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(54) OLD-35 AS AN INFLAMMATORY AGENT

Publication Classification

(76) Inventors: Paul B. Fisher, Scarsdale, NY (US); Devanand Sarkar, Elmsford, NY (US)

Correspondence Address:
BAKER BOTTS L.L.P.
30 ROCKEFELLER PLAZA
44TH FLOOR
NEW YORK, NY 10112-4498 (US)

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C12Q 1/68 (2006.01)
A61P 25/28 (2006.01)
A61K 31/7088 (2006.01)

(52) U.S. Cl. 424/130.1; 435/6; 436/94; 514/44; 536/23.1; 800/12; 800/9

(21) Appl. No.: 11/784,096

(57)

ABSTRACT

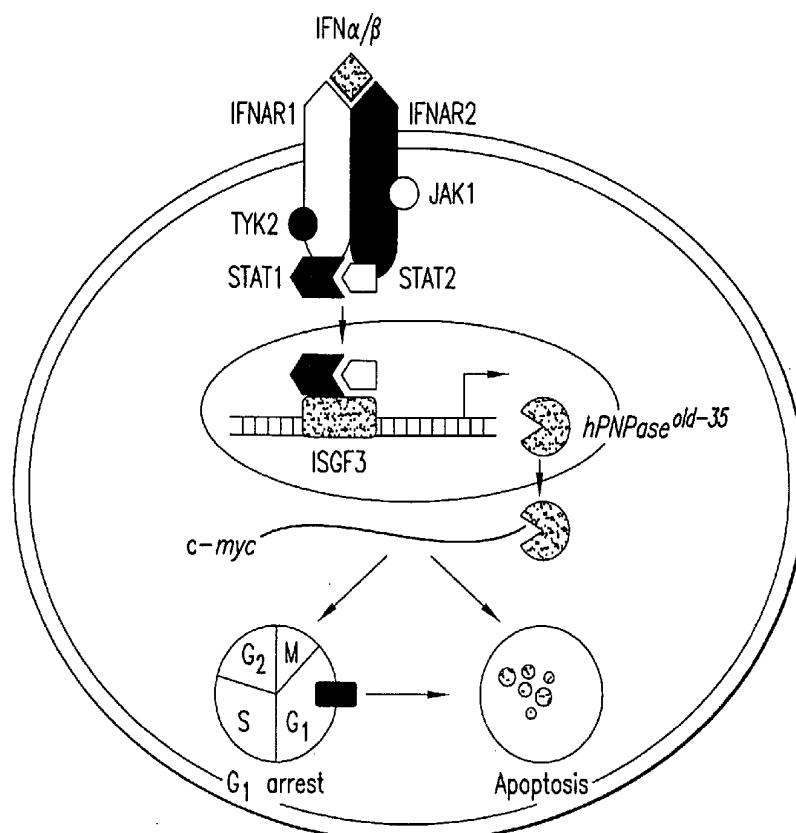
(22) Filed: Apr. 5, 2007

The present invention relates to the discovery that OLD-35, at least in part through the generation of reactive oxygen species, induces a number of inflammatory cytokines and promotes nuclear translocation and binding of the transcriptional activator NF- κ B. Accordingly, the present invention provides for assay systems (which either utilize the old-35 promoter or the old-35 gene) that may be used to identify new anti-inflammatory agents; model systems of inflammation based on over-expression of the old-35 gene in cells and tissues (including specific model systems for arthritis, atherosclerosis and Alzheimer's disease); methods and kits for diagnosing old-35 associated inflammatory conditions, and methods of treatment and anti-inflammatory compositions that utilize agents that antagonize OLD-35 activity.

Related U.S. Application Data

(63) Continuation of application No. PCT/US05/36409, filed on Oct. 7, 2005.

(60) Provisional application No. 60/616,774, filed on Oct. 7, 2004.



