding position in TL2 (Met247 in FIG. 17 [SEQ ID NOS: 7 and 8]) so that this residue was now a cysteine. Both non-mutated and mutated expression 10 line. plasmids of TL1 and TL2 were transiently transfected into COS7 cells, cell supernatants containing the recombinant proteins were harvested, and samples were subjected to both reducing and non-reducing SDS/PAGE electrophoresis and subsequent Western blotting.

FIG. 18 shows the Western blots under non-reducing conditions of both non-mutated and mutated TL1 and TL2 proteins, revealing that the TL1/CYS⁻ mutant runs as a dimer much like TL2 and that the TL2/CYS+ mutant is able to form a trimer, as well as higher-order multimers, more 20 expression by mammalian cells, these recombinant proteins like TL1. When the two mutant proteins were tested for their ability to induce phosphorylation in TIE-2 expressing cells, the TL1/CYS- mutant was able to activate the TIE-2 receptor, whereas the TL2/CYS+ mutant was not.

Thus, when the cysteine residue (residue 265 in FIGS. 25 4A-4D (SEQ ID NO: 2); residue 245 in FIG. 17 [SEQ ID NOS: 7 and 8] of TL1 was genetically altered to a serine, it was found that the covalent structure of TL1 became similar to that of TL2, i.e., primarily dimeric. The modified TL1 molecule still behaved as an agonist, thus the trimeric and/or 30 higher order multimeric structure was not the determining factor giving TL1 the ability to activate. Although the removal of the cysteine did make a molecule with more desirable properties, it did not improve the production level of TL1.

23.2. Domain deletions-The nucleotide sequences encoding TL1 and TL2 share a genetic structure that can be divided into three domains, based on the amino acid sequences of the mature proteins. The last approximately 215 amino acid residues of each mature protein contains six 40 cysteines and bears strong resemblance to a domain of fibrinogen. This region was thus denoted the "fibrinogenlike" domain or "F-domain." A central region of the mature protein containing approximately 205 residues had a high probability of assuming a "coiled-coil" structure and was 45 denoted the "coiled-coil" domain or "C-domain." The amino-terminal approximately 55 residues of the mature protein contained two cysteines and had a low probability of having a coiled-coil structure. This region was designated the "N-terminal" domain or "N-domain." The modified 50 ligands described herein are designated using a terminology wherein N=N-terminal domain, C=coiled-coil domain, F=fibrinogen-like domain and the numbers 1 and 2 refer to TL1 and TL2 respectively. Thus 1N indicates the N-terminal domain from TL1, 2F indicates the fibrinogen-like domain 55 23.3. Swapping constructs (Chimeras): of TL2, and so forth.

In order to test whether the fibrinogen-like domain (F-domain) of the TIE2 ligands contained TIE-2 activating activity, expression plasmids were constructed which deleted the coiled-coil and N-terminal domains, leaving only 60 that portion of the DNA sequence encoding the F-domain (for TL1, beginning in FIGS. 4A-4D (SEQ ID NOS: 1 and 2) at about nucleotide 1159, amino acid residue ARG284; for TL2, corresponding to about nucleotide 1200 in FIGS. 6A-6D (SEQ ID NOS: 5 and 6), amino acid residue 282). 65 This mutant construct was then transiently transfected into COS cells. The supernatant containing the recombinant

protein was harvested. The TL1/F-domain mutant was tested for its ability to bind the TIE-2 receptor. The results showed that, as a monomer, the TL1/F-domain mutant was not able to bind TIE-2 at a detectable level.

But when the TL1/F-domain monomer was myc-tagged and subsequently clustered with an antibody directed against the myc tag, it exhibited detectable binding to TIE-2. However, the antibody-clustered TL1/F-domain mutant was not able to induce phosphorylation in a TIE-2 expressing cell

Thus it was determined that the F-domain of the TIE-2 ligands is involved in binding the receptor but that a truncation consisting of just the F-domain alone is not sufficient for receptor binding. This raised the possibility that the 15 coiled-coil domain was responsible for holding together several fibrinogen-like domains, which might be essential for receptor binding. In an attempt to confirm this hypothesis, the F-domain was fused with the Fc section of human antibody IgG1. Because Fc sections dimerize upon mimicked the theoretical configuration of the F-domains were the native ligands to dimerize. This F-domain-Fc construct bound but failed to activate the receptor. Apparently, multimerization caused by other regions of the ligands is necessary to enable the ligands to bind the TIE receptor. In addition, some other factor outside of the F-domain must contribute to phosphorylation of the receptor

Mutants were then constructed which were missing the fibrinogen-like domain, and therefore contained only the N-terminal and coiled-coil domains. They were not capable of binding to the receptor. To assess the role of the N-terminal domain in receptor binding and activation, the ligands were truncated to just their C- and F-domains and 35 tagged with a FLAG tag at the N-terminus, creating constructs termed FLAG-1C1F and FLAG-2C2F. Although these molecules stained robustly in COS7 cells transfected transiently to express the TIE receptor, they failed to respond in a phosphorylation assay. Thus the N-domain does contain an essential factor for receptor activation although, as disclosed infra, the ability of chimeric molecule 2N2C1F to activate the receptor shows that even the N-domain of an inactive ligand can fill that role.

The differences in behavior between the myc-tagged F-domain truncation and the Fc-tagged F-domain truncation described previously suggested that the TIE ligands can only bind in dimeric or higher multimeric forms. Indeed, nonreducing SDS-PAGE showed that the TIE ligands exist naturally in dimeric, trimeric, and multimeric forms. That the FLAG-1C1F and FLAG-2C2F truncations can bind to the TIE-2 receptor without dimerization by a synthetic tag (such as Fc), whereas the F truncations cannot, suggests that the C-region is at least partly responsible for the aggregation of the F-domains.

Applicants had noted that the level of production of TL1 in COS7 cells was approximately tenfold lower than production of TL2. Therefore, chimeras of TL1 and TL2 were constructed in an attempt to explain this difference and also to further characterize the agonist activity of TL1 as compared to the antagonist activity of TL2.

Four chimeras were constructed in which either the N-terminal domain or the fibrinogen domain was exchanged between TL1 and TL2 and were designated using the terminology described previously such that, for example, 1N1C2F refers to a chimera having the N-terminal and coiled-coil domains of TL1, together with the fibrinogen-

like domain from TL2. The four chimeras were constructed as follows:

chimera 1 -	1N1C2F	
chimera 2 -	2N2C1F	
chimera 3 -	1N2C2F	
chimera 4 -	2N1C1F	

The nucleotide and amino acid sequences of chimeras 1-4 10are shown in FIGS. 24A-24C (SEQ ID NOS: 19 and 20), FIGS. 25A-25C (SEQ ID NOS: 21 and 22), FIGS. 26A-26C (SEQ ID NOS: 23 and 24), and FIGS. 27A-27C (SEQ ID NOS: 25 and 26) respectively.

Each chimera was inserted into a separate expression 15 vector pJFE14. The chimeras were then transfected into COS7 cells, along with the empty pJFE14 vector, native TL1, and native TL2 as controls, and the culture supernatants were collected.

of expression of the ligands, a 1:5 dilution and a 1:50 dilution of the COS7 supernatants were dot-blotted onto nitrocellulose. Three ligands that contained the TL1Ndomain (i.e. native TL1, 1N2C2F and 1N1C2F) were then probed with a rabbit antibody specific to the N-terminus of 25 TL1. Three ligands containing the TL2 N-domain, (i.e. native TL2, 2N1C1F and 2N2C1F) were probed with a rabbit antibody specific for the N-terminus of TL2. The results demonstrated that the COS7 cells were expressing any molecule containing the N-domain of TL2 at roughly ten 30 23.4. Proteolytic resistant constructs-Based on the obsertimes the level of any molecule containing the TL1 N-domain, regardless of the makeup of the rest of the protein. The conclusion was that the N-domain must principally control the level of expression of the ligand.

The next question addressed was the chimeras' ability or 35 inability to activate the TIE-2 receptor. EAhy926 cells were challenged with the four chimeras, as well as TL1 as a positive control for phosphorylation and TL2 or an empty pJFE14-transfected COS7 cell supernatant as negative controls for phosphorylation. The cells were lysed, and the 40 TIE-2 receptor was immunoprecipitated out of the cell lysate and run on an SDS-PAGE. The samples were Western blotted and probed with an anti-phosphotyrosine antibody to detect any receptors that had been phosphorylated. Surprisingly, only the constructs containing the TL1 45 fibrinogen-like domain (2N1C1F and 2N2C1F) could phosphorylate the TIE-2 receptor. Thus, although the N-terminal region of TL1 is essential for activation, it can be replaced by the N-terminal region of TL2, i.e., the information that determines whether the ligand is an agonist or an antagonist 50 to confer protease resistance on the molecule. Finally, this is actually contained in the fibrinogen-like domain. Thus it was determined that the F-domain, in addition to binding the TIE-2 receptor, is responsible for the phosphorylation activity of TL1. Further, when TL2, an otherwise inactive molecule, was altered by replacing its F-domain with the 55 was expected to be activating, well expressed, dimeric, and TL1 F-domain, the altered TL2 acted as an agonist.

The 2N1C1F construct was somewhat more potent, however. The signal caused by chimera 2N1C1F appeared slightly stronger than that of chimera 2N2C1F, leading to speculation that the C-domain of TL1, though not crucial for 60 phosphorylation, might enhance the potency of TL1. However, since the samples used for the phosphorylation assay were not normalized in terms of the concentration of ligand, it was possible that a stronger phosphorylation signal only indicated the presence of more ligand. The phospho-65 rylation assay was therefore repeated with varying amounts of ligand to determine whether the active chimeras displayed

different potencies. The concentration of ligand in the COS7 supernatants of ligand transfections was determined through BIAcore biosenser technology according to methods previously described (Stitt, T. N., et al. (1995) Cell 80: 661-670). BIAcore measured the binding activity of a supernatant to the TIE-2 receptor in arbitrary units called resonance units (RU). Fairly good correlation between RU's and ligand concentration has been generally observed, with 400 RU of activity corresponding to about 1 μ g of protein per mL of supernatant. Samples were diluted to concentrations of 100 RU, 20 RU, and 5 RU each and the phosphorylation assay was repeated. The results demonstrated that chimera 2N2C1F was clearly more potent than either the native TL1 or chimera 1N1C2F at the same concentrations.