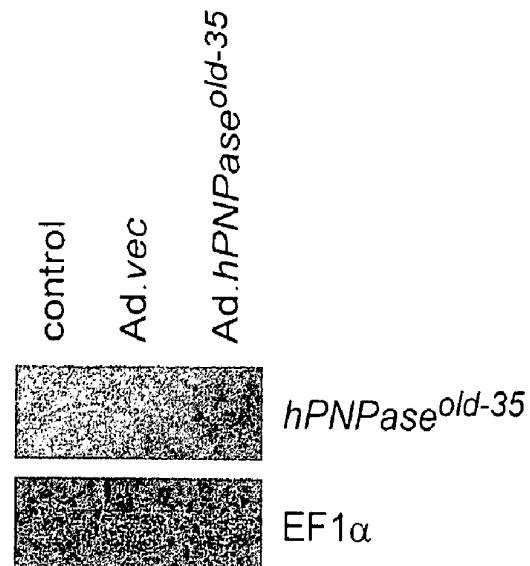


FIG. 1A

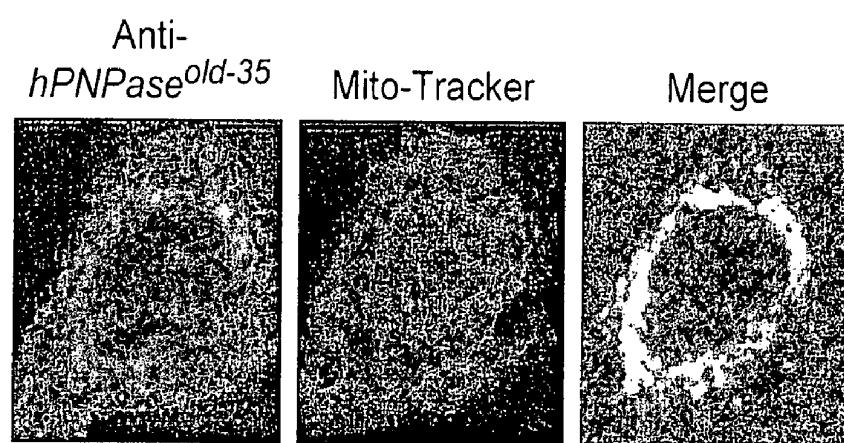


FIG. 1B

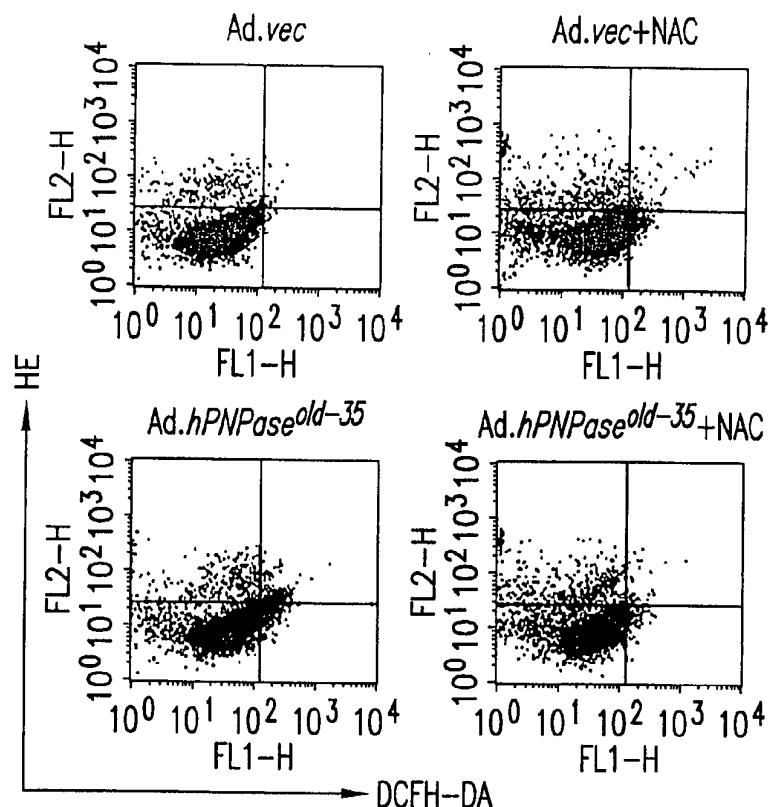


FIG. 1C

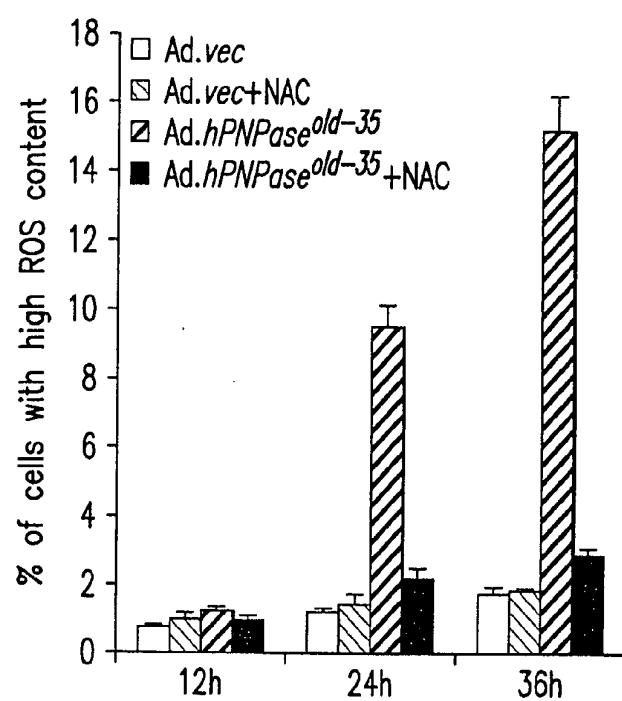


FIG. 1D

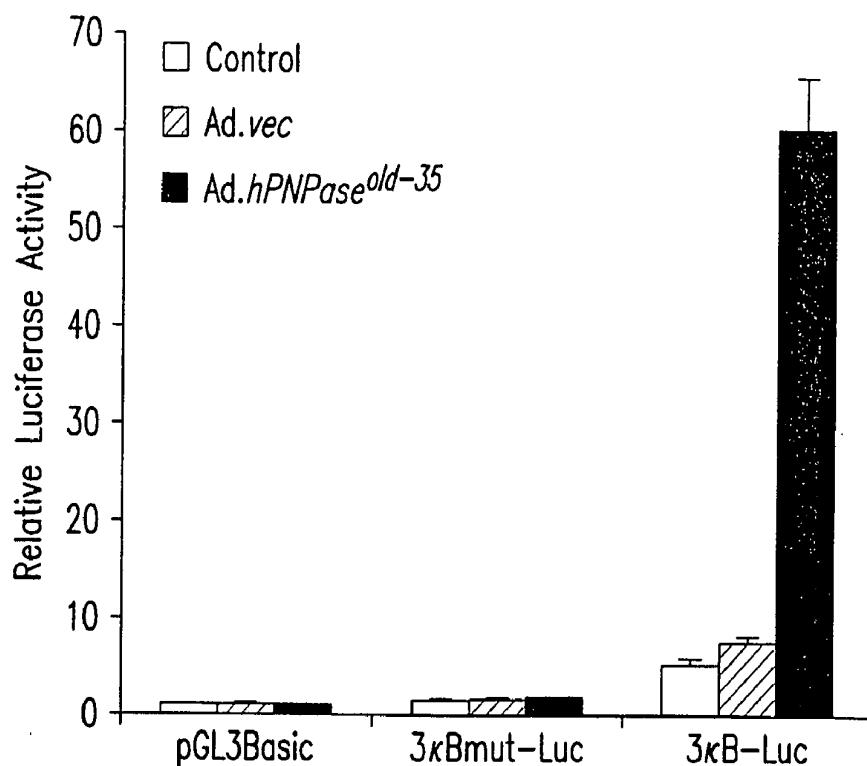


FIG.2A

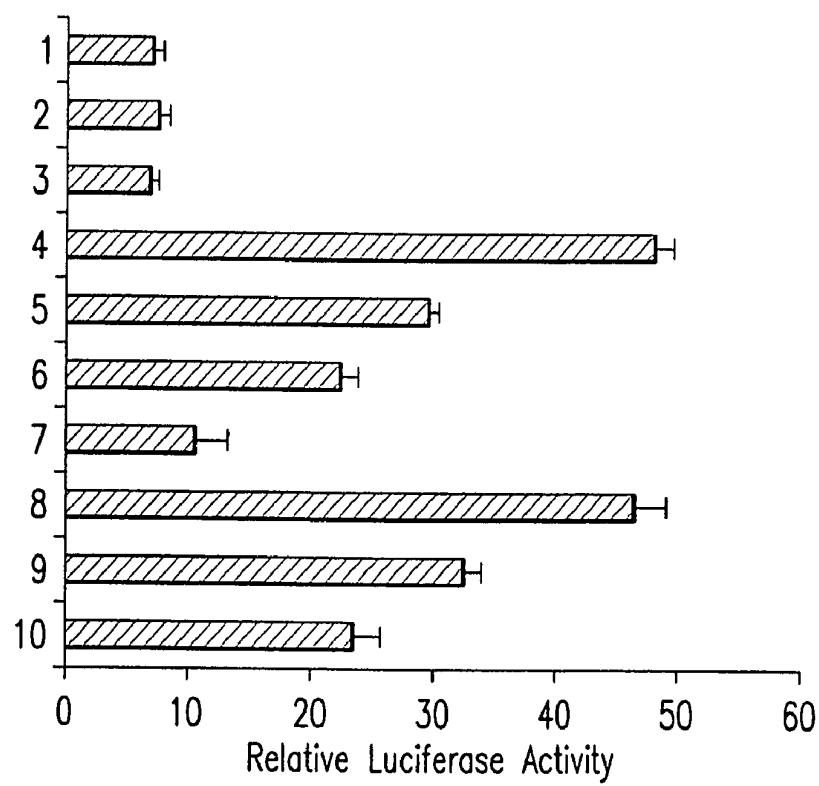


FIG.2B

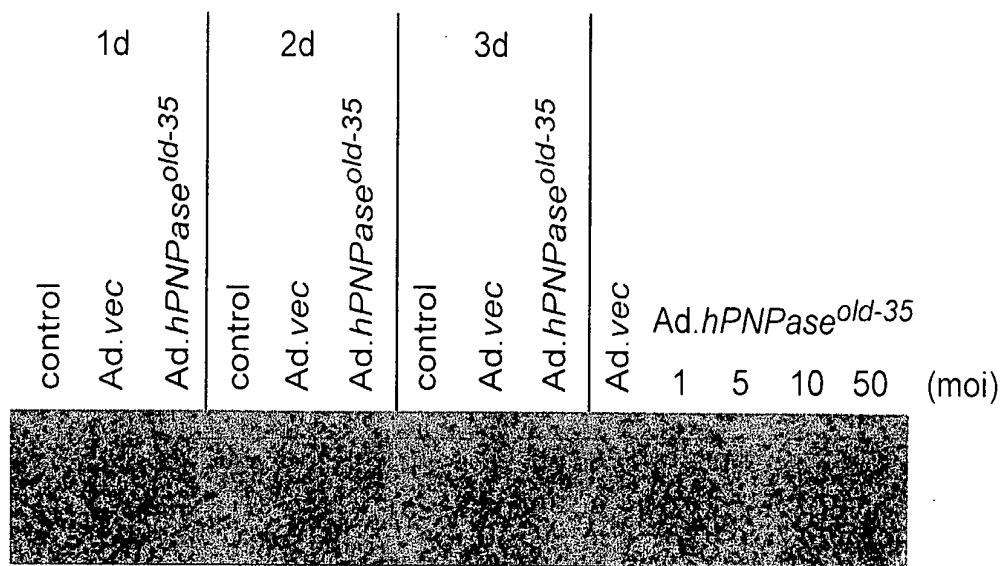


FIG. 3A

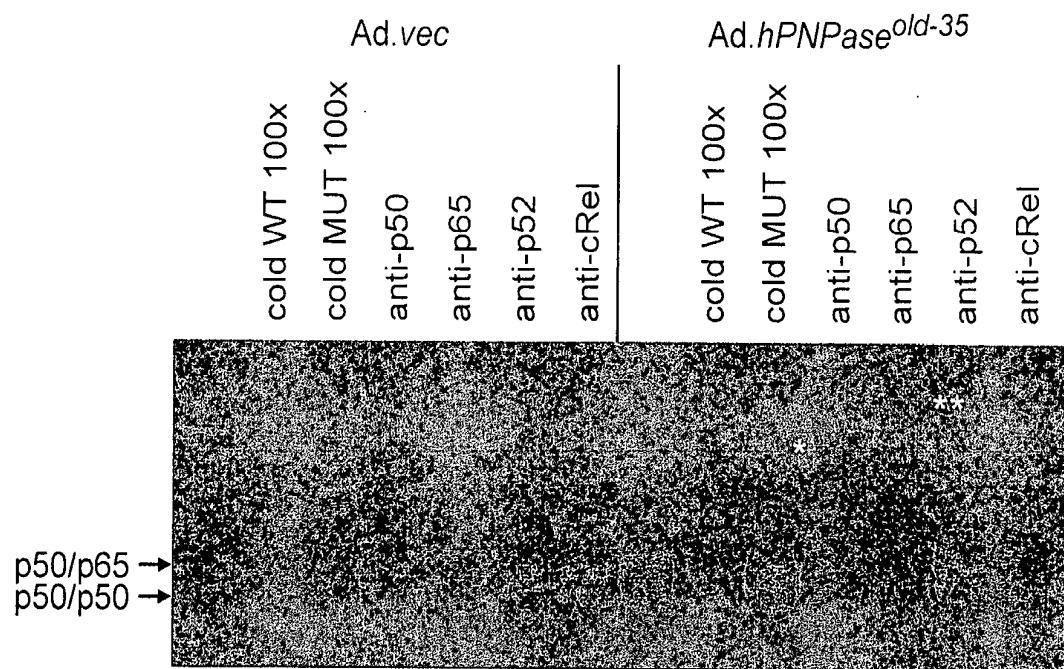


FIG. 3B

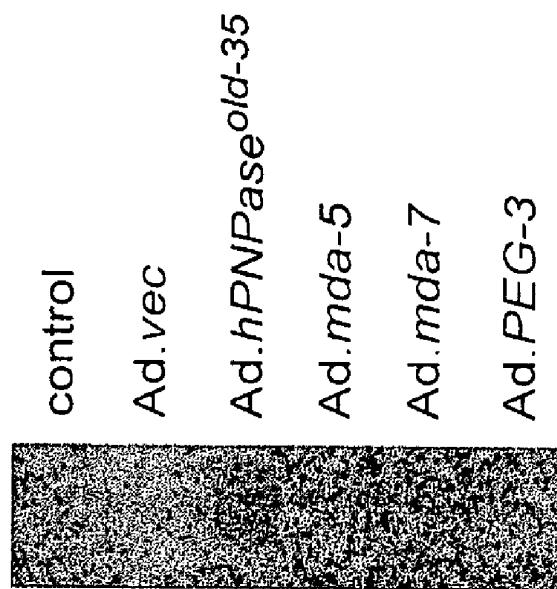


FIG.3C

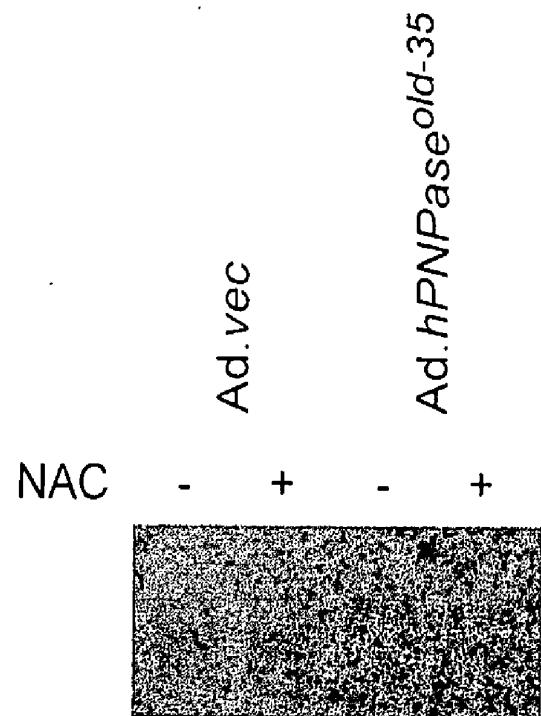


FIG.3D

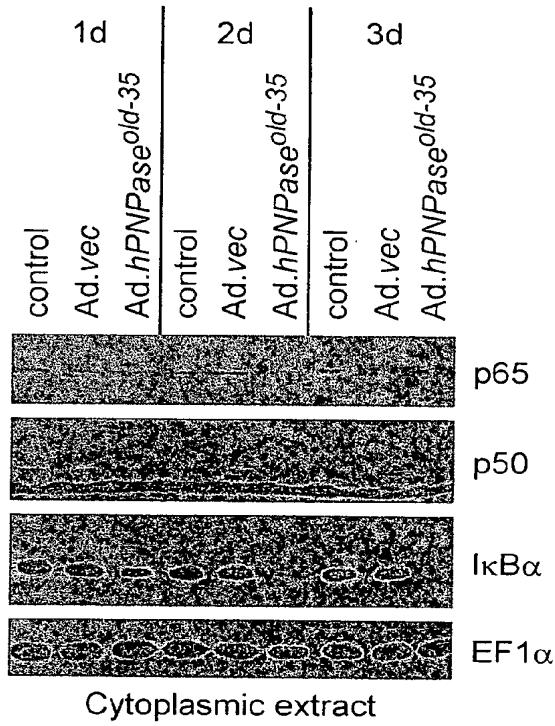


FIG.4A

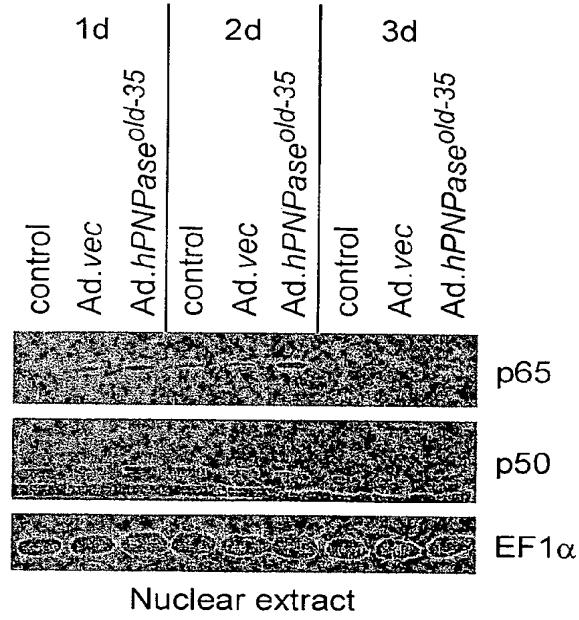


FIG.4B

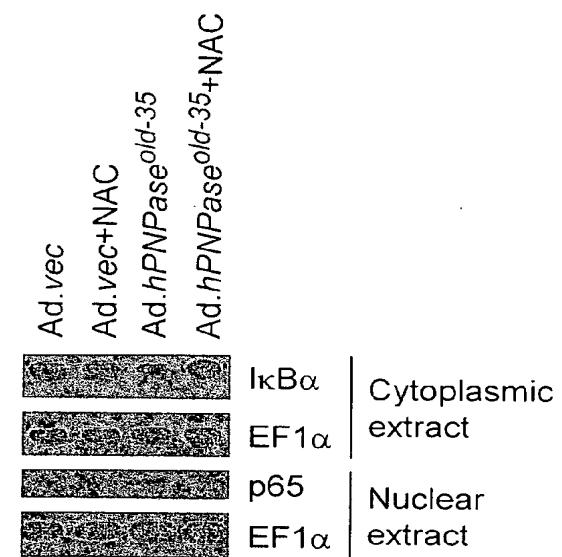


FIG.4C

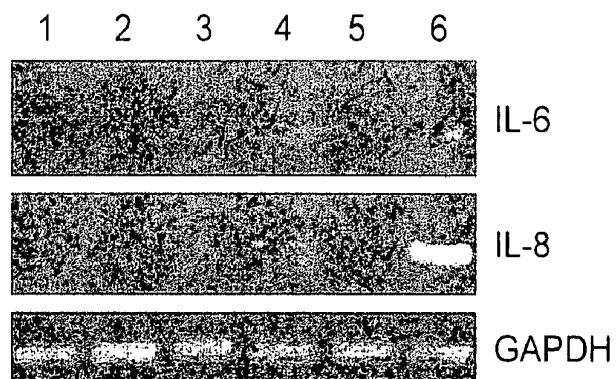


FIG.5A

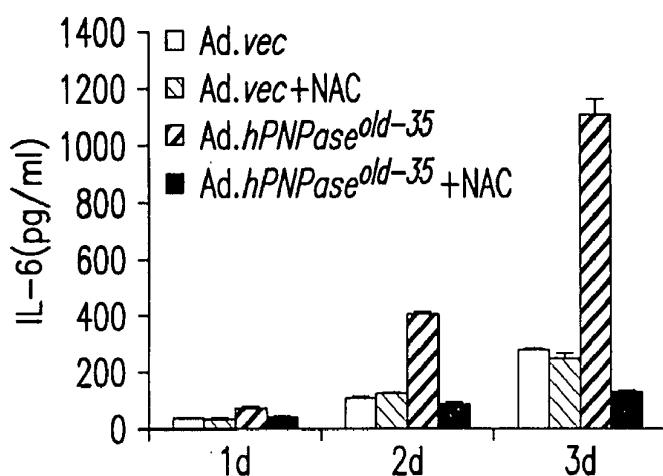


FIG.5B

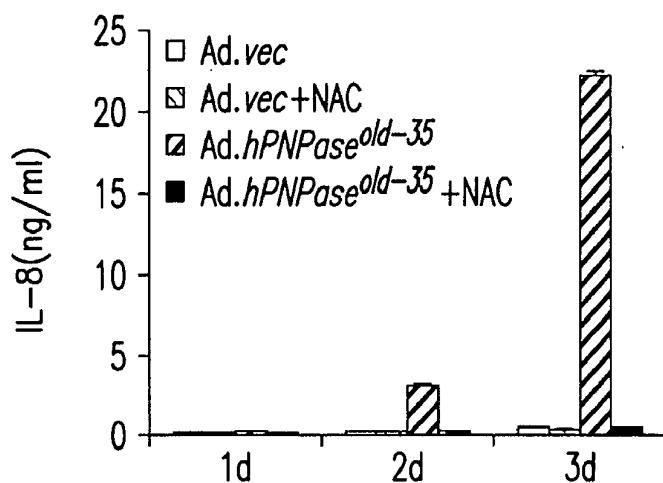


FIG.5C

Apol/Fas	Leptin	Rantes*	ICAM-1	IL-2	IL-7	Pos
CTLA	MIP1 α	TGF β	VCAM-1	IL-3	IL-8*	Pos
Eotaxin	MIP1 β	IFN γ	VEGF	IL-4	IL-10	Neg
GM-CSF	MIP4	TNF α	IL-1 α	IL-5	IL-12 (p40)	Neg
EGF	MIP-5	TNFRI*	IL-1 β	IL-6*	IL-15	Pos
IP-10	MMP3*	TNFRII	IL-1R α	IL-6R	IL-17	Pos