Another interesting aspect of these exchange constructs is in their levels of expression. Each of the four chimeras was tested for its level of production in COS cells, its ability to bind to TIE2, and its ability to phosphorylate TIE2. The results of these experiments showed that chimeras 1 and 3 In order to determine how the swapping affected the level 20 were produced at levels comparable to TL1, whereas chimeras 2 and 4 were produced at levels comparable to TL2. Thus a high level of protein production was correlated with the TL2 N-terminal domain. Additionally, when tested on endothelial EAhy926 cells, chimeras 2 and 4 were active, whereas 1 and 3 were not. Thus activity (phosphorylation of the receptor) correlates with the TL1 fibrinogen-like domain. Chimeras 2 and 4 therefore each had the desirable properties of high production levels as well as agonist activity.

> vation that a large fraction of TL1 preparations was often proteolytically cleaved near the N-terminus, it was proposed that an arginine residue located at position 49 of the mature protein (see FIG. 17 (SEQ ID NO: 7 and SEQ ID NO: 8) was a candidate cleavage site that might be involved in the regulation of the protein's activity in vivo, and that replacing the arginine with a serine (R49 \rightarrow S) might increase the stability of the protein without necessarily affecting its activity. Such a mutant of TL1 was constructed and was found to be about as active as the native TL1 but did not exhibit resistance to proteolytic cleavage.

> 23.5. Combination mutants-The most potent of the chimeric constructs, 2N1C1F, was additionally altered so that the cysteine encoded by nucleotides 784-786 as shown in FIGS. 27A-27C (SEQ ID NOS: 25 and 26) was converted to a serine. This molecule (denoted 2N1C1F (C246S)) was expressed well, potently activated the receptor, was resistant to proteolytic cleavage and was primarily dimeric, rather than higher-order multimeric. Thus the 2N domain appeared molecule was further altered to eliminate the potentially protease sensitive site encoded by nucleotides 199-201 as shown in FIGS. 27A-27C (SEO ID NOS: 25 and 26), to give a molecule (denoted 2N1C1F (R51 \rightarrow S,C246 \rightarrow S)) which protease resistant.

> Table 1 summarizes the modified TIE-2 ligand constructs that were made and characterizes each of them in terms of ability to bind the TIE-2 receptor, ability to activate the TIE-2 receptor, the type of structure formed (monomer, dimer, etc.) and their relative production levels. Unmodified TL1 (plain) and TL2 (striped) are shown with the three domains as boxes. Thus striped boxes indicate domains from TL2. The cysteine located at position 245 of the mature TL1 protein is indicated by a "C." An "X" through the "C" indicates that that cysteine residue was substituted for by another amino acid as in, for example, the TL1 CYS-mutant.

Similarly, an "X" through the "R" in the last construct indicates the substitution for an Arg residue at position 49 of the mature TL1 protein. The "C" is present in one modified TL2 construct showing the TL2 CYS+ mutant. Constructs having Fc tails or flag tagging are also indicated.

Based upon the teachings herein, one of skill in the art can readily see that further constructs may be made in order to create additional modified and chimeric TIE-2 ligands which have altered properties. For example, one may create a construct comprised of the N-terminal domain of TL2 and the F-domain of TL1 fused with the Fc section of human antibody IgG1. This construct would be expected to bind and activate the TIE-2 receptor. Similarly, other constructs may be created using the teachings herein and are therefore considered to be within the scope of this invention.

23.6. Materials and Methods

Construction of Chimeras

Swapping constructs were inserted into a pJFE14 vector in which the XbaI site was changed to an AscI site. This vector was then digested with AscI and Noti yielding an 20 AscI-NotI backbone. DNA fragments for the chimeras were generated by PCR using appropriate oligonucleotides.

The FLAG-1C1F and FLAG-2C2F inserts were subcloned into a pMT21 vector backbone that had been digested with EcoRI and NotI. The "CF" truncations were obtained 25 through PCR, and the FLAG tag and a preceding trypsin signalling sequence were constructed by annealing synthetic oligonucleotides.

Transfections

All constructs were transfected transiently into COS7 30 cells using either DEAE-Dextran or LipofectAMINE according to standard protocols. Cell cultures were harvested 3 days after the transfection and spun down at 1000 rpm for 1 minute, and the supernatants were transferred to fresh tubes and stored at -20° C.

Staining of FLAG-1C1F-Transfected and FLAG-2C2F-Transfected Cells

6-well dishes of COS7 cells were transfected transiently with the TIE-2 receptor. The COS7 supernatant from various ⁵ ligand tansfections was incubated on the cells for 30 minutes, followed by two washes with Phosphate Buffered Saline (PBS) without magnesium or calcium. The cells were fixed in -20° C. methanol for 3 minutes, washed once with PBS, and incubated with anti-FLAG M2 antibody (IBI;1:3000 dilution) in PBS/10% Bovine Calf Serum (BCS) for 30 minutes. The cells were washed once with PBS and incubated with goat anti-mouse IgG Alkaline Phosphatase (AP) conjugated antibody (Promega;1:1000) in PBS/10% BCS. The cells were washed twice with PBS and 15 incubated with the phosphate substrate, BCIP/NBT, with 1 mM levamisole.

Phosphorylation Assays

Dilution of COS7 supernatants for the dose response study was done in the supernatants of COS7 cells transfected with the empty vector pJFE14. EA cells that naturally express the TIE-2 receptor were starved for >2 hours in serum-free medium, followed by challenge with the appropriate COS7 supernatant for 10 minutes at 37° C. in an atmosphere of 5% CO2. The cells were then rinsed in ice-cold PBS and lysed with 1% NP40 lysis buffer containing protease inhibitors (10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM PMSF) followed by immunoprecipitation with an antibody specific for the TIE-2 receptor. Samples were then subjected to immunoblot analysis, using anti pTyr antibodies.

Dot Blots

Samples were applied to a nitrocellulose membrane, which was blocked and probed with the appropriate antibodies.

TABLE .	L
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	MUTATION ANALYSIS OF TIE LIGANDS					
	N COILED-COIL FIBRINOGEN- LIKE	TIE2 Binding	TIE2 Activation	Multimeric Structure	Production Levels	
TL1	С	+	+	HIGHER ORDER	LOW	
TL2		+	-	DIMER	HIGH	
		+	+	DIMER	LOW	
		+	-	HIGHER ORDER	HIGH	
	С	-	N.D.	N.D.	LOW	
		-	N.D.	N.D.	HIGH	
		-	-	MONOMER	HIGH	
		-	-	MONOMER	HIGH	
TABLE 1-continued



*HIGHEST PRODUCTION OF RU

****MOST POTENTLY ACTIVATING**

N.D. = NOT DETERMINED

DEPOSITS

The following have been deposited with the American 55 Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. 20852 in accordance with the Budapest Treaty. A plasmid clone encoding a TIE-2ligand was deposited with the ATCC on Oct. 7, 1994 and designated as "pJFE14 encoding TIE-2 ligand" under ATCC Accession No. 75910. 60 Recombinant *Autographa californica* baculovirus encoding TIE-2 receptorbody was deposited with the ATCC on Oct. 7, 1994 and designated as "vTIE-2 receptorbody" under ATCC Accession No. VR2484. A lambda phage vector containing human tie-2 ligand DNA was deposited with the ATCC on 65 Oct. 26, 1994 and designated as " λ gt10 encoding htie-2 ligand 1" under ATCC Accession No. 75928. A plasmid

clone encoding a second TIE-2 ligand was deposited with the ATCC on Dec. 9, 1994 and designated as "pBluescript KS encoding human TIE 2 ligand 2" under ATCC Accession No. 75963. *E. coli* strain DH10B containing plasmid pBe-LoBac11 with a human TL-4 gene insert encoding human TIE ligand-4 was deposited with the ATCC on Jul. 2, 1996 and designated as "hTL-4" under ATCC Accession No. 98095.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims. <160> NUMBER OF SEQ ID NOS: 30

SEQUENCE LISTING

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Glu	Glu	Leu	Asp 210	Thr	Leu	Lys	Glu	Glu 215	Lys	Glu	Asn	Leu	Gln 220	Gly	Leu	
gtt Val																1023
aga Arg	-					-	-		-		-				-	1071
atg Met . 255																1119
tta Leu																1167
gca Ala .	-	-			-					-						1215
tat Tyr				-		-			-			-		-	-	1263
gtc Val .							-				-	-	-		5	1311
cta Leu 335																1359
ccc Pro																1407
agt Ser	-		-		-		-				-	-		-		1455
aac Asn .																1503
caa Gln .				-									-			1551
cag Gln 415																1599
gat Asp .																1647
tgg Trp																1695
act Thr .																1743
ttc Phe				-				-				-	-		-	1791
cct Pro 495		-		tgaa	aagco	gca a	atgto	cagaa	ag co	gatta	atgaa	a ago	caaca	aaag		1843
aaat	ccgo	jag a	aagc	tgeea	ag gi	tgaga	aaact	: gti	tgaa	aaac	ttca	agaaq	gca a	aacaa	atattg	1903
tctc	cctt	cc a	agcaa	ataa	gt go	gtag	ttato	g tga	aagto	cacc	aag	gttc	ttg a	accgi	cgaatc	1963

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tgga	agec	gtt †	tgag [.]	ttca	ca a	gagto	ctcta	a cti	zgggg	gtga	cag	tgct	cac (gtggo	etegae	2023
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aati	ttc															2149
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Tyr	Asn	Arg 35	Ile	Gln	His	Gly	Gln 40	Cys	Ala	Tyr	Thr	Phe 45	Ile	Leu	Pro	
Glu	His 50	Asp	Gly	Asn	Cys	Arg 55	Glu	Ser	Thr	Thr	Asp 60	Gln	Tyr	Asn	Thr	
Asn 65	Ala	Leu	Gln	Arg	Asp 70	Ala	Pro	His	Val	Glu 75	Pro	Asp	Phe	Ser	Ser 80	
Gln	Lys	Leu	Gln	His 85	Leu	Glu	His	Val	Met 90	Glu	Asn	Tyr	Thr	Gln 95	Trp	
Leu	Gln	Lys	Leu 100	Glu	Asn	Tyr	Ile	Val 105	Glu	Asn	Met	Lys	Ser 110	Glu	Met	
Ala	Gln	Ile 115	Gln	Gln	Asn	Ala	Val 120	Gln	Asn	His	Thr	Ala 125	Thr	Met	Leu	
Glu	Ile 130	Gly	Thr	Ser	Leu	Leu 135	Ser	Gln	Thr	Ala	Glu 140	Gln	Thr	Arg	Lys	
Leu 145	Thr	Asp	Val	Glu	Thr 150	Gln	Val	Leu	Asn	Gln 155	Thr	Ser	Arg	Leu	Glu 160	
Ile	Gln	Leu	Leu	Glu 165	Asn	Ser	Leu	Ser	Thr 170	Tyr	Lys	Leu	Glu	L y s 175	Gln	
Leu	Leu	Gln	Gln 180	Thr	Asn	Glu	Ile	Leu 185	Lys	Ile	His	Glu	L y s 190	Asn	Ser	
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Leu	Asp 210	Thr	Leu	Lys	Glu	Glu 215	Lys	Glu	Asn	Leu	Gln 220	Gly	Leu	Val	Thr	
Arg 225	Gln	Thr	Tyr	Ile	Ile 230	Gln	Glu	Leu	Glu	L y s 235	Gln	Leu	Asn	Arg	Ala 240	
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Lys	Gly	Gly 275	Lys	Arg	Glu	Glu	Glu 280	Lys	Pro	Phe	Arg	Asp 285	Cys	Ala	Asp	
Val	Ty r 290	Gln	Ala	Gly	Phe	Asn 295	Lys	Ser	Gly	Ile	T y r 300	Thr	Ile	Tyr	Ile	
Asn 305	Asn	Met	Pro	Glu	Pro 310	Lys	Lys	Val	Phe	С у в 315	Asn	Met	Asp	Val	Asn 320	
Gly	Gly	Gly	Trp	Thr 325	Val	Ile	Gln	His	Arg 330	Glu	Asp	Gly	Ser	Leu 335	Asp	