FIG.6A

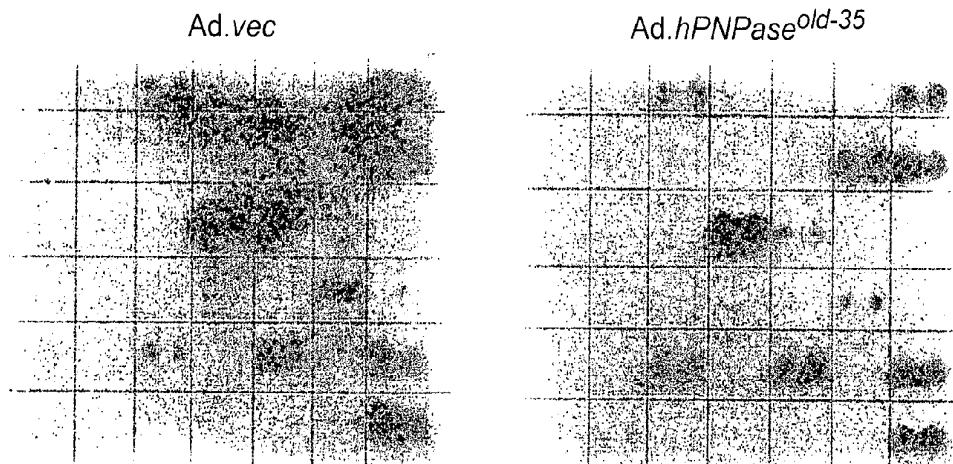


FIG.6B

FIG.6C

CTTATAGTCT ATACAAAGCA GTTTAAATT TATCCTAAA GATTTAGGAA
GCCACTGAAA AAATTAATC AGGATGGAGA CACAATTCTA TTTGTGATTG
AGAAAGAAGA TCCTTCTGGT AGTGGTAG CACAGACTT CTTAAAGTTG
AGTAGGCTAC ATTCTTCAA GAGAAGAATT TTTCTGAA CACTTCAGGG
ACTGCTGATA CTTTAATT TAATGTTACT ATATAACAAA ATATAACTTC
CATATTGTTA CAGCTTTAG TCATAAAAGC AAATAAAATG TATATCCTGT
CTAAACTAAA CTACAAACTA AATTAAAATA TAGTTAAAGG GCCGGGCTCT
GTGGCTCACG CCTGTAATCC CAGCATTTG TGAGGCCAAG GCAGGCAGAT
CACCTGAGGG TGGGAGTTGG AGATCAGCCT GGCCAACATG GTGAAACCC
GTCTCTATTA AAAGTACAAA AATTAGCTGG GCGCGGTGGC GCACGTC
AGTCCCAGCT ACTTGGGAGG CTGAGGTAGG AGAATTGCTT GAACCCAGGA
GGTGGAGGTT GCAGTGAGCC AAGATTGTGC CACTGCACTC CAGCTTGGC
AACAGAGCAA GACTCCATCT CAAAAAAA AAAAGTAGTT AACATAATT
TAATGCAATG GTGTTTACT TAACGTAGTT GCTTATTCA AACATGCAGT
GGTATTAACT GTTTGATGC AGGTTCATCA GAGTTGAAG AAAAGGGAAA
AGGAGATCTG AGAAGTTGCT GGTGGATTAA AGTTGGATCA TTTTAATGT
CCTTCAGCAG TCATAGGTAA ATAAATCCCT TTAAATTAT ATTTTGGCTG
TTGAACATACA CTTGACATAA CTCTACTTGC AGAATAACAT CTCAGACTGA
ACCTCACCGA TTATAGCTTC AGTAGTAACA ACTACAAGAA TTAAACTTA
TTATATTTTT CTTTCTACGC TAATCTAAA ATGCTTAATA TAAACATATA
AACGCATTCT GTATCATCTA TATCTTCATA ATAAAGCTCT GCTGCAAGAT
TTTAGTGCCT ACATTGCTT TTGAGTTAC TATCCAATT TAATCTATTG
AATTATATCA GATTGTTG AGGAAATGGG GGTGGGGGAT TCTTAATAGG
TAAATGCTGC TTTCCATCAC TTCTGGCTGG ATAAAGCTA ACCGATTCAA
GGAGTTCAAG GAGTTCTGAA GAAGAGAAAA ATGTTCAGAA TACCTGGAAA
CTGGAGGAAG ATTAAGAAA AGTCTGGATA AGCCTGGCG CGGTGGCTCA
TGCTGTAA CCCAGCACTT TGGGAGGCCG TGGCGGGTGG ATTGCCTGAG
GTCAGGAGTT CAAGACCAAC CTGGCCAACA TGATGAAACC CCATCTCTAC
TTAAATCCA AAAAATTCCC CGGGTTTGT GGGCAGCCCT TTACTCTCCA
ACTTGGAGG GGGGCTGAGG GGGGGAATT TGCATCCCCC CGGGACGCCG
GTGTTCCAGT GAGCTGAGAT CACGCCACTG CACTCTAGCC TGGGCAACAA
GAGAAACT CTGCTTGAA AAAACGAAA AGGCTGGATG AAAGCTAAAT
TTACCATTTA TTGAGTGTG ACAATGTGCT AGGTACTATT ATATCATCTG
ACACTTTTTT TTGAGTGTG ACAATGTGCT AGGTACTATT ATATCATCTG
CAGGCCGGAC TGCGGACTGC AGTGGCGCAA TCTCGGCTCA CTGCAAGCTC
CGCTCCCGG GTTCACGCCA TTCTCTGCC TCAGCCTCCC GAGTAGCTGG
GAATACAGGC GCCCGCTACC GCGCCCGCT AATTTTTGT ATTTTTAGTA
GAGACGGGGT TTCACCTGT TAGCCAGGAT GGTCTCAATC TCCTGACCTC
ATGATCCACC CGCCTCGGCC TCCCAAAGTG CTAGGATTAC AGGCAGTGAGC
CACCGCGCCC GGCGACACT TTTAATCCTT ACAAAAACCC TATTAGATT
ATATTACTAT CTGTTTTAT GGGGAAACTG AGACTCAGAG AGCTAGAGTC
ACACAACTGG TAATTAACAG AGACAAGATT GGAACCAGCT GGGACTAACT

FIG.7A

CCAAGTCTAT GCTCTAACT ATTAGGCAAT ACTGCCAAA TGAAAATGAT
TACATAGTAA TTAAGTATAT TAACAAAAG ATGATTTGT ATTCTTACTC
TATAAATCAA AAGTCATGTT CAATTGTGGC AAGTAGGTT TCTGAGAGAT
TTATCTTCAT GAGAGATTTC AATAGATTGG TCTGGAAAAC TCTGTGGCAA
CTCAAGGTAAGTAAAAGGG AGGAGACGCA AAAACTGCTT TAACACTAAA
AATCTGTAAA CAAAGTTAAT GGTAAATCCCT CTATTGGAAT GGAAATCCCA
AATCAGTAAT AATAGCTCAT GAAAGCAAGG TGTCAGAAAA CTGCTTGCC
AGCAAATCAT TTGTCCTGTT AGAGCTCATC ATTCTTTTTT CTTTTTTAAA
TCATCACGGA TCAACATTAA TAACTTTTTT TTTTTTTTT TTTTTTTTAA
GATGGAGTCT CGGTCTGGAG TGCACTGGCG CGATCTCGGC TCACTGCAAC
CTCCGCCTCC TGGATTCAAG CGATTCTCAT GCCTCAGCCT CTGGAGTAGC
TGGGATTACA GGCACGCGCC ACCACACCAGA GCTAATTTTT GTATTTTTAG
TAGAGACGCG GTTTCAGCAT GTTGGCCAGG ATGCCCTGGG TCTCTTGACC
TCGTGATCCA CCCGCCTCTG CCTCCCAGCG TGCTGGGATT ACAGGCATGA
GCCACTGCGC CCGGACTTTT TTTTTTTTT TTTTTTTTT TTTTTTTTAA
TTGAGACCGA GTTTCACTCT TGTTGCAATG ACGCGATCTC GGCTCACTGC
AACCTCCGCC TCCCAGGTT AAGCGATTCC CCTGCCTCAG CCTCCCGAGT
AGCTGGGATT ACAGGCATAA GCCACCATGC CCGGCTAGTT TTGTATTTTT
AGTAGAGACG GGGTTCTCT GTGTTGGTCA GGCTGGTCTC GAACTCAGGT
GATCCGCCCG CCTCGGCCTC CCAAAGTGCT GGGATTACAG GCGTGAGCCA
CAGCGTCCAG CCAACGTTT TAATTCTAA AACTGTGTTT GAATGAGTGT
TGGGTTTTG TTGTTGGTT TTCTTGGGG TAAGGCGGTT ATCACCTTAC
ACAAAAAATT TACATCATAA TATGAAAATG CCCATTGGTT CATTCAAGGA
AAAACATAAA GAATTTCTAA CAGAATGGAT TAATGGGGTG GAAGAAATT
ATCCATTCTC TAAAAATTAA TCAAATCCCA AAAACTTTTT TTTTTCTTAA
TACAAAATAT TTACTTCAA CAGTAGCTGG CATTGGTCA TTAAGAGTTC
GACCAAAAGG GACAGAAATA TAAATTGCTA ATTATTAGCA ATGGAAATGA
AAATGAATCA GAAGTGACAA TAAAGATTTC TAAAATTACA AGTCGATGAA
AAATGAATT ACTGTAATT GGTAAACACA AATCGCTGTA GTAACATATC
TGCTATGTGA AATTTTTAAA ATAAAAACCA GGGCGGAGAA GGGGAGAAGG
AGATCTCTT CCAAGGCCA CCACCCAAAT TCTAATTCTT AGTTCACAGA
TCCAAATAAT AATAATGTGT TGTTCTATGA CTTCTAGTAT TAAAAGTAAA
TATAAATTAA CTCTGCATAT TAAACATTAA CATTATTCTA GGACTTCTCC
TTTAAATTAC GATAGTACAC ACACAAAAAC CTTATTATC TAATTGCCTT
TCGTTGGCAC ATAAAGACAA AGATCGTTG CAACATAATC GCGTTCTGAT
ACGTTTGACC CTCTCTAGTC TTCGTAGAAG GTGGCCAAGG TTTCCGTTAC
CCCGGCGTCC CTCGGGCACC GCGGAAACGA AACTCCATCA GGCTCCGCC
CACGGTCTGC GGAGTGAGCC AATCAGGGCA CAGCCTGCGI TGACCCGCG
CCGGGTGTCA TGGCGGCCTG CAGGTACTGC TGCTCGTGCC TCCGGCTCCG
GC