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Tyr Arg Leu Tyr Leu Lys Gly His Thr Gly Thr Ala Gly Lys Gln Ser 405 407 408 409 409 409 409 409 409 409 409											-	con	tin	ued					
35 360 365 Arg Gin Tyr Met Leu Arg The Glu Leu Met Ang Trp Glu Gly Ann Arg 370 310 9370 370 310 Aha Tyr Ser Gin Tyr Apa Arg Phe His Ile Gly Ann Glu Lys Gln Ann 395 310 9277 Arg Leu Tyr Leu Lys Gly His Thr Gly Thr Ala Gly Lys Gln Ser 400 415 828 Leu His Gly Ala Asp Phe Ser Thr Lys Asp Ala Asp Ann 410 415 829 Arg Ann Cys Met Cys Lys Cys Ala Leu And Leu Thr Gly Gly Trp Trp 413 445 430 435 445 445 445 445 446 440 450 Ann Hia Gly Lys Leu Ann Gly He Tym The Ala 460 445 410 Ho Tym The Lys 449 445 440 4210 Ho Ann Hia Gly Lys Leu Ann Gly He Tym The Tym Phe Lys 449 445 4210 SKG ID NO 3 445 445 4215 ORMANISH: Formo sequens 490 495 495 4215 ORMANISH: Formo sequens 490 495	Phe Gli	n Arg		Trp	Lys	Glu	Tyr	_	Met	Gly	Phe	Gly		Pro	Ser				
370 375 380 Alls Tyr Ser Ghn Tyr Ang Ang Phe His Ile Cly Ann Glu Lys Cln Asn 390 400 Tyr Arg Leu Tyr Leu Lys Cly His Thr Cly Thr Ala Cly Lys Qln Asn 410 410 Arg Ann Cys Met Cys Lye Cys Ala Leu Men Leu Thr Gly Gly Trp Trp 415 415 Asp Ann Cys Met Cys Lye Cys Ala Leu Men Glu Met Phe Tyr Thr Ala 450 425 Yer Ann His Gly Lye Leu Ann Gly He Tyr The His Tyr Phe Lys 445 440 400 455 470 401 455 470 402 475 480 410 455 470 420 455 445 430 455 410 480 411 450 420 475 421 480 421 480 421 480 421 480 421 480 421 480 421 480 421 480 421 480 421 480 421 480 421 480 421 480	Gly Gl	_	Trp	Leu	Gly	Asn		Phe	Ile	Phe	Ala		Thr	Ser	Gln				
385 390 395 400 Typ Arg Leu Tyr Leu Lye Gly His Thr Gly Thr Ala Gly Lys Gln Ser 400 405 401 Ser Leu Iie Leu His Gly Ala Asp Phe Ser Thr Lys Asp Ala Asp Asn 420 405 401 Aap Ann Cys Ket Cys Lys Cys Ala Leu Ket Leu Thr Gly Gly Trp Trp 435 400 445 She Asp Ala Cys Cly Pro Ser Asn Leu Asn Gly Het Phe Tyr Thr Ala 450 400 460 Cly Gin Aan His Cly Lys Leu Asn Gly Iie Lys Trp His Tyr Phe Lys 445 400 460 Cly Fro Ser Tyr Ger Chr Thr Thr Met Net Ile Arg Pro Leu 445 470 400 All DuRCHH: 2146 470 490 495 All DuRCH: 2146 475 490 495 All DuRCH: 2146 </td <td>-</td> <td>_</td> <td>Met</td> <td>Leu</td> <td>Arg</td> <td></td> <td>Glu</td> <td>Leu</td> <td>Met</td> <td>Asp</td> <td>-</td> <td>Glu</td> <td>Gly</td> <td>Asn</td> <td>Arg</td> <td></td> <td></td> <td></td> <td></td>	-	_	Met	Leu	Arg		Glu	Leu	Met	Asp	-	Glu	Gly	Asn	Arg				
405 410 415 Ser Leu Ile Leu His Gly Ala Amp Phe Ser Thr Lys Amp Ala Amp Amn 425 425 427 440 425 427 440 445 Amp Am Cys Met Cys Lys Cys Ala Leu Net Leu Thr Gly Gly Trp Trp 445 Amp Am Cys Met Cys Ily Cys Ala Leu Xet Leu Thr Gly Gly Trp Trp 445 Phe Amp Ala Cys Gly Pro Ser Am Leu Amn Gly Met Phe Tyr Thr Ala 450 450 470 485 Algo Am His Cly Typ Leu Amn Gly Ile Lys Trp His Tyr Phe Lys 445 445 Algo Thr Ser Tyr Ser Leu Arg Ser Thr Thr Met Net Ile Arg Pro Leu 495 450 470 485 Amp Phe 2210 SKR TWR 1244 4213 TYPE: DNA 425 4210 SKR THR THR Sepines 426 4220 TYPE: DNA 4223 MIR/HYR: CDS 4220 CHART MEROMMITION: 41803 4223 MIR/HYR: CDS 4220 CHART MEROMMITION: 41803 4225 MIR/HYR: CDS 4225 MIR/HYR: CDS 4225 MIR/HYR: CDS 4226 THR THR THR AMP	Ala Ty: 385	r Ser	Gln	Tyr		Arg	Phe	His	Ile		Asn	Glu	Lys	Gln					
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The App Ala Cys Gly Pro Ser Aan Leu Aan Cly Met Phe Tyr Thr Ala 450 451 Gli Aan His Gly Lys Leu Aan Gly IIe Lys Trp His Tyr Phe Lys 465 470 475 475 475 475 475 475 475 475	Asp Asi		Met	Cys	Lys	Cys			Met	Leu	Thr	_		Trp	Trp				
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Amp Phe 2210 SEQ ID NO 3 2211 SLENGTH: 2146 2212 TIPE: DNA 2213 OCCANIEN: Homo sapiens 2205 FEATURE: 2221 DOCTORINE: 2222 DOCTORINE: 2223 DOTHER INFORMATION: 2233 OTHER INFORMATION: 2233 OTHER INFORMATION: 2235 OTHER INFORMATION: 2230 SEQUENCE: 3 Cagedgacet aggeaggete catgetgace ggteacacag agaggaaaca ataaatetea 60 gedactatge aataatate toaagttta acgaagaaaa acateatige agigaaataa 120 aaaattttaa aatttagaa caaagetaac aaatggetag tttteatig attettetta 180 aacgettet tigaggggga aagagteaaa caaacaagea gtttgeaga aggaeggaag agagtgetget 300 ggeagtaca atg aggt the off the off tiget the off ged agg agg aggaeggaa ggagtgegg 330 ggeagtaca atg aggt the off the off the Ser Phe Ala Phe Leu Ala Ala Ile Leu 1 1 0 5 10 1 act cac ata ggg tge age aat cag ege cga agt cca gaa aac agt ggg 15 2 20 Ca Can Arg Arg Ser Pro Glu Ans Ser Gly 15 2 20 Ca can Can Arg Arg Ser Pro Glu Ans Ser Gly 16 2 2 5 2 00 aga aga tat aac egg at caa cat ggg caa tgt gee taa caft the att att att ast 15 2 0 0 70 70 70 75 75 75 75 75 75 75 75 75 75 75 75 75		o Ser	Tyr			Arg	Ser	Thr			Met	Ile	Arg						
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gaa cac tcc ctc tcg aca aac aaa ttg gaa aaa cag att ttg gac cag Glu His Ser Leu Ser Thr Asn Lys Leu Glu Lys Gln Ile Leu Asp Gln 165 170 175	887
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Gln	Val	Leu	Glu	Asn 85	Ile	Met	Glu	Asn	Asn 90	Thr	Gln	Trp	Leu	Met 95	Lys					
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Asn	Leu	Leu	Thr 260	Met	Met	Ser	Thr	Ser 265	Asn	Ser	Ala	Lys	Asp 270	Pro	Thr					
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Ser 385	Leu	Tyr	Glu	His	Phe 390	Tyr	Leu	Ser	Ser	Glu 395	Glu	Leu	Asn	Tyr	Arg 400					
Ile	His	Leu	Lys	Gly 405	Leu	Thr	Gly	Thr	Ala 410	Gly	Lys	Ile	Ser	Ser 415	Ile					
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Ser	Leu	Leu 115	Ser	Gln	Thr	Ala	Glu 120	Gln	Thr	Arg	Lys	Leu 125	Thr	Asp	Val
Glu	Thr 130	Gln	Val	Leu	Asn	Gln 135	Thr	Ser	Arg	Leu	Glu 140	Ile	Gln	Leu	Leu
Glu 145	Asn	Ser	Leu	Ser	Thr 150	Tyr	Lys	Leu	Glu	L y s 155	Gln	Leu	Leu	Gln	Gln 160
Thr	Asn	Glu	Ile	Leu 165	Lys	Ile	His	Glu	L y s 170	Asn	Ser	Leu	Leu	Glu 175	His
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Ile	Ile 210	Gln	Glu	Leu	Glu	L y s 215	Gln	Leu	Asn	Arg	Ala 220	Thr	Thr	Asn	Asn
Ser 225	Val	Leu	Gln	Lys	Gln 230	Gln	Leu	Glu	Leu	Met 235	Asp	Thr	Val	His	Asn 240
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His	Gly	Ala	Asp	Phe 405	Ser	Thr	Lys	Asp	Ala 410	Asp	Asn	Asp	Asn	Сув 415	Met
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Gly	Pro	Ser 435	Asn	Leu	Asn	Gly	Met 440	Phe	Tyr	Thr	Ala	Gly 445	Gln	Asn	His
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Leu	Glu	Asn	Tyr	Ile 85	Gln	Asp	Asn	Met	Lys 90	Lys	Glu	Met	Val	Glu 95	Ile
Gln	Gln	Asn	Ala 100	Val	Gln	Asn	Gln	Thr 105	Ala	Val	Met	Ile	Glu 110	Ile	Gly
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Val	Glu 130	Ala	Gln	Val	Leu	Asn 135	Gln	Thr	Thr	Arg	Leu 140	Glu	Leu	Gln	Leu
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Lys	Lys	Val	Leu 180	Ala	Met	Glu	Asp	L y s 185	His	Ile	Ile	Gln	Leu 190	Gln	Ser
Ile	Lys	Glu 195	Glu	Lys	Asp	Gln	Leu 200	Gln	Val	Leu	Val	Ser 205	Lys	Gln	Asn
Ser	Ile 210		Glu	Glu	Leu	Glu 215		Lys	Ile	Val	Thr 220		Thr	Val	Asn
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	Ser Thr Ser Asn	235 240 Ser Ala Lys Asp Pro Thr
245 Val Ala Lys Glu Glu Glr	250 I Ile Ser Phe Arg 2	255 Asp Cys Ala Glu Val Phe
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275 275	280	Thr Leu Thr Phe Pro Asn 285
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Ser Ser Sei	: Leu Gln 260	Gln Gl	n Gln	Gln 265	Gln	Leu	Thr	Glu	Phe 270	Val	Gln
Arg Leu Va 27		Val Al	a Gln 280	Asp	Gln	His	Pro	Val 285	Ser	Leu	Lys
Thr Pro Ly: 290	s Pro Val	Phe Gl 29	-	Cys	Ala	Glu	Ile 300	Lys	Arg	Ser	Gly
Val Asn Thi 305	: Ser Gly	Val Ty 310	r Thr	Ile	Tyr	Glu 315	Thr	Asn	Met	Thr	Lys 320
Pro Leu Ly:	s Val Phe 325	_	p Met	Glu	Thr 330	Asp	Gly	Gly	Gly	Trp 335	Thr
Leu Ile Gli	n His Arg 340	Glu As	p Gly	Ser 345	Val	Asn	Phe	Gln	Arg 350	Thr	Trp
Glu Glu Ty: 35!		Gly Ph	e Gly 360	Asn	Val	Ala	Arg	Glu 365	His	Trp	Leu
Gly Asn Glu 370	ı Ala Val	His Ar 37	-	Thr	Ser	Arg	Thr 380	Ala	Tyr	Leu	Leu
Arg Val Glu 385	ı Leu His	Asp Tr 390	p Glu	Gly	Arg	Gln 395	Thr	Ser	Ile	Gln	Tyr 400
Glu Asn Phe	e Gln Leu 405		er Glu	Arg	Gln 410	Arg	Tyr	Ser	Leu	Ser 415	Val
Asn Asp Sei	Ser Ser 420	Ser Al	a Gly	Arg 425	Lys	Asn	Ser	Leu	Ala 430	Pro	Gln
Gly Thr Ly: 43		Thr Ly	rs Asp 440	Met	Asp	Asn	Asp	Asn 445	Суз	Met	Cys
Lys Cys Ala 450	a Gln Met	Leu Se 45		Gly	Trp	Trp	Phe 460	Asp	Ala	Суз	Gly
Leu Ser Ası 465	n Leu Asn	Gly I1 470	e Tyr.	Tyr	Ser	Val 475	His	Gln	His	Leu	His 480
Lys Ile Ası	n Gl y Ile 485		p His	Tyr	Phe 490	Arg	Gly	Pro	Ser	T y r 495	Ser
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His	Arg	Gly	Pro 20	Glu	Ala	Gly	Gly	His 25	Arg	Gln	Ile	His	Gln 30	Val	Arg					
Arg	Gly	Gln 35	Cys	Ser	Tyr	Thr	Phe 40	Val	Val	Pro	Glu	Pro 45	Asp	Ile	Cys					
Gln	Leu 50	Ala	Pro	Thr	Ala	Ala 55	Pro	Glu	Ala	Leu	Gly 60	Gly	Ser	Asn	Ser					
Leu 65	Gln	Arg	Asp	Leu	Pro 70	Ala	Ser	Arg	Leu	His 75	Leu	Thr	Asp	Trp	Arg 80					
Ala	Gln	Arg	Ala	Gln 85	Arg	Ala	Gln	Arg	Val 90	Ser	Gln	Leu	Glu	L y s 95	Ile					
Leu	Glu	Asn	Asn 100	Thr	Gln	Trp	Leu	Leu 105	Lys	Leu	Glu	Gln	Ser 110	Ile	Lys					
Val	Asn	Leu 115	Arg	Ser	His	Leu	Val 120	Gln	Ala	Gln	Gln	Asp 125	Thr	Ile	Gln					
Asn	Gln 130	Thr	Thr	Thr	Met	Leu 135	Ala	Leu	Gly	Ala	Asn 140	Leu	Met	Asn	Gln					
Thr 145	Lys	Ala	Gln	Thr	His 150	Lys	Leu	Thr	Ala	Val 155	Glu	Ala	Gln	Val	Leu 160					
Asn	Gln	Thr	Leu	His 165	Met	Lys	Thr	Gln	Met 170	Leu	Glu	Asn	Ser	Leu 175	Ser					
Thr	Asn	Lys	Leu 180	Glu	Arg	Gln	Met	Leu 185	Met	Gln	Ser	Arg	Glu 190	Leu	Gln					
Arg	Leu	Gln 195	Gly	Arg	Asn	Arg	Ala 200	Leu	Glu	Thr	Arg	Leu 205	Gln	Ala	Leu					
Glu	Ala 210	Gln	His	Gln	Ala	Gln 215	Leu	Asn	Ser	Leu	Gln 220	Glu	Lys	Arg	Glu					
Gln 225	Leu	His	Ser	Leu	Leu 230	Gly	His	Gln	Thr	Gly 235	Thr	Leu	Ala	Asn	Leu 240					
Lys	His	Asn	Leu	His 245	Ala	Leu	Ser	Ser	Asn 250	Ser	Ser	Ser	Leu	Gln 255	Gln					
Gln	Gln	Gln	Gln 260	Leu	Thr	Glu	Phe	Val 265	Gln	Arg	Leu	Val	Arg 270	Ile	Val					
Ala	Gln	A sp 275	Gln	His	Pro	Val	Ser 280	Leu	Lys	Thr	Pro	L y s 285	Pro	Val	Phe					
Gln	Asp 290	Сув	Ala	Glu	Ile	L y s 295	Arg	Ser	Gly	Val	Asn 300	Thr	Ser	Gly	Val					
T y r 305	Thr	Ile	Tyr	Glu	Thr 310	Asn	Met	Thr	Lys	Pro 315	Leu	Lys	Val	Phe	C y s 320					
Asp	Met	Glu	Thr	Авр 325	Gly	Gly	Gly	Trp	Thr 330	Leu	Ile	Gln	His	Arg 335	Glu					
Asp	Gly	Ser	Val 340	Asn	Phe	Gln	Arg	Thr 345	Trp	Glu	Glu	Tyr	Lys 350	Glu	Gly					
Phe	Gly	Asn 355	Val	Ala	Arg	Glu	His 360	Trp	Leu	Gly	Asn	Glu 365	Ala	Val	His					
Arg	Leu 370	Thr	Ser	Arg	Thr	Ala 375	-	Leu	Leu	Arg	Val 380	Glu	Leu	His	Asp					
Trp 385	Glu	Gly	Arg	Gln	Thr 390	Ser	Ile	Gln	Tyr	Glu 395	Asn	Phe	Gln	Leu	Gly 400					
Ser	Glu	Arg	Gln	Arg 405	Tyr	Ser	Leu	Ser	Val 410	Asn	Asp	Ser	Ser	Ser 415	Ser					
Ala	Gly	Arg	Lys	Asn	Ser	Leu	Ala	Pro	Gln	Gly	Thr	Lys	Phe	Ser	Thr					

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Lys	Asp	Met 435	Asp	Asn	Asp	Asn	Cys 440	Met	Cys	Lys	Cys	Ala 445	Gln	Met	Leu
Ser	Gly 450	Gly	Trp	Trp	Phe	Asp 455	Ala	Cys	Gly	Leu	Ser 460	Asn	Leu	Asn	Gly
Ile 465	Tyr	Tyr	Ser	Val	His 470	Gln	His	Leu	His	L y s 475	Ile	Asn	Gly	Ile	Arg 480
Trp	His	Tyr	Phe	Arg 485	Gly	Pro	Ser	Tyr	Ser 490	Ile	His	Gly	Thr	Arg 495	Met
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Gln	Cys	Ala 35	Tyr	Thr	Phe	Ile	Leu 40	Pro	Glu	His	Asp	Gly 45	Asn	Суз	Arg
Glu	Ser 50	Thr	Thr	Asp	Gln	T y r 55	Asn	Thr	Asn	Ala	Leu 60	Gln	Arg	Asp	Ala
Pro 65	His	Val	Glu	Pro	Asp 70	Phe	Ser	Ser	Gln	L y s 75	Leu	Gln	His	Leu	Glu 80
His	Val	Met	Glu	Asn 85	Tyr	Thr	Gln	Trp	Leu 90	Gln	Lys	Leu	Glu	Asn 95	Tyr
Ile	Val	Glu	Asn 100	Met	Lys	Ser	Glu	Met 105	Ala	Gln	Ile	Gln	Gln 110	Asn	Ala
Val	Gln	Asn 115	His	Thr	Ala	Thr	Met 120	Leu	Glu	Ile	Gly	Thr 125	Ser	Leu	Leu
	Gln 130					135					140				
145	Leu				150	-				155					160
	Ser		-	165			-		170					175	
	Leu	-	180			-		185					190		
	Met	195	-	-		-	200			-		205	-		
-	Glu 210				-	215			-		220	-			
225	Leu				230					235					240
	Lys			245				-	250					255	
	Суз		260					265	-	-	-	-	270		
Lys	Pro	Phe 275	Arg	Asp	Суз	Ala	Asp 280	Val	Tyr	Gln	Ala	Gly 285	Phe	Asn	Lys