FIG. 7B

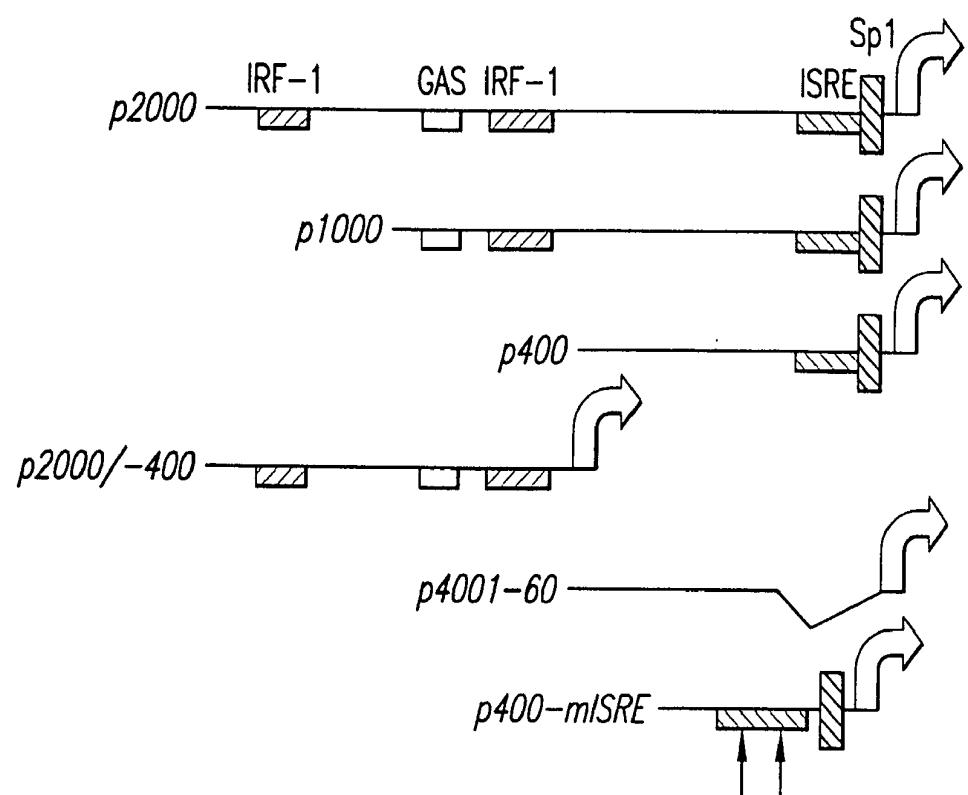


FIG.8

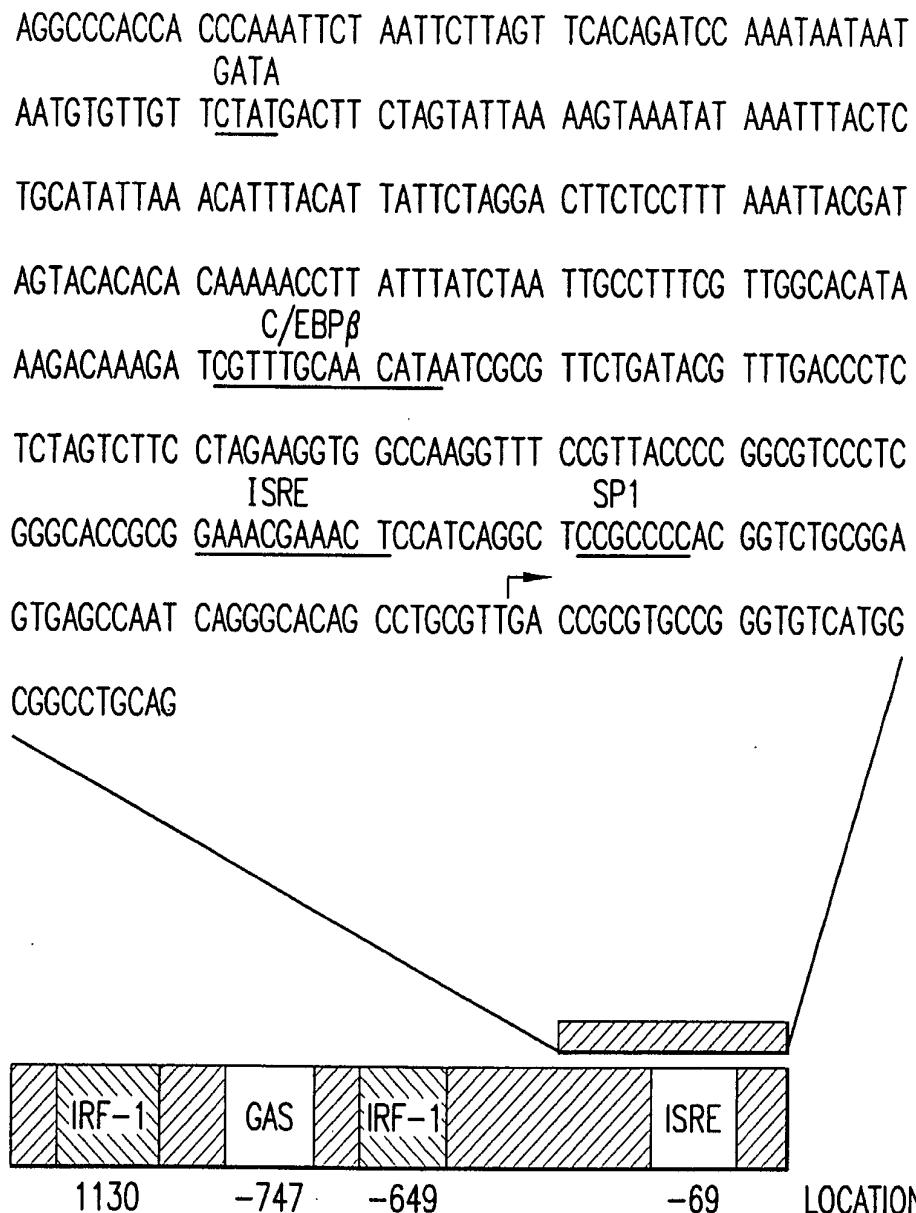


FIG.9

Consensus ISRE GAAANGAAA
old-35 ISRE GAAACGAAA

FIG. 10

1 gcgtgccggg tgtcatggcg gcctgcagg actgctgctc gtgcctccgg ctccggcccc
 61 tgagcgatgg tccttcctt ctgccacggc gggatcgggc actcacccag ttgcaagtgc
 121 gagcaactatg gagtagcgca gggtctcgag ctgtggccgt ggacttaggc aacagggaaat
 181 tagaaatatac ttctggaaag ctggccagat ttgcagatgg ctctgctgta gtacagttag
 241 gtgacactgc agtaatggtc acagcggtca gtaaaaacaaa accttcccct tcccaagttt
 301 tgccttgggt ggttactac agacaaaaag ctgctgcagc aggttagaatt cccacaaaact
 361 atctgagaag agaggttgtt acttctgata aagaaattct aacaagtcga ataataagatc
 421 gttcaatttag accgctctt ccagctggct acttcttatga tacacagggtt ctgtgtatc
 481 tggtagcagt agatggtgta aatgagcctg atgtccttagc attaatggc gcttccgtag
 541 ccctctcatt atcagatatt ccttggaaatg gacctgttgg ggcagtagcga attaggaaataa
 601 ttgatggaga atatgttgtt aacccaacaa gaaaagaaat gtcttcttagt actttaaatt
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 721 ttttacagca ggacttttgc catgctatca aagtggaggt gaaatataacc caacaatcaa
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 961 taagattaga tacggaggaa caactaaaag aaaaatttcc agaagccgat ccataatgaaa
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 1081 acaaaaagggtg cgatggtcgg gatttgactt cacttagaa tgtaagtgtt gaggtagata
 1141 tggtaaaaac ccttcatttgc tcagcattat ttcaaaagagg acaaacacag gtgcttgc
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 1441 tccttagagtc aaatgggtca tcttcattgg catctgcatt tggcgaaatg tttagcattaa
 1501 tggattcagg gggttcaatt tcatttcatttgc ttgcaggcgt agcaatagga ttggtcacca
 1561 aaaccgatcc tgagaagggt gaaatagaag attatcggtt gctgacagat attttggaa
 1621 ttgaagatta caatggtgac atggacttca aaatagctgg cactaataaa ggaataactg
 1681 cattacaggc tgatattaaa ttacctggaa taccaataaa aattgtgtat gaggctattc
 1741 aacaagcttc agtggcaaaa aaggagatatt tacagatcat gaacaaaact atttcaaaac
 1801 ctcgagcatc tagaaaaagaa aatggacctg ttgttagaaac tggtcagggtt ccattatcaa
 1861 aacggccaaa atttgggttgc cctgggtgc ataacttaaa aaaacttcag gctgaaacag
 1921 gtgtactat tagtcagggtt gatgaaagaaa cgtttctgt atttgacca acacccagt
 1981 ttatgcatttgc ggcagagac ttcatctgtt aaatctgcattt ggtgtatgg ggcggccat
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 2101 taaaattata tccaaatatg actgcgtac tgcttcataa cacacaactt gatcaacgaa
 2161 agattaaaca tcctactgcc ctaggattt aagttggcca agaaatttcag gtgaaatact
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 2521 ttttaatttgc gagtaacccca tattttttttttt attttttttt cattataat caagaaatatt
 2581 ttattattaa aagtaagtca ttatataatc tttaga

FIG. 11A

MAACRYCCSCLRLRPLSDGPFLPDRRALTQLQVRALWSSAGSRAAVADLGNRKLEIS
SGKLARFADGSAVVQSGDTAVMVTAVSKTPSPSQFMPLVVDYRQAAAAGRIPTNYLR
REVGTSDEILTSRIIDRSIRPLFPAGFYDTQVLCNLLAVDGVNEPDVLAINGASVAL
SLSDIPWNPGPGAVRIGIIDGEYVNPNTRKEMSSSTLNLVVAGAPKSQIVMLEASAENI
LQQDFCHAIVGVVKYTQQIIQGIQQLVKETGVTKRTPQKLFTPSPEIVKYTHKLAMERL
YAVFTDYEHDKVSREAVNKIRLDTEEQLKEKFPEADPYEIIESFNVVAKEVFRSIVLN
EYKRCDGRLTLSRNVSCEVDMFKTLHGSALFQRGQTQVLCTVTFDSLESGIKSDQVIT
AINGIKDKNFMHYEFPPYATNEIGKVTGLNRRELGHGALAEKALYPVIPRDPFTIRV
TSEVLESNGSSMASACGGSLALMDSGVPPISSAVAGVAIGLVTKTDPKGIEDYRLLT
DILGIEDYNGDMDFKIAGTNKGITALQADIKLPGIPIKIVMEAIIQQASVAKKEILQIMN
KTISKPRASRKENGPVVETVQVPLSKRAKFVPGGGYNLKKLQAETGVTISQVDEETFSV
FAPTPSVMHEARDFITEICKDDQEQQLEFGAVYTATITEIRDGVMVKLYPNMTAVLLH
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SIVMGEPISQSSNSQ