												con	tin	ueu	
Ser	Gly 290	Ile	Tyr	Thr	Ile	Tyr 295	Ile	Asn	Asn	Met	Pro 300	Glu	Pro	Lys	Lys
Val 305	Phe	Cys	Asn	Met	Asp 310	Val	Asn	Gly	Gly	Gly 315	Trp	Thr	Val	Ile	Gln 320
His	Arg	Glu	Asp	Gly 325	Ser	Leu	Asp	Phe	Gln 330	Arg	Gly	Trp	Lys	Glu 335	Tyr
Lys	Met	Gly	Phe 340	Gly	Asn	Pro	Ser	Gly 345	Glu	Tyr	Trp	Leu	Gly 350	Asn	Glu
Phe	Ile	Phe 355	Ala	Ile	Thr	Ser	Gln 360	Arg	Gln	Tyr	Met	Leu 365	Arg	Ile	Glu
Leu	Met 370	Asp	Trp	Glu	Gly	Asn 375	Arg	Ala	Tyr	Ser	Gln 380	Tyr	Asp	Arg	Phe
His 385	Ile	Gly	Asn	Glu	L y s 390	Gln	Asn	Tyr	Arg	Leu 395	Tyr	Leu	Lys	Gly	His 400
Thr	Gly	Thr	Ala	Gly 405	Lys	Gln	Ser	Ser	Leu 410	Ile	Leu	His	Gly	Ala 415	Asp
Phe	Ser	Thr	Lys 420	Asp	Ala	Asp	Asn	Asp 425	Asn	Суз	Met	Сув	Lys 430	Сув	Ala
Leu	Met	Leu 435	Thr	Gly	Gly	Trp	Trp 440	Phe	Asp	Ala	Cys	Gly 445	Pro	Ser	Asn
Leu	Asn 450		Met	Phe	Tyr	Thr 455	Ala	Gly	Gln	Asn	His 460	Gly	Lys	Leu	Asn
Gly 465	Ile	Lys	Trp	His	T y r 470		Lys	Gly	Pro	Ser 475		Ser	Ile	Arg	Ser 480
	Thr	Met	Met	Ile 485		Pro	Leu	Asp	Phe 490	115					100
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Arg	Ser	Pro	Glu 20	Asn	_				10	-	сув	Thr	Thr	Gln 15	Arg
Gln	Cys	mh w			Ser	Gly	Arg	Arg 25		_				15	-
		35	Tyr	Thr		_	-	25	Phe	Asn	Arg	Ile	Gln 30	15 His	Gly
Glu	Ser 50	35	-		Phe	Ile	Leu 40	25 Pro	Phe Glu	Asn Gln	Arg Asp	Ile Gly 45	Gln 30 Asn	15 His Cys	Gly Arg
		35 Thr	- Thr	Asp	Phe Gln	Ile Tyr 55	Leu 40 Asn	25 Pro Thr	Phe Glu Asn	Asn Gln Ala	Arg Asp Leu 60	Ile Gly 45 Gln	Gln 30 Asn Arg	15 His Cys Asp	Gly Arg Ala
Pro 65	50	35 Thr Val	Thr Glu	Asp Gln	Phe Gln Asp 70	Ile Tyr 55 Phe	Leu 40 Asn Ser	25 Pro Thr Phe	Phe Glu Asn Gln	Asn Gln Ala Lys 75	Arg Asp Leu 60 Leu	Ile Gly 45 Gln Gln	Gln 30 Asn Arg His	15 His Cys Asp Leu	Gly Arg Ala Glu 80
Pro 65 His	50 His	35 Thr Val Met	Thr Glu Glu	Asp Gln Asn 85	Phe Gln Asp 70 Tyr	Ile Tyr 55 Phe Thr	Leu 40 Asn Ser Gln	25 Pro Thr Phe Trp	Phe Glu Asn Gln Leu 90	Asn Gln Ala Lys 75 Gln	Arg Asp Leu 60 Leu Lys	Ile Gly 45 Gln Gln Leu	Gln 30 Asn Arg His Glu	15 His Cys Asp Leu Ser 95	Gly Arg Ala Glu 80 Tyr
Pro 65 His Ile	50 His Val	35 Thr Val Met Glu	Thr Glu Glu Asn 100	Asp Gln Asn 85 Met	Phe Gln Asp 70 Tyr Lys	Ile Tyr 55 Phe Thr Ser	Leu 40 Asn Ser Gln Glu	25 Pro Thr Phe Trp Met 105	Phe Glu Asn Gln Leu 90 Ala	Asn Gln Ala Lys 75 Gln Gln	Arg Asp Leu 60 Leu Lys Leu	Ile Gly 45 Gln Leu Gln	Gln 30 Asn Arg His Glu Gln 110	15 His Cys Asp Leu Ser 95 Asn	Gly Arg Ala Glu 80 Tyr Ala
Pro 65 His Ile Val	50 His Val Val	35 Thr Val Met Glu Asn 115	Thr Glu Glu Asn 100 His	Asp Gln Asn 85 Met Thr	Phe Gln Asp 70 Tyr Lys Ala	Ile Tyr 55 Phe Thr Ser Thr	Leu 40 Asn Ser Gln Glu Met 120	25 Pro Thr Phe Trp Met 105 Leu	Phe Glu Asn Gln Leu 90 Ala Glu	Asn Gln Ala Lys 75 Gln Gln Ile	Arg Asp Leu 60 Leu Lys Leu Gly	Ile Gly 45 Gln Gln Leu Gln Thr 125	Gln 30 Asn Arg His Glu Glu Gln 110 Ser	15 His Cys Asp Leu Ser 95 Asn Leu	Gly Arg Ala Glu 80 Tyr Ala Leu
Pro 65 His Ile Val Ser	50 His Val Gln Gln	35 Thr Val Met Glu Asn 115 Thr	Thr Glu Glu Asn 100 His Ala	Asp Gln Asn 85 Met Thr Glu	Phe Gln Asp 70 Tyr Lys Ala Gln	Ile Tyr 55 Phe Thr Ser Thr 135	Leu 40 Asn Ser Gln Glu Met 120 Arg	25 Pro Thr Phe Trp Met 105 Leu Lys	Phe Glu Asn Gln Leu 90 Ala Glu Leu	Asn Gln Ala Lys 75 Gln Gln Ile Thr	Arg Asp Leu 60 Leu Lys Leu Gly Asp 140	Ile Gly 45 Gln Gln Leu Gln Thr 125 Val	Gln 30 Asn Arg His Glu Gln 110 Ser Glu	15 His Cys Asp Leu Ser 95 Asn Leu Thr	Gly Arg Ala Glu S0 Tyr Ala Leu Gln
Pro 65 His Ile Val Ser Val 145	50 His Val Val Gln Gln 130	35 Thr Val Met Glu Asn 115 Thr Asn	Thr Glu Glu Asn 100 His Ala Gln	Asp Gln Asn 85 Met Thr Glu Thr	Phe Gln Asp 70 Tyr Lys Ala Gln Ser 150	Ile Tyr 55 Phe Thr Ser Thr Thr 135 Arg	Leu 40 Asn Ser Gln Glu Met 120 Arg Leu	25 Pro Thr Phe Trp Met 105 Leu Lys Glu	Phe Glu Asn Gln Leu 90 Ala Glu Leu Ile	Asn Gln Ala Lys 75 Gln Gln Ile Thr Gln 155	Arg Asp Leu 60 Leu Lys Leu Gly Asp 140 Leu	Ile Gly 45 Gln Leu Gln Leu Val Leu	Gln 30 Asn Arg His Glu Glu Glu Glu Glu	15 His Cys Asp Leu Ser 95 Asn Leu Thr Asn	Gly Arg Ala Glu 80 Tyr Ala Leu Gln Ser 160

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Ile	Leu	Lys	Ile 180	His	Glu	Lys	Asn	Ser 185	Leu	Leu	Glu	His	Lys 190	Ile	Leu
Glu	Met	Glu 195	Glu	Arg	His	Lys	Glu 200	Glu	Met	Asp	Thr	Leu 205	Lys	Glu	Glu
Lys	Glu 210	Asn	Leu	Gln	Gly	Leu 215	Val	Thr	Arg	Gln	Ser 220	Tyr	Ile	Ile	Gln
Glu 225	Leu	Glu	Lys	Gln	Leu 230	Asn	Lys	Ala	Thr	Thr 235	Asn	Asn	Ser	Val	Leu 240
Gln	Lys	Gln	Gln	Leu 245	Glu	Leu	Met	Asp	Thr 250	Val	His	Thr	Leu	Ile 255	Thr
Leu	Сув	Ser	Lys 260	Glu	Gly	Val	Leu	Leu 265	Lys	Asn	Ala	Lys	Arg 270	Glu	Glu
Glu	Lys	Pro 275	Phe	Arg	Asp	Сув	Ala 280	Asp	Val	Tyr	Gln	Ala 285	Gly	Phe	Asn
Lys	Ser 290	Gly	Ile	Tyr	Thr	Ile 295	Tyr	Ile	Asn	Asn	Val 300	Ser	Asp	Pro	Lys
L y s 305	Val	Phe	Сув	Asn	Met 310	Asp	Val	Asn	Gly	Gly 315	Gly	Trp	Thr	Val	Ile 320
Gln	His	Arg	Glu	Asp 325	Gly	Ser	Leu	Asp	Phe 330	Gln	Lys	Gly	Trp	Lys 335	Glu
Tyr	Lys	Met	Gly 340	Phe	Gly	Ser	Pro	Ser 345	Gly	Glu	Tyr	Trp	Leu 350	Gly	Asn
Glu	Phe	Ile 355	Phe	Ala	Ile	Thr	Ser 360	Gln	Arg	Gln	Tyr	Ser 365	Leu	Arg	Ile
Glu	Leu 370	Met	Asp	Trp	Glu	Gly 375	Asn	Arg	Ala	Tyr	Ser 380	Gln	Tyr	Asp	Arg
Phe 385	His	Ile	Gly	Asn	Glu 390	Lys	Gln	Asn	Tyr	Arg 395	Leu	Tyr	Leu	Lys	Gl y 400
His	Ser	Gly	Thr	Ala 405	Gly	Lys	Gln	Ser	Ser 410	Leu	Ile	Leu	His	Gly 415	Ala
Glu	Phe	Ser	Thr 420	Lys	Asp	Ala	Asp	Asn 425	Asp	Asn	Сув	Met	Cys 430	Lys	Сув
Ala	Leu	Met 435	Leu	Thr	Gly	Gly	Trp 440	Trp	Phe	Asp	Ala	С у в 445	Gly	Pro	Ser
Asn	Leu 450	Asn	Gly	Met	Phe	T y r 455	Thr	Ala	Gly	Gln	Asn 460	His	Gly	Lys	Leu
Asn 465	Gly	Ile	Lys	Trp	His 470	Tyr	Phe	Lys	Gly	Pro 475	Arg	Tyr	Ser	Ile	Arg 480
Ser	Thr	Thr	Met	Met 485	Ile	Arg	Pro	Leu	Asp 490	Phe					
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Tyr	Asn	Arg 35	Ile	Gln	His	Gly	Gln 40	Сув	Ala	Tyr	Thr	Phe 45	Ile	Leu	Pro
Glu	His	Asp	Gly	Asn	Суз	Arg	Glu	Ser	Thr	Thr	Asp	Gln	Tyr	Asn	Thr

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Asn 65	Ala	Leu	Gln	Arg	Asp 70	Ala	Pro	His	Val	Glu 75	Pro	Asp	Phe	Ser	Ser 80
Gln	Lys	Leu	Gln	His 85	Leu	Glu	His	Val	Met 90	Glu	Asn	Tyr	Thr	Gln 95	Trp
Leu	Gln	Lys	Leu 100	Glu	Asn	Tyr	Ile	Val 105	Glu	Asn	Met	Lys	Ser 110	Glu	Met
Ala	Gln	Ile 115	Gln	Gln	Asn	Ala	Val 120	Gln	Asn	His	Thr	Ala 125	Thr	Met	Leu
Glu	Ile 130	Gly	Thr	Ser	Leu	Leu 135	Ser	Gln	Thr	Ala	Glu 140	Gln	Thr	Arg	Lys
Leu 145	Thr	Asp	Val	Glu	Thr 150	Gln	Val	Leu	Asn	Gln 155	Thr	Ser	Arg	Leu	Glu 160
Ile	Gln	Leu	Leu	Glu 165	Asn	Ser	Leu	Ser	Thr 170	Tyr	Lys	Leu	Glu	L y s 175	Gln
Leu	Leu	Gln	Thr 180	Asn	Glu	Ile	Leu	L y s 185	Ile	His	Glu	Lys	Asn 190	Ser	Leu
Leu	Glu	His 195	Lys	Ile	Leu	Glu	Met 200	Glu	Gly	Lys	His	L y s 205	Glu	Glu	Met
Asp	Thr 210	Leu	Lys	Glu	Glu	L y s 215	Glu	Asn	Leu	Gln	Gly 220	Leu	Val	Ser	Arg
Gln 225	Ser	Phe	Ile	Ile	Gln 230	Glu	Leu	Glu	Lys	Gln 235	Leu	Ser	Arg	Ala	Thr 240
Asn	Asn	Asn	Ser	Ile 245	Leu	Gln	Lys	Gln	Gln 250	Leu	Glu	Leu	Met	Asp 255	Thr
Val	His	Asn	Leu 260	Ile	Ser	Leu	Суз	Thr 265	Lys	Glu	Gly	Val	Leu 270	Leu	Lys
Gly	Gly	L y s 275	Arg	Glu	Glu	Glu	L y s 280	Pro	Phe	Arg	Asp	C y s 285	Ala	Asp	Val
Tyr	Gln 290	Ala	Gly	Phe	Asn	L y s 295	Ser	Gly	Ile	Tyr	Thr 300	Ile	Tyr	Phe	Asn
Asn 305	Val	Pro	Glu	Pro	L y s 310	Lys	Val	Phe	Сув	Asn 315	Met	Asp	Val	Asn	Gly 320
Gly	Gly	Trp	Thr	Val 325	Ile	Gln	His	Arg	Glu 330	Asp	Gly	Ser	Leu	Asp 335	Phe
Gln	Lys	Gly	Trp 340	Lys	Glu	Tyr	Lys	Met 345	Gly	Phe	Gly	Ser	Pro 350	Ser	Gly
Glu	Tyr	Trp 355	Leu	Gly	Asn	Glu	Phe 360	Ile	Phe	Ala	Ile	Thr 365	Ser	Gln	Arg
Gln	Tyr 370	Met	Leu	Arg	Ile	Glu 375	Leu	Met	Asp	Trp	Glu 380	Gly	Asn	Arg	Ala
T y r 385	Ser	Gln	Tyr	Asp	Arg 390	Phe	His	Ile	Gly	Asn 395	Glu	Lys	Gln	Asn	Tyr 400
Arg	Leu	Tyr	Leu	L y s 405	Gly	His	Thr	Gly	Thr 410	Ala	Gly	Lys	Gln	Ser 415	Ser
Leu	Ile	Leu	His 420	Gly	Ala	Asp	Phe	Ser 425	Thr	Lys	Asp	Ala	Asp 430	Asn	Asp
Asn	Суз	Met 435	Суз	Lys	Суз	Ala	Leu 440	Met	Leu	Thr	Gly	Gly 445	Trp	Trp	Phe
Asp	Ala 450	Сув	Gly	Pro	Ser	Asn 455	Leu	Asn	Gly	Met	Phe 460	Tyr	Thr	Ala	Gly
Gln 465	Asn	His	Gly	Lys	Leu 470	Asn	Gly	Ile	Lys	Trp 475	His	Tyr	Phe	Lys	Gly 480

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Arg Tyr Arg Ile Gln Asn Gly Pro Cys Ala Tyr Thr Phe Leu Leu Pro354045
Glu Thr Asp Ser Gly Arg Ser Ser Ser Ser Thr Tyr Met Thr Asn Ala 50 55 60
Val Gln Arg Asp Ala Pro Pro Asp Tyr Glu Asp Ser Val Gln Ser Leu 65 70 75 80
Gln Leu Glu Asn Val Met Glu Asn Tyr Thr Gln Trp Leu Met Lys 85 90 95
Leu Glu Asn Tyr Ile Gln Asp Asn Met Lys Lys Glu Met Ala Glu Ile 100 105 110
Gln Gln Asn Val Gln Asn His Thr Ala Val Met Ile Glu Ile Gly 115 120 125
Thr Ser Leu Leu Ser Gln Thr Ala Glu Gln Thr Arg Lys Leu Thr Asp 130 135 140
Val Glu Thr Gln Val Leu Asn Gln Thr Thr Arg Leu Glu Leu Gln Leu 145 150 155 160
Leu Gln His Ser Ile Ser Thr Tyr Lys Leu Glu Lys Gln Ile Leu Asp 165 170 175
Gln Thr Ser Glu Ile Asn Lys Ile His Asn Lys Asn Ser Phe Leu Glu 180 185 190
Gln Lys Val Leu Asp Met Glu Gly Lys His Ser Glu Glu Met Gln Thr 195 200 205
Met Lys Glu Gln Lys Asp Glu Leu Gln Val Leu Val Ser Lys Gln Ser 210 215 220
Ser Val Ile Asp Glu Leu Glu Lys Lys Leu Val Thr Ala Thr Val Asn225230235240
Asn Ser Leu Leu Gln Lys Gln Gln His Asp Leu Met Asp Thr Val Asn 245 250 255
Ser Leu Leu Thr Met Met Ser Ser Pro Asn Ser Lys Ser Ser Leu Ala 260 265 270
Ile Arg Arg Glu Glu Gln Thr Thr Phe Arg Asp Cys Ala Asp Val Phe275280285
LysAla GlyLeuThrLysSerGlyIleThrLeuThrProAsn290295300
Ser Pro Glu Glu Ile Lys Ala Tyr Cys Asn Met Asp Val Gly Gly Gly305310315320
Gly Trp Thr Val Ile Gln His Arg Glu Asp Gly Ser Leu Asp Phe Gln 325 330 335
Lys Gly Trp Lys Glu Tyr Lys Met Gly Phe Gly Asn Pro Leu Gly Glu 340 345 350

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Tyr Trp	Leu Gly 355	Asn	Glu	Phe	Ile 360	Ser	Gln	Ile	Thr	Gly 365	Gln	His	Arg
Tyr Val 370	Leu Lys	Ile	Gln	Leu 375	Lys	Asp	Trp	Glu	Gly 380	Asn	Glu	Ala	His
Ser Leu 385	Tyr Asp	His	Phe 390	Tyr	Ile	Ala	Gly	Glu 395	Glu	Ser	Asn	Tyr	Arg 400
Ile His I	Leu Thr	Gly 405	Leu	Thr	Gly	Thr	Ala 410	Ala	Lys	Ile	Ser	Ser 415	Ile
Ser Gln 3	Pro Gly 420	Ser	Asp	Phe	Ser	Thr 425	Lys	Asp	Ser	Asp	Asn 430	Asp	Lys
Cys Ile	Сув Lув 435	Cys	Ser	Leu	Met 440	Leu	Thr	Gly	Gly	Trp 445	Trp	Phe	Азр
Ala Cys 450	Gly Pro	Ser	Asn	Leu 455	Asn	Gly	Gln	Phe	Tyr 460	Pro	Gln	Lys	Gln
Asn Thr . 465	Asn Lys	Phe	Asn 470	Gly	Ile	Lys	Trp	T y r 475	Tyr	Trp	Lys	Gly	Ser 480
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Ala Ala '	T y r Asn 20	Asn	Phe	Arg	Lys	Ser 25	Met	Asp	Ser	Ile	Gly 30	Lys	Lys
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Gln Leu I	Leu Glu	Asn 85	Val	Met	Glu	Asn	Tyr 90	Thr	Gln	Trp	Leu	Met 95	Lys
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Gln Gln .	Asn Ala 115	Val	Gln	Asn	His 120	Thr	Ala	Val	Met	Ile 125	Glu	Ile	Gly
Thr Ser 1 130	Leu Leu	Ser	Gln	Thr 135	Ala	Glu	Gln	Thr	Arg 140	Lys	Leu	Thr	Asp
Val Glu 145	Thr Gln	Val	Leu 150	Asn	Gln	Thr	Thr	Arg 155	Leu	Glu	Leu	Gln	Leu 160
Leu Gln :	His Ser	Ile 165	Ser	Thr	Tyr	Lys	Leu 170	Glu	Lys	Gln	Ile	Leu 175	Азр
Gln Thr	Ser Glu 180	Ile	Asn	Lys	Ile	His 185	Asp	Lys	Asn	Ser	Phe 190	Leu	Glu
Lys Lys '	Val Leu 195	Asp	Met	Glu	Asp 200	Lys	His	Ile	Ile	Glu 205	Met	Gln	Thr
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Val	Ala	Arg 275	Glu	Glu	Gln	Ile	Ser 280	Phe	Arg	Asp	Cys	Ala 285	Asp	Val	Phe				
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Gly	Trp	Thr	Ile	Ile 325	Gln	Arg	Arg	Glu	Asp 330	Gly	Ser	Leu	Asp	Phe 335	Gln				
Lys	Gly	Trp	Lys 340	Glu	Tyr	Lys	Val	Gly 345	Phe	Gly	Ser	Pro	Ser 350	Gly	Glu				
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Tyr	Val 370	Leu	Lys	Ile	His	Leu 375	Lys	Asp	Trp	Glu	Gly 380		Glu	Ala	Tyr				
Ser 385	Leu	Tyr	Asp	His	Phe 390	Tyr	Ile	Ser	Gly	Glu 395	Glu	Leu	Asn	Tyr	Arg 400				
Ile	His	Leu	Lys	Gly 405	Leu	Thr	Gly	Thr	Ala 410	Ala	Lys	Ile	Ser	Ser 415	Ile				
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Сув	Ile	C y s 435	Lys	Cys	Ser	Leu	Met 440	Leu	Thr	Gly	Gly	Trp 445	Trp	Phe	Asp				
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														atc Ile		336
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-			-		-	-			-	-			-	caa Gln	-	1152