FIG. 11B

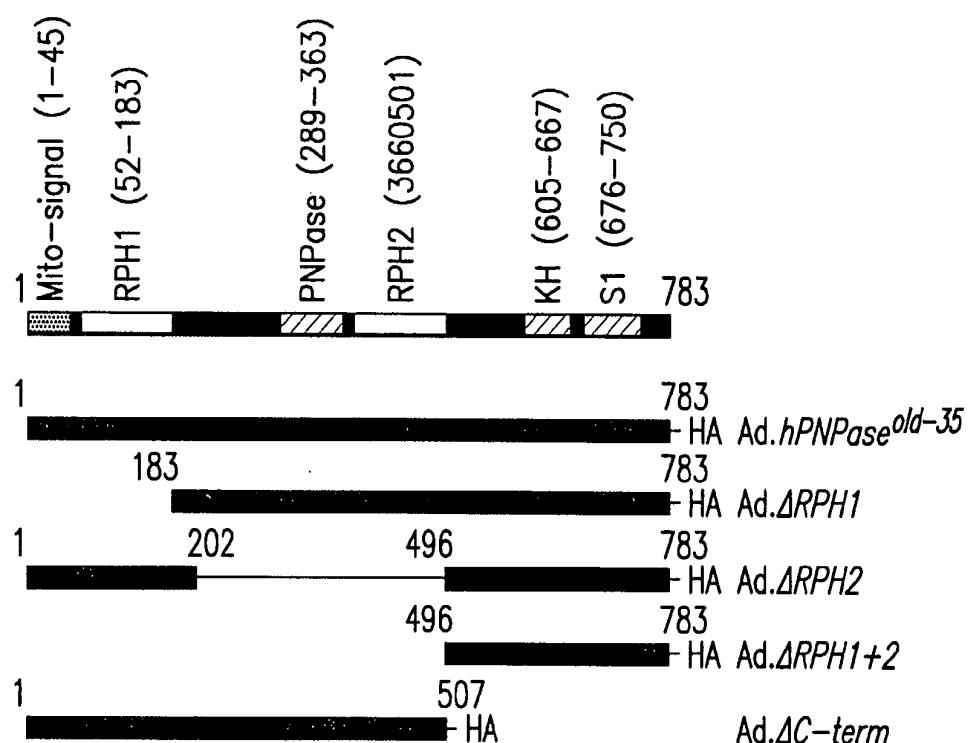


FIG. 12A

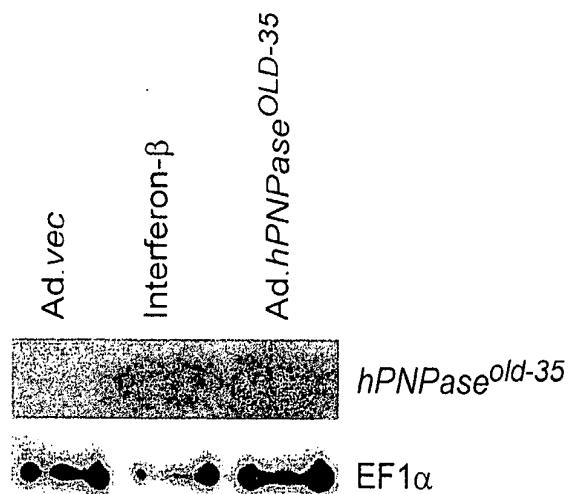


FIG. 12B

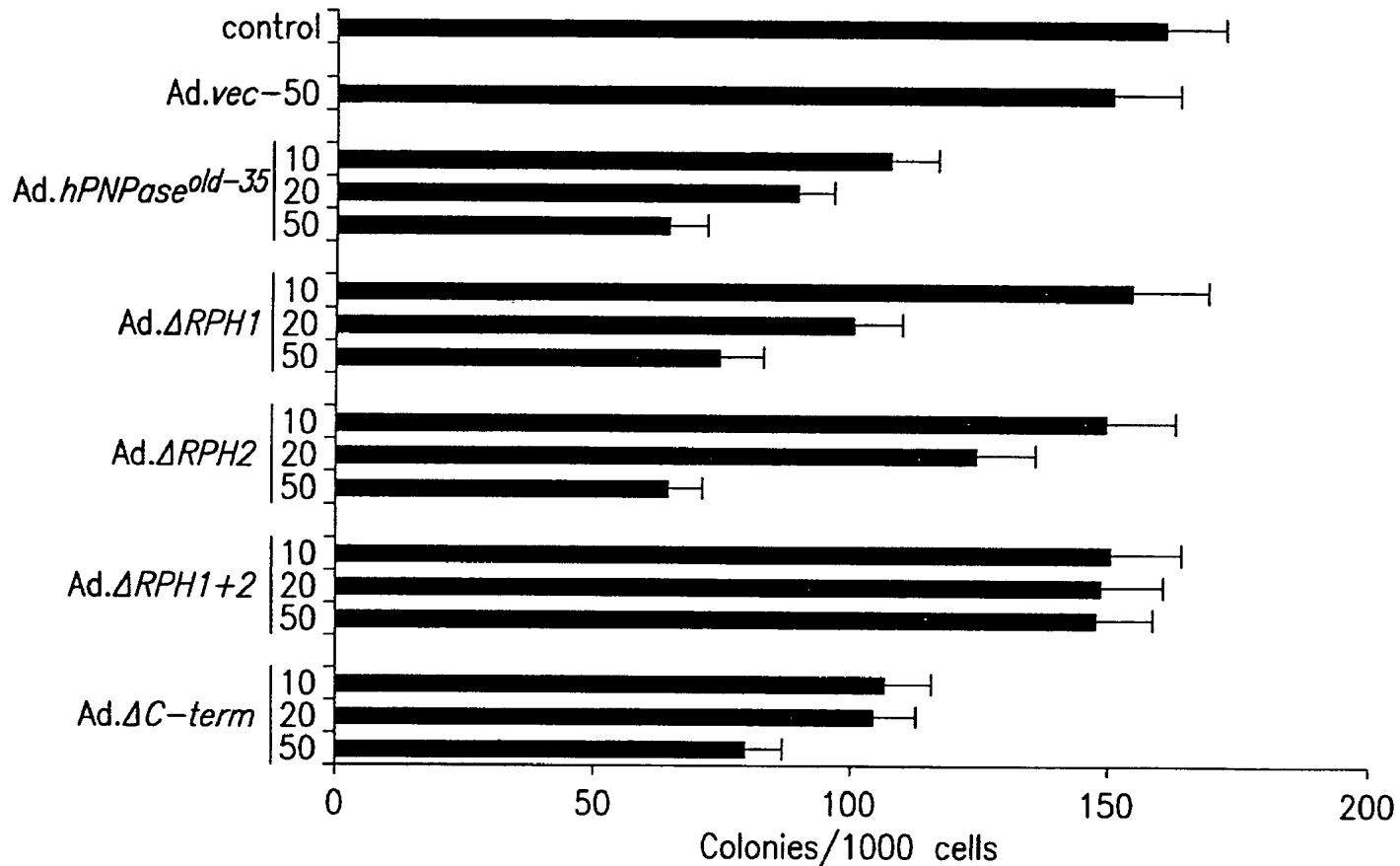


FIG. 12C

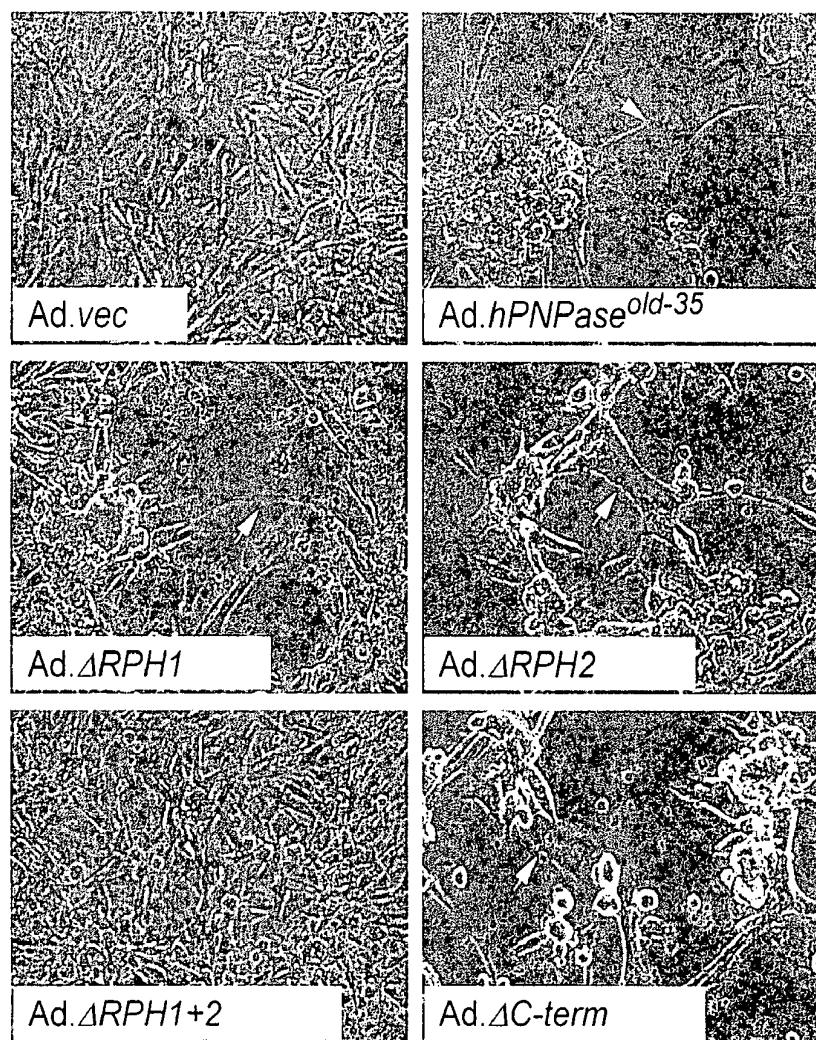


FIG. 12D

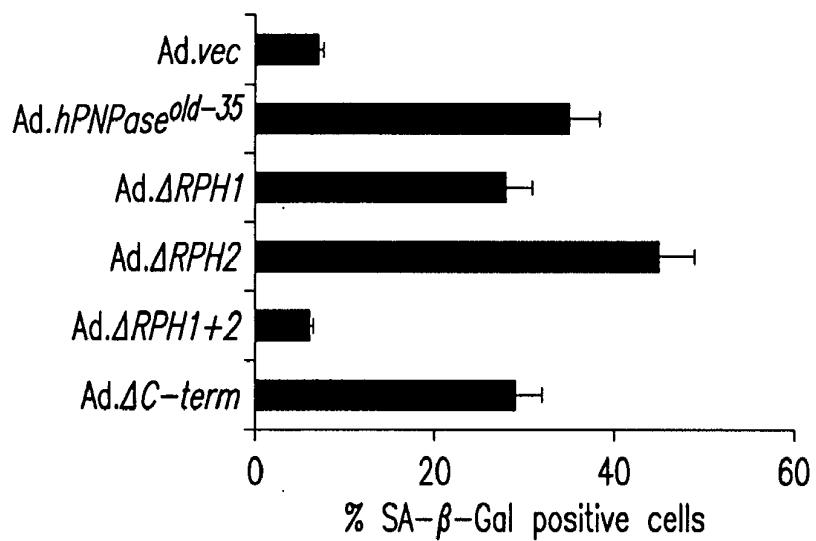


FIG. 12E

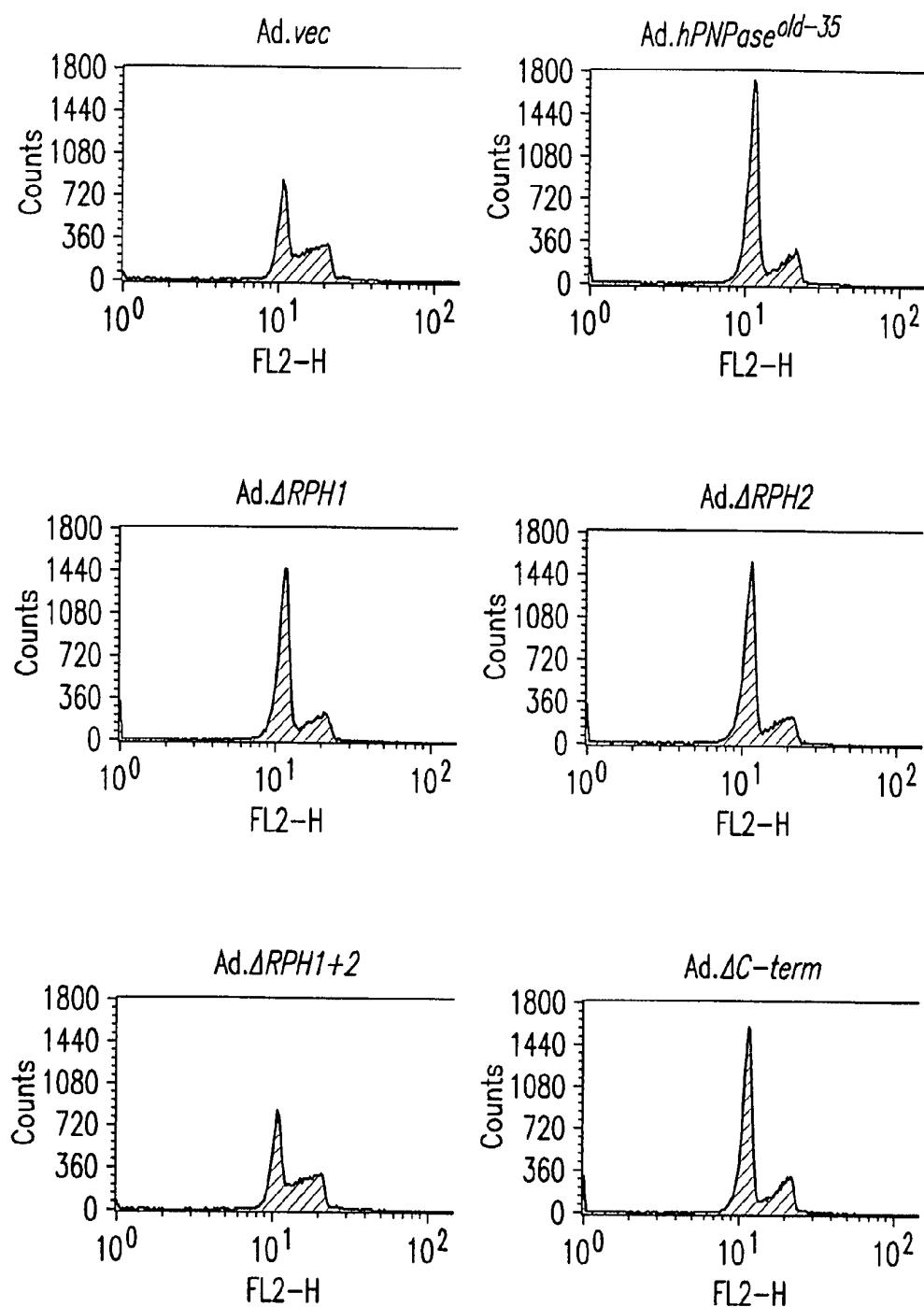


FIG.13A

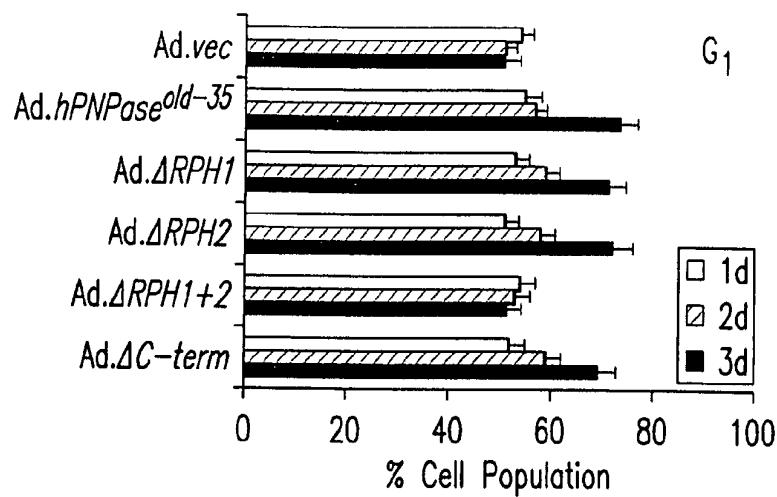


FIG. 13B

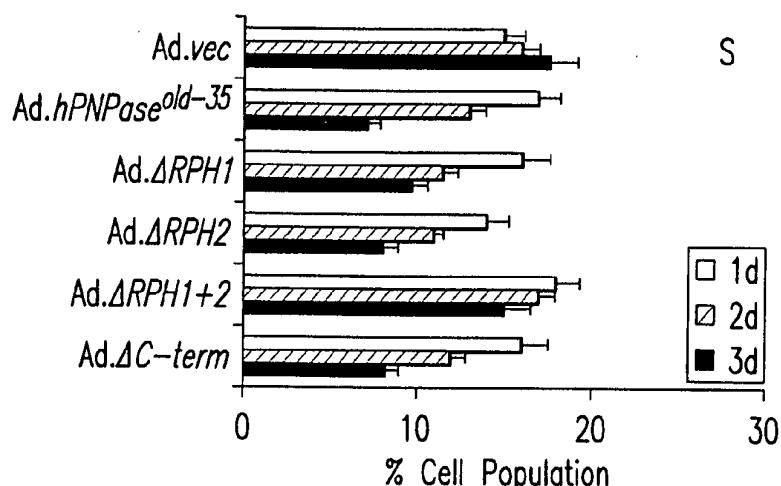