_	С	o	n	t	i	n	u	е	d	

<pre>try and args are set args are the per case has man cat the case of g gues</pre>												_	con	CTI	ueu		
Ser Glu Aan Chi Leen Tyr Arg Lee Ser Val Val Gly Tyr Ser Gly Ser 410 410 410 410 410 411 412 411 412 411 412 412 412	Trp	-				Ala		-	-		Glu				-	Gly	1200
Ala Giy Arg Oin Ser Ser Lei Val Lei Cin Aen Thr Ser Phe Ser Thr 430 Ctt gac taa gac aac gac cac tgt ctt ga aag tgt goc cag gtg atg 1344 Leu Aep Ser Aep Aen Aep Bic Cys Leu Cys Lys Cys Ala Gin Val Met 1344 tot gag gt gg tgg tgt tg ac goc tgt ggc ctg ta aac ctc aac ggc 1392 ser Giy Giy Trp Trp Phe Asp Ala Cys Giy Leu Ser Aen Leu Aen Ciy 1400 465 410 440 465 410 410 465 410 410 465 410 410 465 410 410 465 410 410 465 410 410 465 410 410 465 410 410 465 410 410 465 410 410 465 410 410 465 410 410 465 410 410 450 420 420 451 420 411 451 420 410 451 420 420 421 420 420					Leu					Val					Gly		1248
Len Åeg Ser Åeg Aen Åeg His c'ys Len c'ys Lys c'ys Åla Gln Val Met 4435 tt dyg dyg tgg tgg tgg tt ga cg oc tgt gg octg tca aac oto aac gg Ser Gly Gly Trp Trp Phe Åeg Ala Cys Gly Leu Ser Aen Leu Aen Gly 450 1392 gto tac tac cac gdt coc gac aac ag tac aag tac ag tg gac gg ot cc ogc 470 470 470 470 470 470 470 470	-		-	Gln	-	-	-	-	Leu	-			-	Phe	-		1296
Ser dily dily Trp Trp Phe Åep Åla Cys dily Leu Ser Aan Leu Aan dily 450 455 460 460 Val Tyr Tyr His Åla Pro Åep Åan Lys Tyr Lys Met Åep Gly Ile Årg 1440 465 470 475 480 1 Tyr Tyr His Åla Pro Åep Åan Lys Tyr Lys Met Åep Gly Ile Årg 1480 1 Typ His Tyr Phe Lys Gly Pro Ser Tyr Ser Leu Årg Åla Ser Årg Net 495 atg ata cgg oct tt gg ac ato taa 1512 atg ata cgg oct tt gg ac ato taa 1512 212> TPE D Leu Åap Tile 500 212> TPE PRT 213> OKGANISM: Homo sepiens <400> SEQUENCE: 18 10 Met Lee Ser Glu Leu Åla Met Leu Glu Gly Ser Leu Leu Leu Val Val 1 10 213< OKGANISM: Homo sepiens			Ser					Cys					Āla				1344
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Asn 225	Thr	Leu	Ser	Arg	Gln 230	Ser	Ala	Ala	Leu	Thr 235	Asn	Ile	Glu	Arg	Gly 240	
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Met Thr Val 1 Ile Gly Cys Tyr Asn Arg	Phe Leu 5 Ser Asn 20 Ile Gln	Gln Arg His Gly	Arg Sen 25 Gln Cys 40	e Leu 10 c Pro s Ala	Ala Glu Tyr	Ala Asn Thr	Ile Ser Phe 45	Leu Gly 30 Ile	Thr 15 Arg Leu	His Arg Pro	
Met Thr Val 1 Ile Gly Cys Tyr Asn Arg 35 Glu His Asp	Phe Leu 5 Ser Asn 20 Ile Gln Gl y Asn	Gln Arg His Gly Cys Arg 55	Arg Sen 25 Gln Cys 40 Glu Sen	e Leu 10 : Pro s Ala : Thr	Ala Glu Tyr Thr	Ala Asn Thr Asp 60	Ile Ser Phe 45 Gln	Leu Gly 30 Ile Tyr	Thr 15 Arg Leu Asn	His Arg Pro Thr	
Met Thr Val 1 Ile Gly Cys Tyr Asn Arg 35 Glu His Asp 50 Asn Ala Leu	Phe Leu 5 Ser Asn 20 Ile Gln Gly Asn Gln Arg	Gln Arg His Gly Cys Arg 55 Asp Ala 70	Arg Sen 25 Gln Cys 40 Glu Sen Pro His	E Leu 10 F Pro S Ala F Thr S Val	Ala Glu Tyr Thr Glu 75	Ala Asn Thr 60 Pro	Ile Ser Phe 45 Gln Asp	Leu Gly 30 Ile Tyr Phe	Thr 15 Arg Leu Asn Ser	His Arg Pro Thr Ser 80	
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Ile	Gln	Leu	Leu	Glu 165	Asn	Ser	Leu	Ser	Thr 170	Tyr	Lys	Leu	Glu	L y s 175	Gln
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Leu	A sp 210	Thr	Leu	Lys	Glu	Glu 215	Lys	Glu	Asn	Leu	Gln 220	Gly	Leu	Val	Thr
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Thr	Val	His	Asn 260	Leu	Val	Asn	Leu	С у в 265	Thr	Lys	Glu	Gly	Val 270	Leu	Leu
Lys	Gly	Gly 275	Lys	Arg	Glu	Glu	Glu 280	Lys	Pro	Phe	Arg	As p 285	Cys	Ala	Glu
Val	Phe 290	Lys	Ser	Gly	His	Thr 295	Thr	Asn	Gly	Ile	Tyr 300	Thr	Leu	Thr	Phe
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Phe	Gln	Arg	Thr 340	Trp	Lys	Glu	Tyr	Lys 345	Val	Gly	Phe	Gly	Asn 350	Pro	Ser
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Gln	Arg 370	Tyr	Val	Leu	Lys	Ile 375	His	Leu	Lys	Asp	Trp 380	Glu	Gly	Asn	Glu
Ala 385	Tyr	Ser	Leu	Tyr	Glu 390	His	Phe	Tyr	Leu	Ser 395	Ser	Glu	Glu	Leu	Asn 400
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	-				aaa Lys	-										912

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-		aca gca gga aaa cag agc agc Thr Ala Gly Lys Gln Ser Ser 410 415	•
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Glu Met Asp Asn 50	Cys Arg Ser Ser 55	Ser Ser Pro Tyr Val Ser Asn 60	Ala
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Gln Val Leu Glu	Asn Ile Met Glu	Asn Asn Thr Gln Trp Leu Met	Lys

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Gln	Gln	Asn 115	Ala	Val	Gln	Asn	Gln 120	Thr	Ala	Val	Met	Ile 125	Glu	Ile	Gly
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Gln	Thr	Ser	Glu 180	Ile	Asn	Lys	Leu	Gln 185	Asp	Lys	Asn	Ser	Phe 190	Leu	Glu
Lys	Lys	Val 195	Leu	Ala	Met	Glu	Asp 200	Lys	His	Ile	Ile	Gln 205	Leu	Gln	Ser
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Tyr	Met 370	Leu	Arg	Ile	Glu	Leu 375	Met	Asp	Trp	Glu	Gly 380	Asn	Arg	Ala	Tyr
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Ile	Leu	His	Gly 420	Ala	Asp	Phe	Ser	Thr 425	Lys	Asp	Ala	Asp	Asn 430	Asp	Asn
Сув	Met	Сув 435	Lys	Суз	Ala	Leu	Met 440	Leu	Thr	Gly	Gly	Trp 445	Trp	Phe	Asp
Ala	C y s 450	Gly	Pro	Ser	Asn	Leu 455	Asn	Gly	Met	Phe	T y r 460	Thr	Ala	Gly	Gln
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	tg aag et Lys						-	-		-	-		-	-	336
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Ğlu I	ta ggg le Gl y 30								-						432
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Leu G	ag tca ln Ser 10			-			-	-		-			-		672
	aa aat ln Asn														720
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cag caa cgc tat gtg ctt aaa ata cac ctt aaa gac tgg gaa ggg aat 1152 Gln Gln Arg Tyr Val Leu Lys Ile His Leu Lys Asp Trp Glu Gly Asn 370 375 380		1152					Asp					Lys					Gln	
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aat tat agg att cac ctt aaa gga ctt aca ggg aca gcc ggc aaa ata 1248 Asn Tyr Arg Ile His Leu Lys Gly Leu Thr Gly Thr Ala Gly Lys Ile 405 410 415		1248		Lys					Thr					His				
agc agc atc agc caa cca gga aat gat ttt agc aca aag gat gga gac 1296 Ser Ser Ile Ser Gln Pro Gly Asn Asp Phe Ser Thr Lys Asp Gly Asp 420 425 430		1296	-		Asp	-		-		Asp					Ser		-	-
aac gac aaa tgt att tgc aaa tgt tca caa atg cta aca gga ggc tgg 1344 Asn Asp Lys Cys Ile Cys Lys Cys Ser Gln Met Leu Thr Gly Gly Trp 435 440 445		1344				Thr		-			Cys		-		-	Lys	-	
tgg ttt gat gca tgt ggt cct tcc aac ttg aac gga atg tac tat cca 1392 Trp Phe Asp Ala Cys Gly Pro Ser Asn Leu Asn Gly Met Tyr Tyr Pro 450 455 460		1392				-	Gly		-			Pro		-	-	-	Phe	
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Ile Gly Cys Ser Asn Gln Arg Arg Ser Pro Glu Asn Ser Gly Arg Arg 20 25 30			Arg	Arg	_	Ser	Asn	Glu	Pro		Arg	Arg	Gln	Asn		Cys	Gly	Ile
Tyr Asn Arg Ile Gln His Gly Gln Cys Ala Tyr Thr Phe Ile Leu Pro			Pro	Leu	Ile	Phe	Thr	Tyr	Ala	Сув	Gln	Gly	His	Gln	Ile	Arg	Asn	Tyr