FIG. 13C

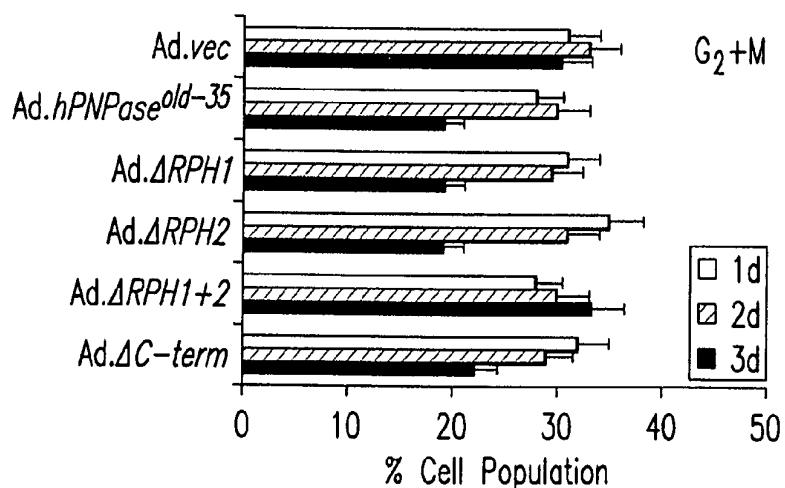


FIG. 13D

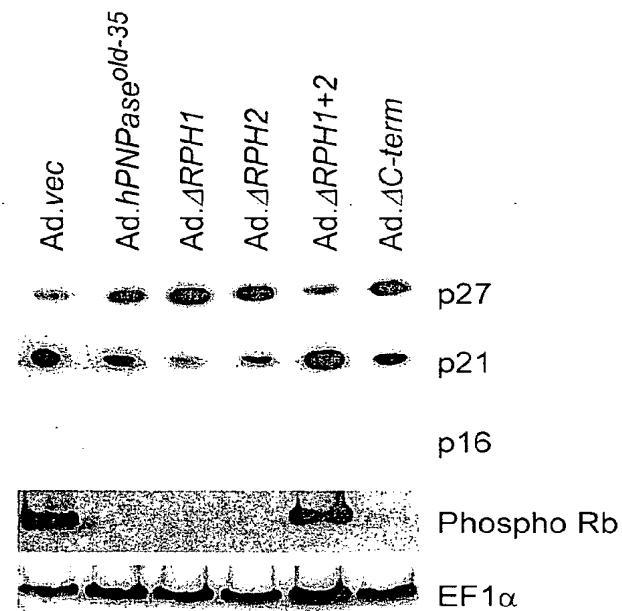


FIG. 14A

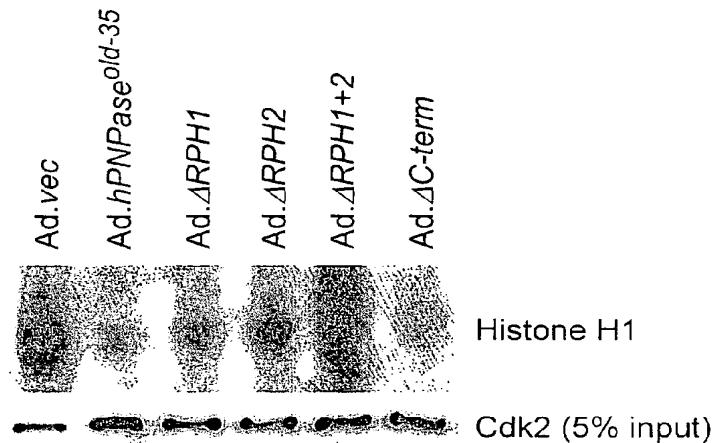


FIG. 14B

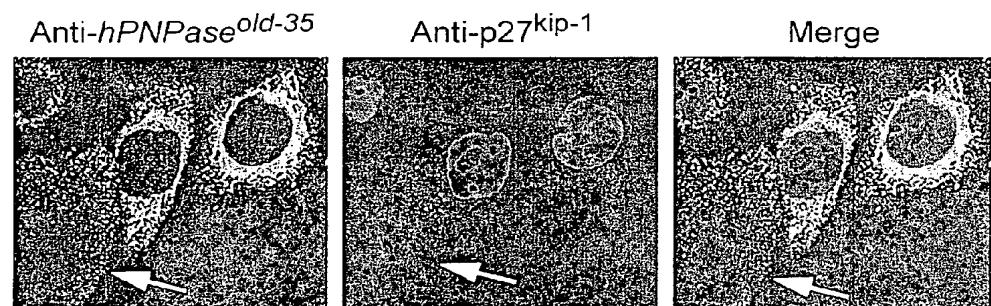


FIG. 14C

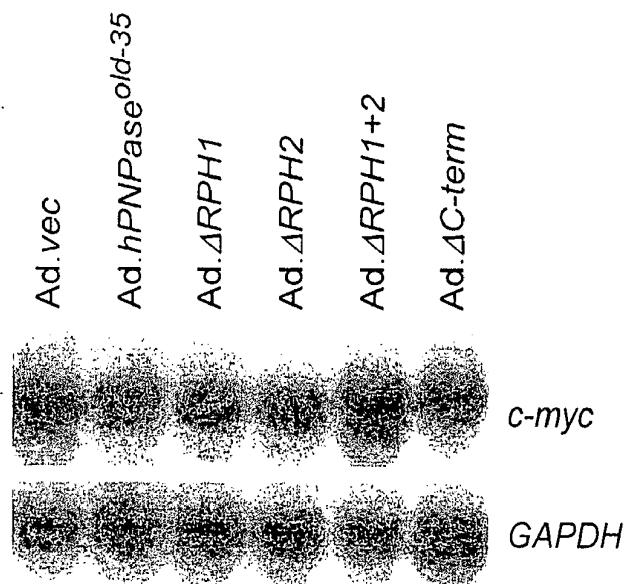


FIG. 15A

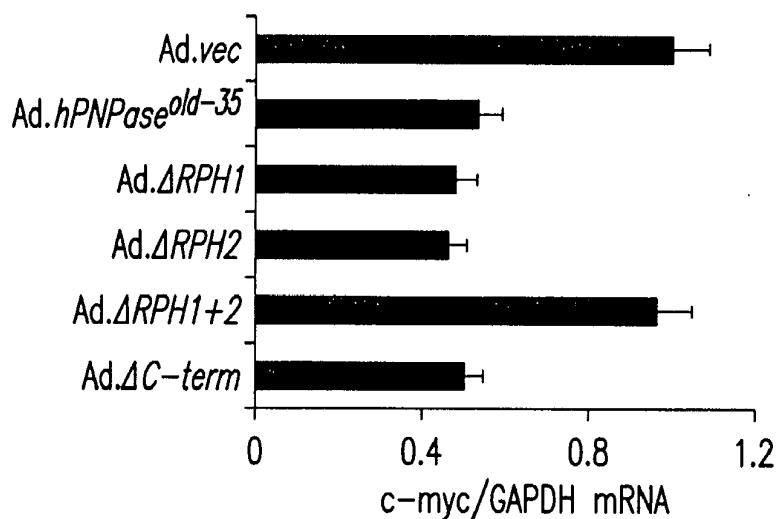


FIG. 15B

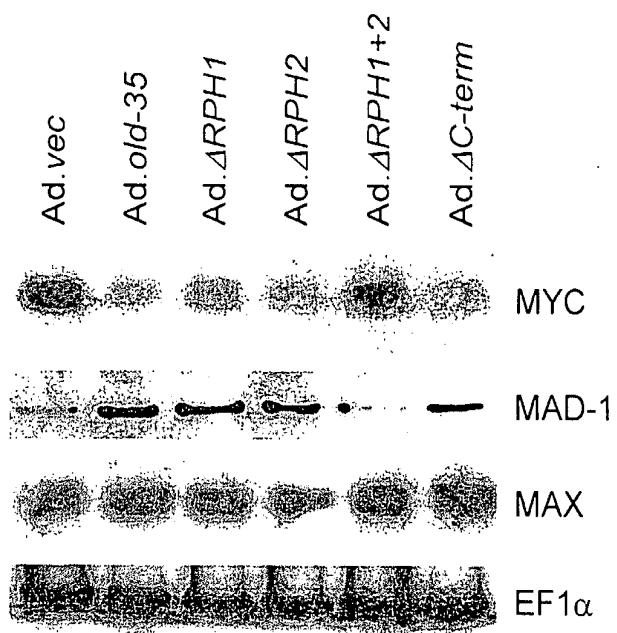


FIG. 15C

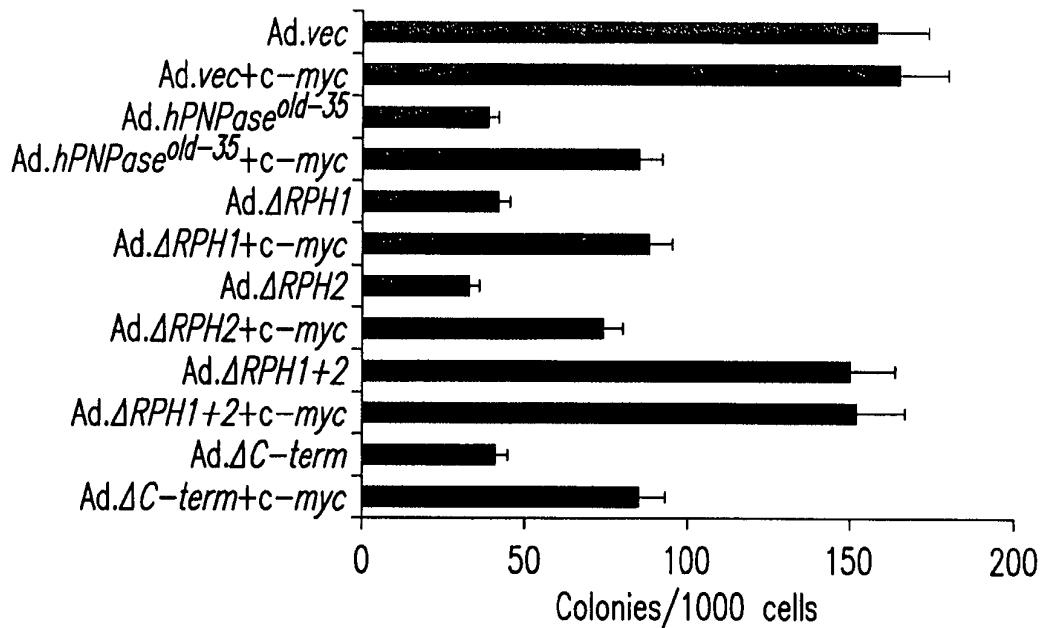


FIG. 15D

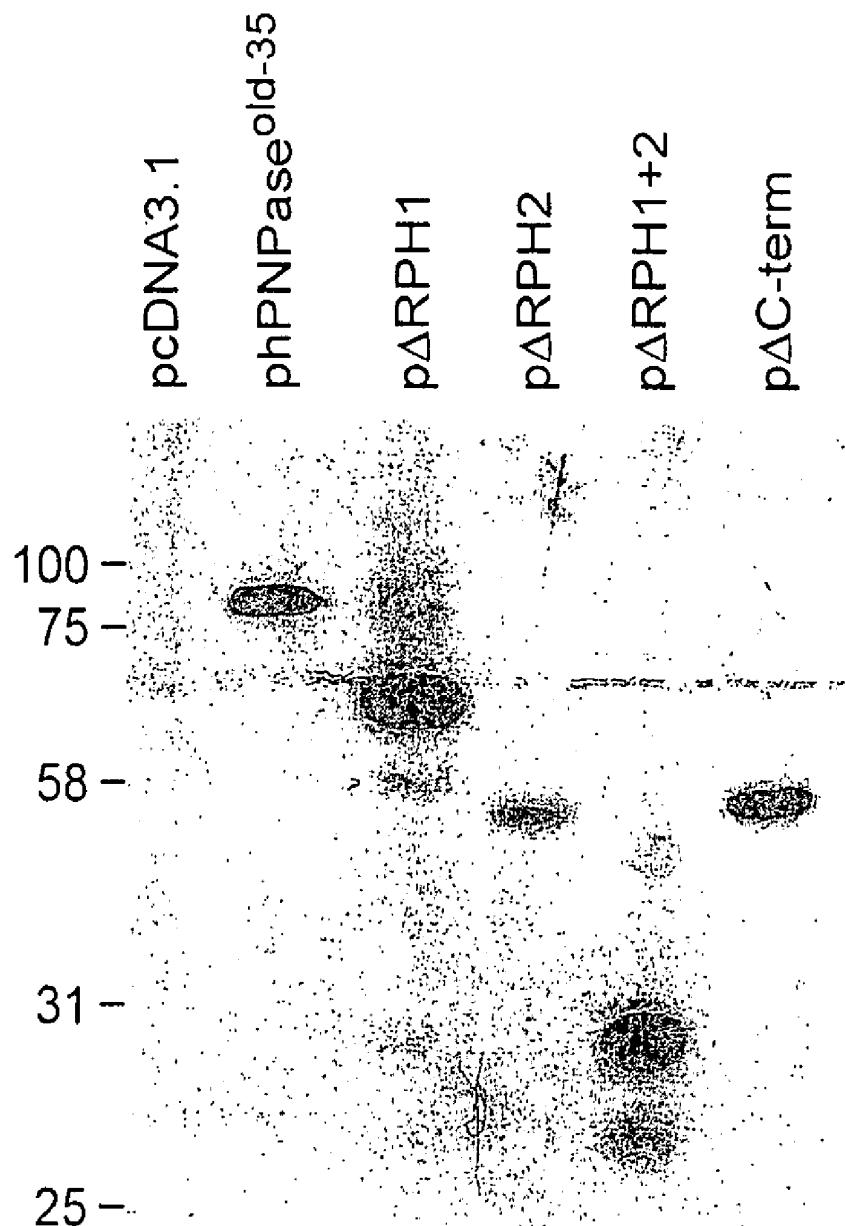


FIG. 16A

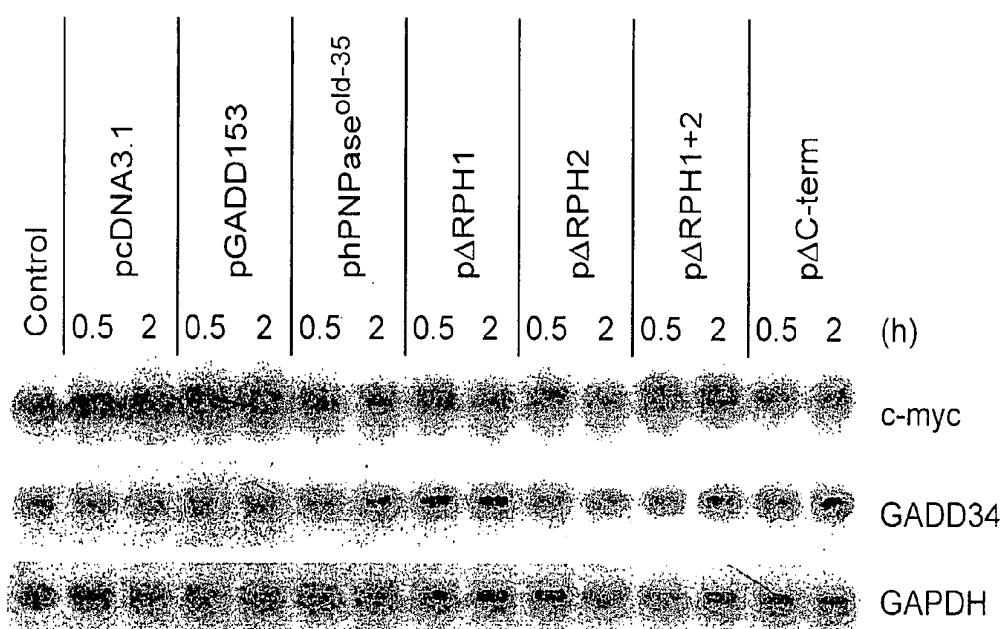


FIG.16B

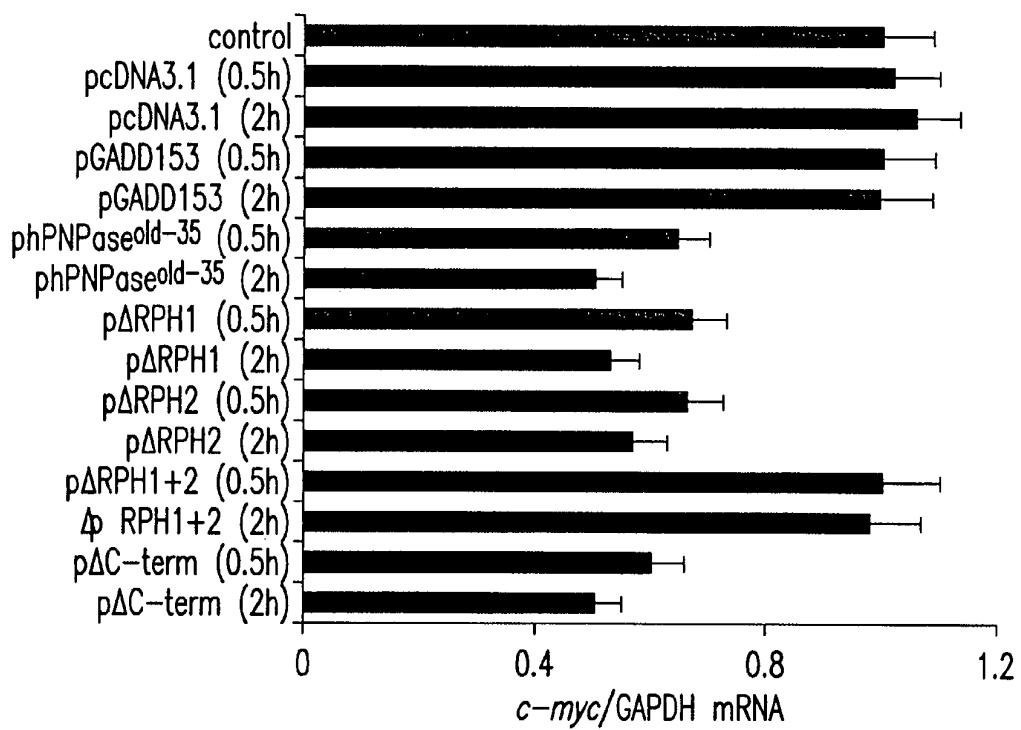


FIG.16C

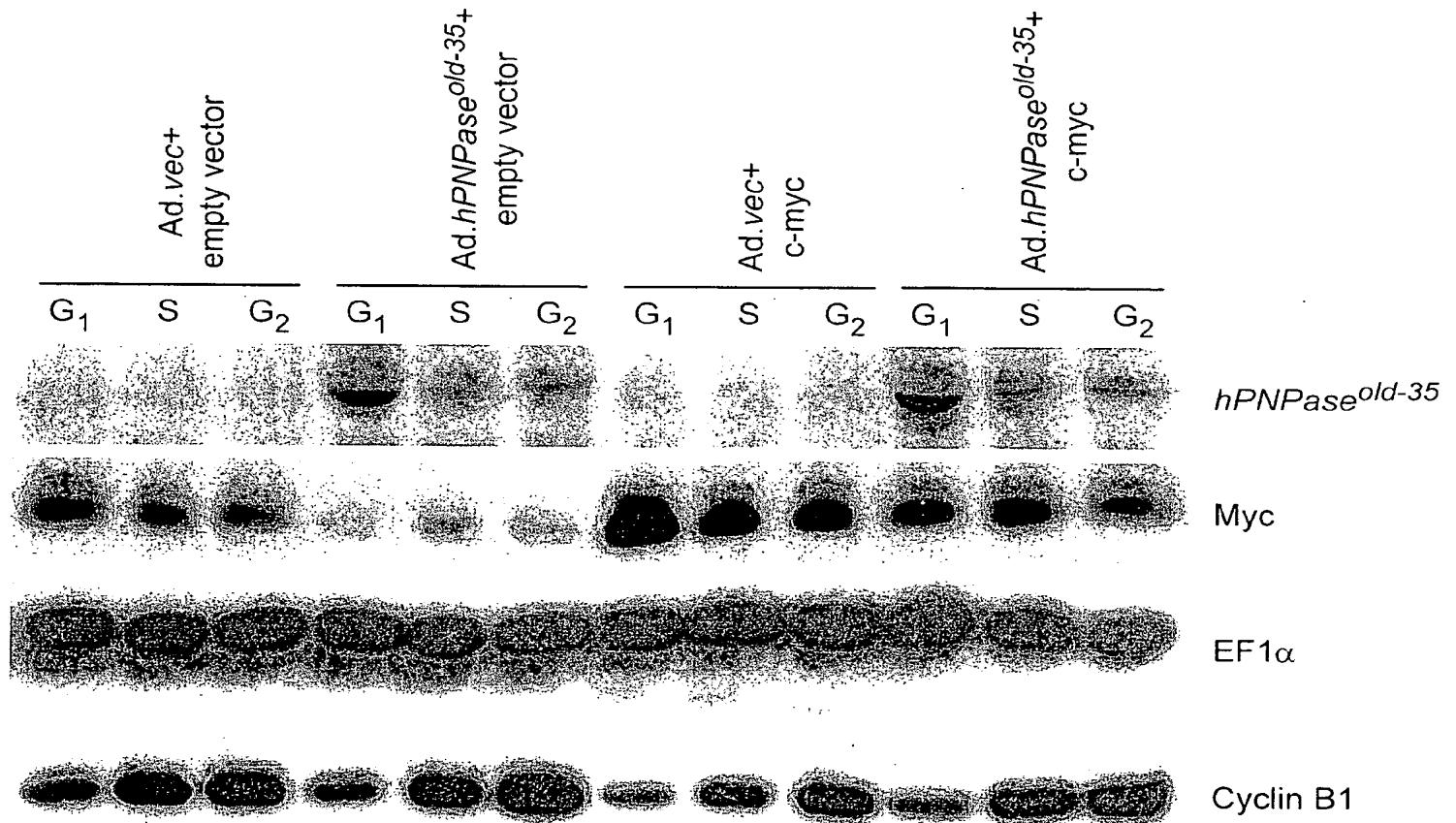


FIG. 17

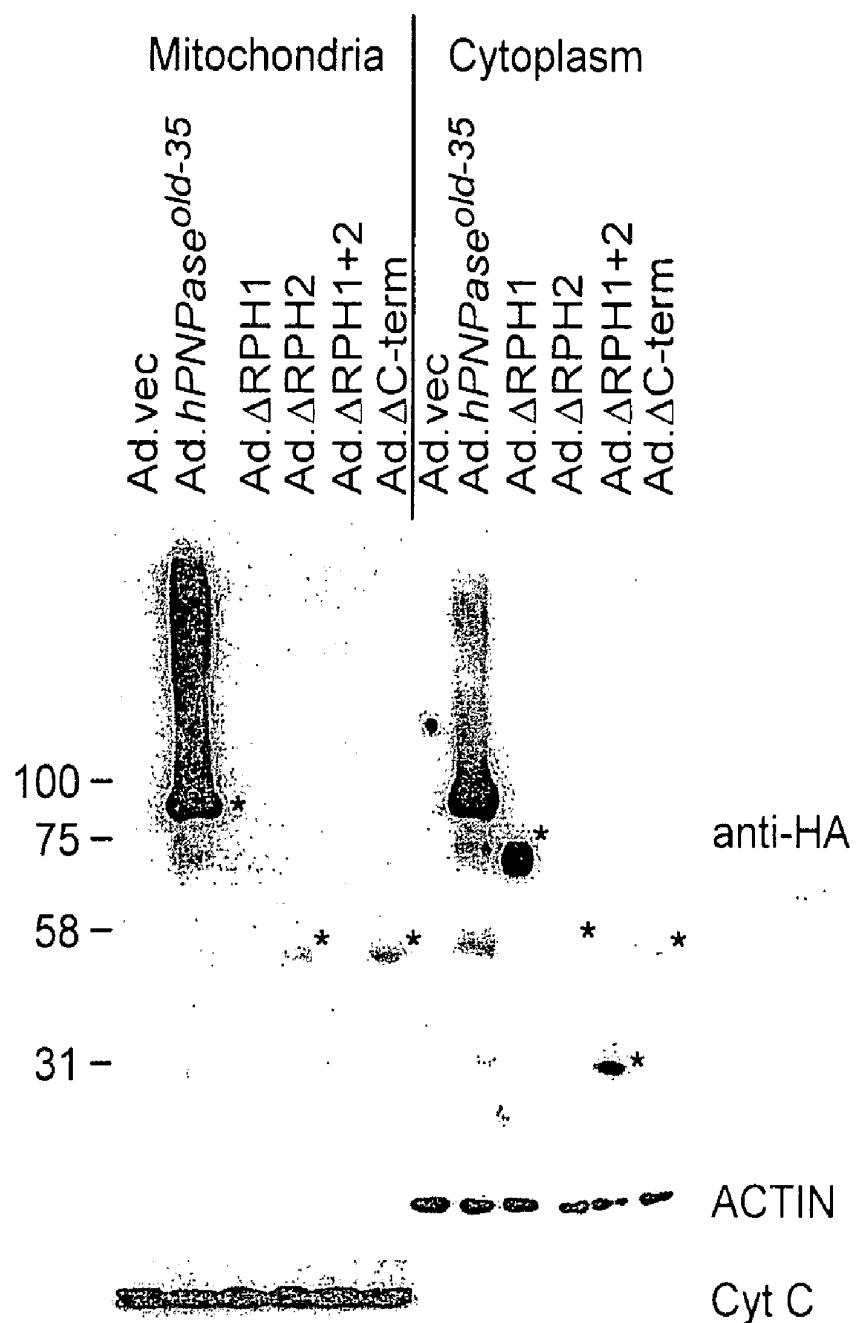


FIG. 18A

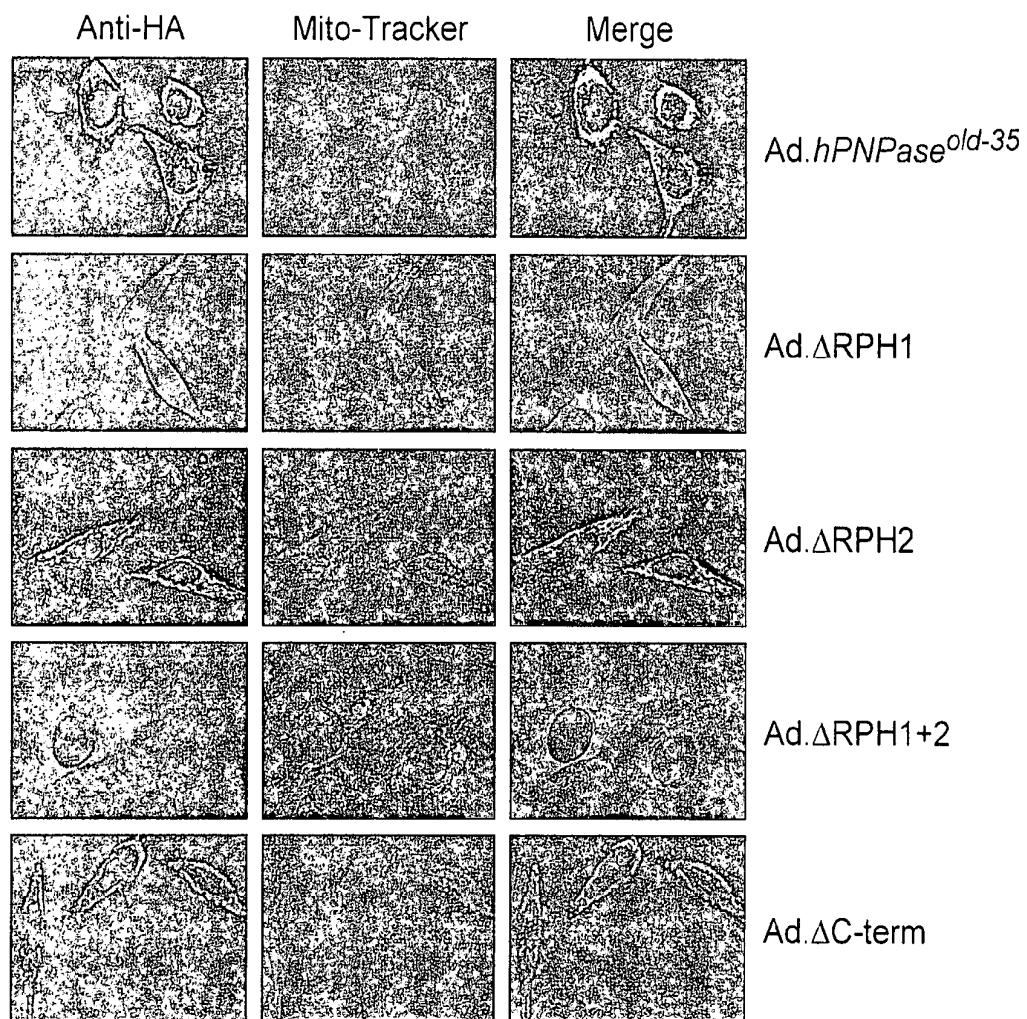


FIG.18B

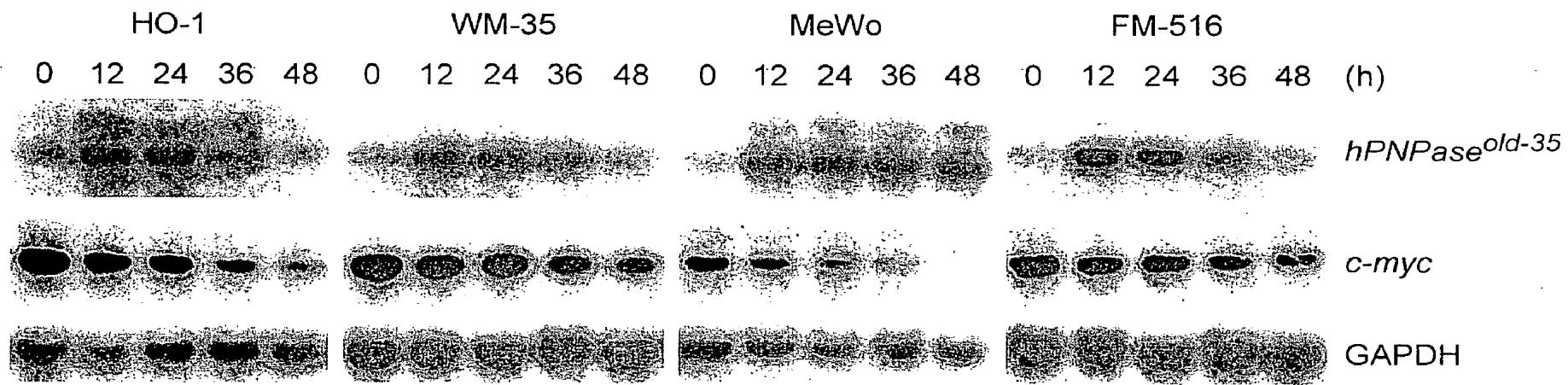