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Asn 65	Ala	Leu	Gln	Arg	Asp 70	Ala	Pro	His	Val	Glu 75	Pro	Asp	Asp	Ser	Val 80
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Glu	Ile 130	Gly	Thr	Asn	Leu	Leu 135	Asn	Gln	Thr	Ala	Glu 140	Gln	Thr	Arg	Lys
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Leu	Gln 210	Ser	Ile	Lys	Glu	Glu 215	Lys	Asp	Gln	Leu	Gln 220	Val	Leu	Val	Ser
L y s 225	Gln	Asn	Ser	Ile	Ile 230	Glu	Glu	Leu	Glu	L y s 235	Lys	Ile	Val	Thr	Ala 240
Thr	Val	Asn	Asn	Ser 245	Val	Leu	Gln	Lys	Gln 250	Gln	His	Asp	Leu	Met 255	Glu
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Asp	Pro	Thr 275	Val	Ala	Lys	Glu	Glu 280	Gln	Ile	Ser	Phe	Arg 285	Asp	Суз	Ala
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Phe 305	Pro	Asn	Ser	Thr	Glu 310	Glu	Ile	Lys	Ala	Tyr 315	Сув	Asp	Met	Glu	Ala 320
Gly	Gly	Gly	Gly	Trp 325	Thr	Ile	Ile	Gln	Arg 330	Arg	Glu	Asp	Gly	Ser 335	Val
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Ser	Gly	Glu 355	Tyr	Trp	Leu	Gly	Asn 360	Glu	Phe	Val	Ser	Gln 365	Leu	Thr	Asn
Gln	Gln 370	Arg	Tyr	Val	Leu	L y s 375	Ile	His	Leu	Lys	Asp 380	Trp	Glu	Gly	Asn
Glu 385	Ala	Tyr	Ser	Leu	T y r 390	Glu	His	Phe	Tyr	Leu 395	Ser	Ser	Glu	Glu	Leu 400
	-	-		405		-	Gly		410	-			-	415	
			420			_	Asn	425				-	430	-	-
Asn	Asp	Lys 435	Суз	Ile	Суз	Lys	Cys 440	Ser	Gln	Met	Leu	Thr 445	Gly	Gly	Trp
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ctt gag aat tac a Leu Glu Asn Tyr I 100				336
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	agt Ser															768
	ctt Leu	-			-			-		-			-			816
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-	ggt Gl y 290				-										-	912
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	act Thr	-				-	-	-		-		-			-	1008
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⁻continued

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Ala Ala Ty	r Asn Asr 20	n Phe Are	g Lys	Ser 25	Met	Asp	Ser	Ile	Gly 30	Lys	Lys
Gln Tyr Gl 35	n Val Glr	h His Gly	y Ser 40	Cys	Ser	Tyr	Thr	Phe 45	Leu	Leu	Pro
Glu Met As 50	p Asn Cys	a Arg Se: 55	s Ser	Ser	Ser	Pro	Tyr 60	Val	Ser	Asn	Ala
Val Gln Ar 65	g Asp Ala	a Pro Le 70	ı Glu	Tyr	Asp	Phe 75	Ser	Ser	Gln	Lys	Leu 80
Gln His Le	u Glu His 85	s Val Me [.]	: Glu	Asn	Tyr 90	Thr	Gln	Trp	Leu	Gln 95	Lys
Leu Glu As	n Tyr Ile 100	e Val Gli	ı Asn	Met 105	Lys	Ser	Glu	Met	Ala 110	Gln	Ile
Gln Gln As 11		. Gln Ası	n His 120	Thr	Ala	Thr	Met	Leu 125	Glu	Ile	Gly
Thr Ser Le 130	u Leu Ser	Gln Th: 13		Glu	Gln	Thr	Arg 140	Lys	Leu	Thr	Asp
Val Glu Th 145	r Gln Val	Leu Ası 150	ı Gln	Thr	Ser	Arg 155	Leu	Glu	Ile	Gln	Leu 160
Leu Glu As	n Ser Leu 165		r Tyr	Lys	Leu 170	Glu	Lys	Gln	Leu	Leu 175	Gln
Gln Thr As	n Glu Ile 180	e Leu Ly:	s Ile	His 185	Glu	Lys	Asn	Ser	Leu 190	Leu	Glu
His Lys Il 19		1 Met Gli	1 Gly 200	Lys	His	Lys	Glu	Glu 205	Leu	Asp	Thr
Leu Lys Gl 210	u Glu Lys	s Glu Ası 21		Gln	Gly	Leu	Val 220	Thr	Arg	Gln	Thr
Tyr Ile Il 225	e Gln Glu	Leu Gli 230	ı Lys	Gln	Leu	Asn 235	Arg	Ala	Thr	Thr	Asn 240
Asn Ser Va	l Leu Glr 245	-	ı Gln	Leu	Glu 250	Leu	Met	Asp	Thr	Val 255	His
Asn Leu Va	l Asn Leu 260	ı Cys Th:	: Lys	Glu 265	Gly	Val	Leu	Leu	L y s 270	Gly	Gly
Lys Arg Gl 27		ı Lys Pro	280 Phe	Arg	Asp	Суз	Ala	Asp 285	Val	Tyr	Gln
Ala Gly Ph 290	e Asn Lys	s Ser Gly 29		Tyr	Thr	Ile	T y r 300	Ile	Asn	Asn	Met
Pro Glu Pr 305	o Lys Lys	s Val Phe 310	e Cys	Asn	Met	Asp 315	Val	Asn	Gly	Gly	Gly 320
Trp Thr Va	l Ile Glr 325		g Glu	Asp	Gly 330	Ser	Leu	Asp	Phe	Gln 335	Arg
Gly Trp Ly	s Glu Tyr 340	: Lys Me [.]	: Gly	Phe 345	Gly	Asn	Pro	Ser	Gly 350	Glu	Tyr
Trp Leu Gl 35	-	1 Phe Ile	e Phe 360	Ala	Ile	Thr	Ser	Gln 365	Arg	Gln	Tyr
Met Leu Ar 370	g Ile Glu	ı Leu Me [.] 37!		Trp	Glu	Gly	Asn 380	Arg	Ala	Tyr	Ser
Gln Tyr As 385	p Arg Phe	e His Ile 390	e Gly	Asn	Glu	L y s 395	Gln	Asn	Tyr	Arg	Leu 400
Tyr Leu Ly	s Gly Hia 405		7 Thr	Ala	Gly 410	Lys	Gln	Ser	Ser	Leu 415	Ile

-COI		

-continued
Leu His Gly Ala Asp Phe Ser Thr Lys Asp Ala Asp Asn Asp Asn Cys 420 425 430
Met Cys Lys Cys Ala Leu Met Leu Thr Gly Gly Trp Trp Phe Asp Ala 435 440 445
Cys Gly Pro Ser Asn Leu Asn Gly Met Phe Tyr Thr Ala Gly Gln Asn 450 455 460
His Gly Lys Leu Asn Gly Ile Lys Trp His Tyr Phe Lys Gly Pro Ser 465 470 475 480
Tyr Ser Leu Arg Ser Thr Thr Met Met Ile Arg Pro Leu Asp Phe 485 490 495
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What is claimed is:

55

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1. An isolated mature modified TIE-2 ligand encoded by an isolated nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of:

- (a) a nucleotide sequence encoding the mature modified TIE-2 ligand as set forth by nucleotides 49–1485 in ⁶⁰ FIG. **27** (SEQ ID NO: 25); and
- (b) a nucleotide sequence that, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a) and which encodes the mature modified TIE-2 ligand of (a).

2. An isolated mature modified TIE-2 ligand encoded by an isolated nucleic acid molecule, which is modified to encode a serine amino acid residue instead of the cysteine amino acid residue encoded by nucleotides 784–786 set forth in FIG. **27** (SEQ ID NO: 25).

3. An isolated mature modified TIE-2 ligand encoded by the isolated nucleic acid molecule of claim **2**, which is further modified to encode a serine amino acid residue instead of the arginine amino acid residue encoded by nucleotides 199–201 set forth in FIG. **27** (SEQ ID NO: 25).

4. An isolated modified TIE-2 ligand encoded by the isolated nucleic acid molecule of claim 2, which is further modified to encode a different amino acid residue instead of the cysteine amino acid residue encoded by nucleotides 784–786 set forth in FIG. 27 (SEQ ID NO: 25), wherein the

different amino acid residue is selected from the group consisting of glycine, threonine, tyrosine, asparagine and glutamine.

5. An isolated modified TIE-2 ligand encoded by the isolated nucleic acid molecule of claim **3**, which is further 5 modified to encode a different amino acid residue instead of

the arginine amino acid residue encoded by nucleotides 199–201 set forth in FIG. **27** (SEQ ID NO: 25), wherein the different amino acid residue is selected from the group consisting of lysine and histidine.

* * * * *

patsnap

专利名称(译)	表达配体 - 血管细胞间信号分子								
公开(公告)号	US6441137	公开(公告)日	2002-08-27						
申请号	US09/709188	申请日	2000-11-09						
[标]申请(专利权)人(译)	再生元医药公司								
申请(专利权)人(译)	REGENERON制药公司.								
当前申请(专利权)人(译)	REGENERON制药公司.								
[标]发明人	DAVIS SAMUEL YANCOPOULOS GEORGE D								
发明人	DAVIS, SAMUEL YANCOPOULOS, GEORGE D.								
IPC分类号	C07K14/515 C07K14/435 C07K14/ A61K48/00 A61K51/00 A61P7/00 A C07K16/22 C07K19/00 C12N1/19 C12N15/63 C12P21/02 C12P21/08	A61P7/02 A61P9/00 A61P17/02 C12N1/21 C12N5/02 C12N5/10							
CPC分类号	C07K14/515 C07K14/71 C12N15/8509 A01K2207/15 A01K2217/00 A01K2217/05 A01K2217/075 A01K2227/105 A01K2227/30 A01K2267/01 A01K2267/03 A01K2267/0331 A01K2267/0375 A01K2267 /0381 A61K38/00 C07K2319/00 C07K2319/02 A61P17/02 A61P27/00 A61P29/00								
助理审查员(译)	DECLOUX , AMY								
外部链接	Espacenet <u>USPTO</u>								

摘要(译)

本发明提供了修饰的TIE-2配体,其通过添加,缺失或取代一个或多个氨基酸或通过标记,例如人IgG-1的Fc部分而改变,但保留了它结合TIE-2 受体的能力。本发明进一步提供了修饰的TIE-2配体,其是嵌合TIE-2配体,其包含至少一部分第一TIE-2配体和一部分第二TIE-2配体,其不同 于第一配体。在一个具体实施方案中,本发明还提供嵌合TIE配体,其包 含至少一部分TIE-2配体-1和一部分TIE-2配体-2。此外,本发明提供了编 码所述修饰的TIE-2配体的分离的核酸分子。本发明还提供治疗组合物以 及阻断血管生长的方法,促进新血管形成的方法,促进表达TIE受体的细 胞生长或分化的方法,阻断生长或分化的方法。表达TIE受体的细胞和减 轻或预防人体肿瘤生长的方法。



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