FIG. 19A

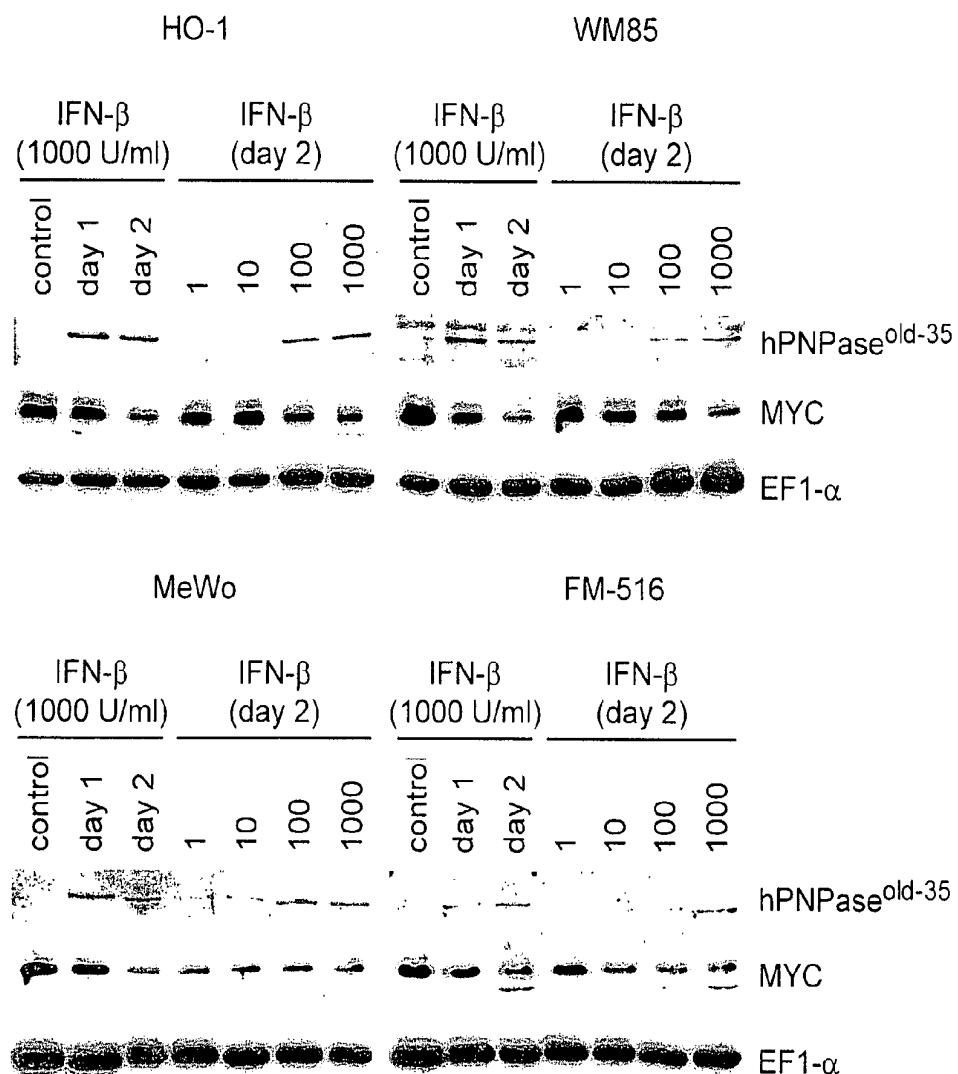


FIG. 19B

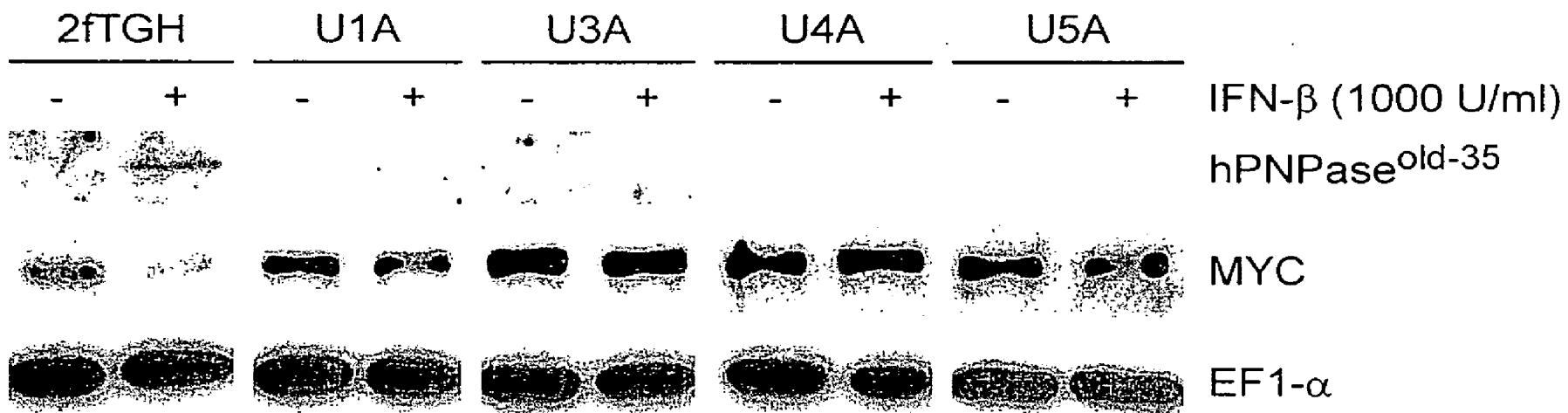


FIG. 19C

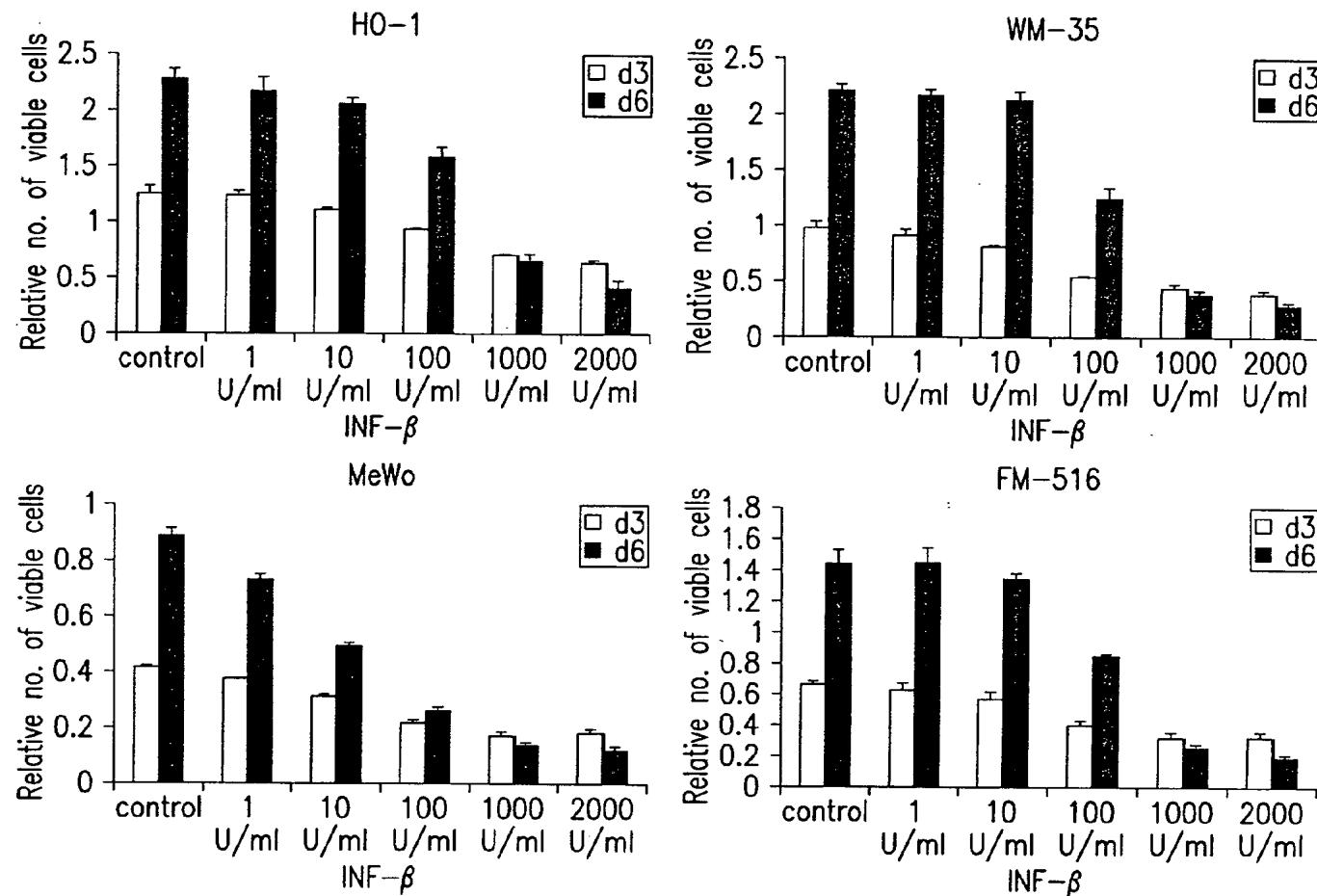
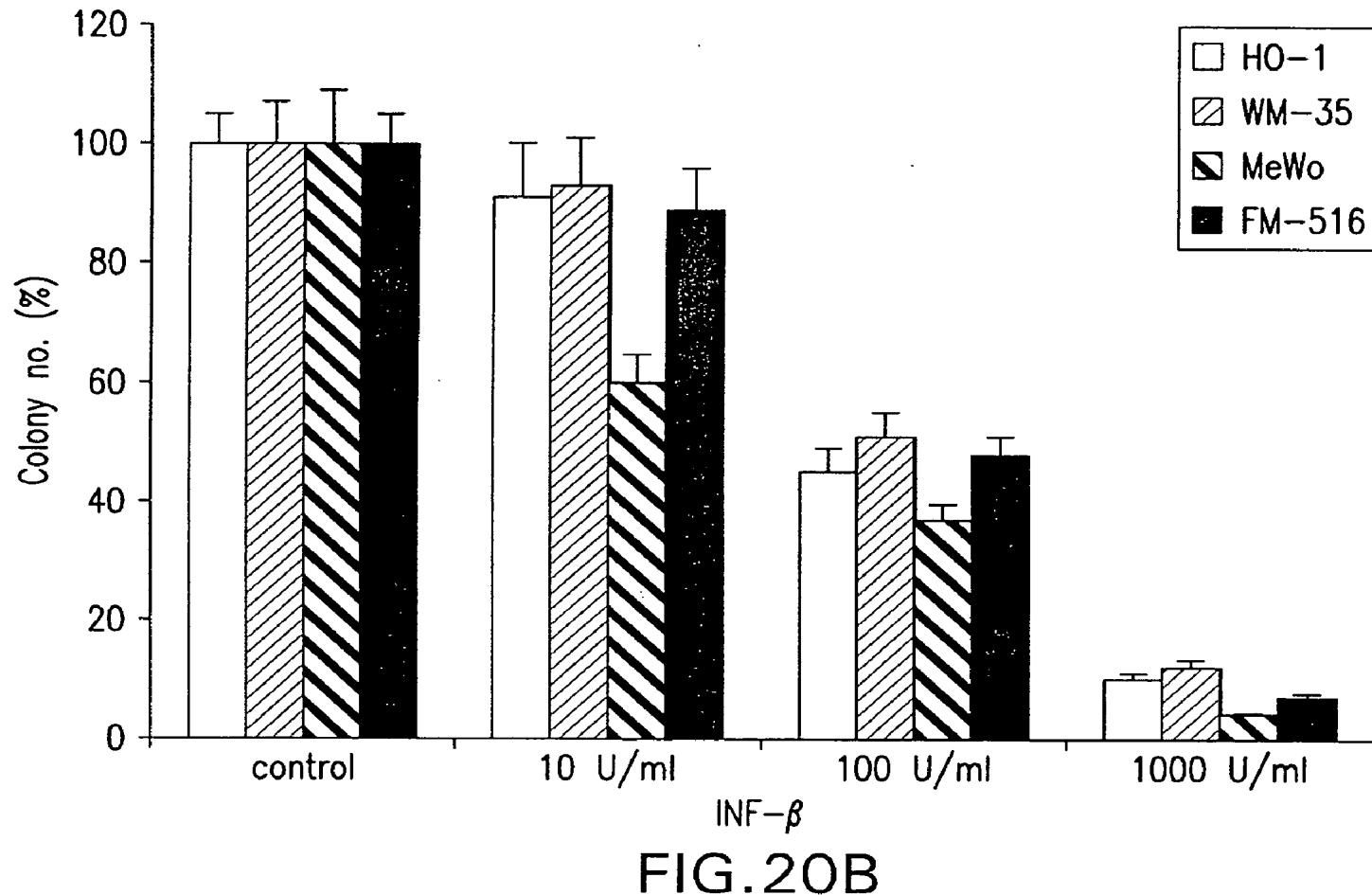


FIG. 20A



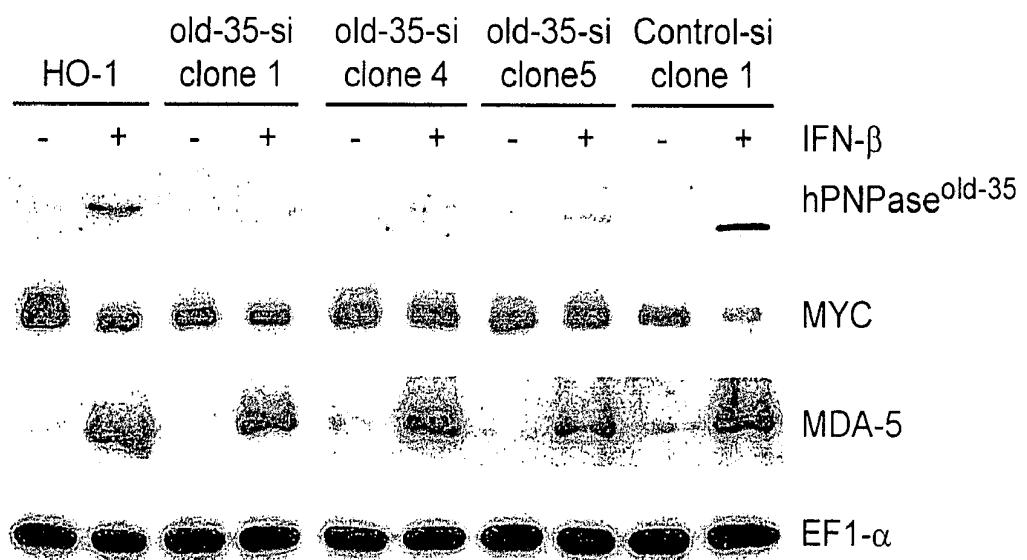


FIG.21A

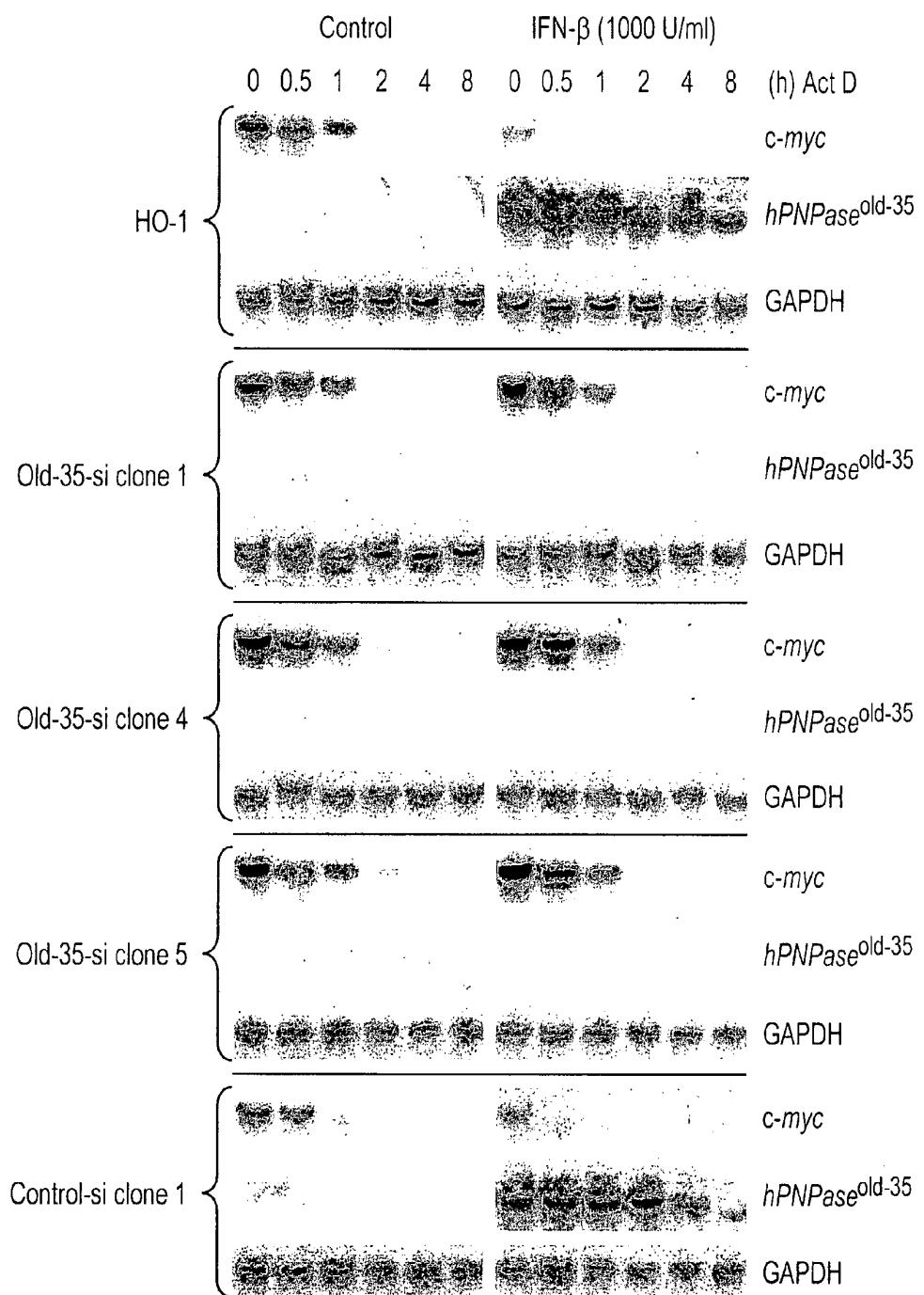


FIG. 21B

Control siRNA

c-myc siRNA

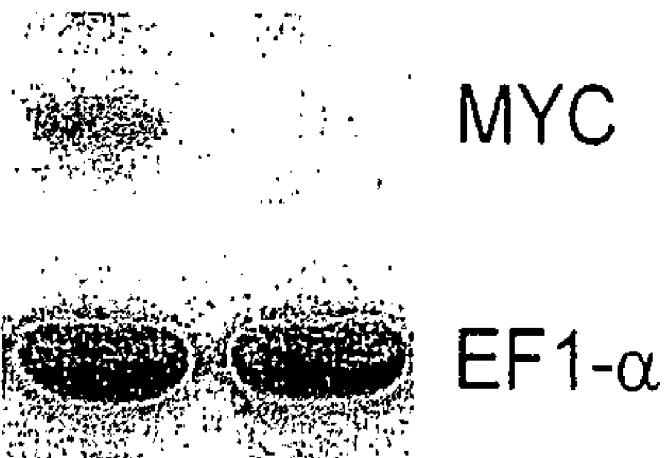


FIG.22A

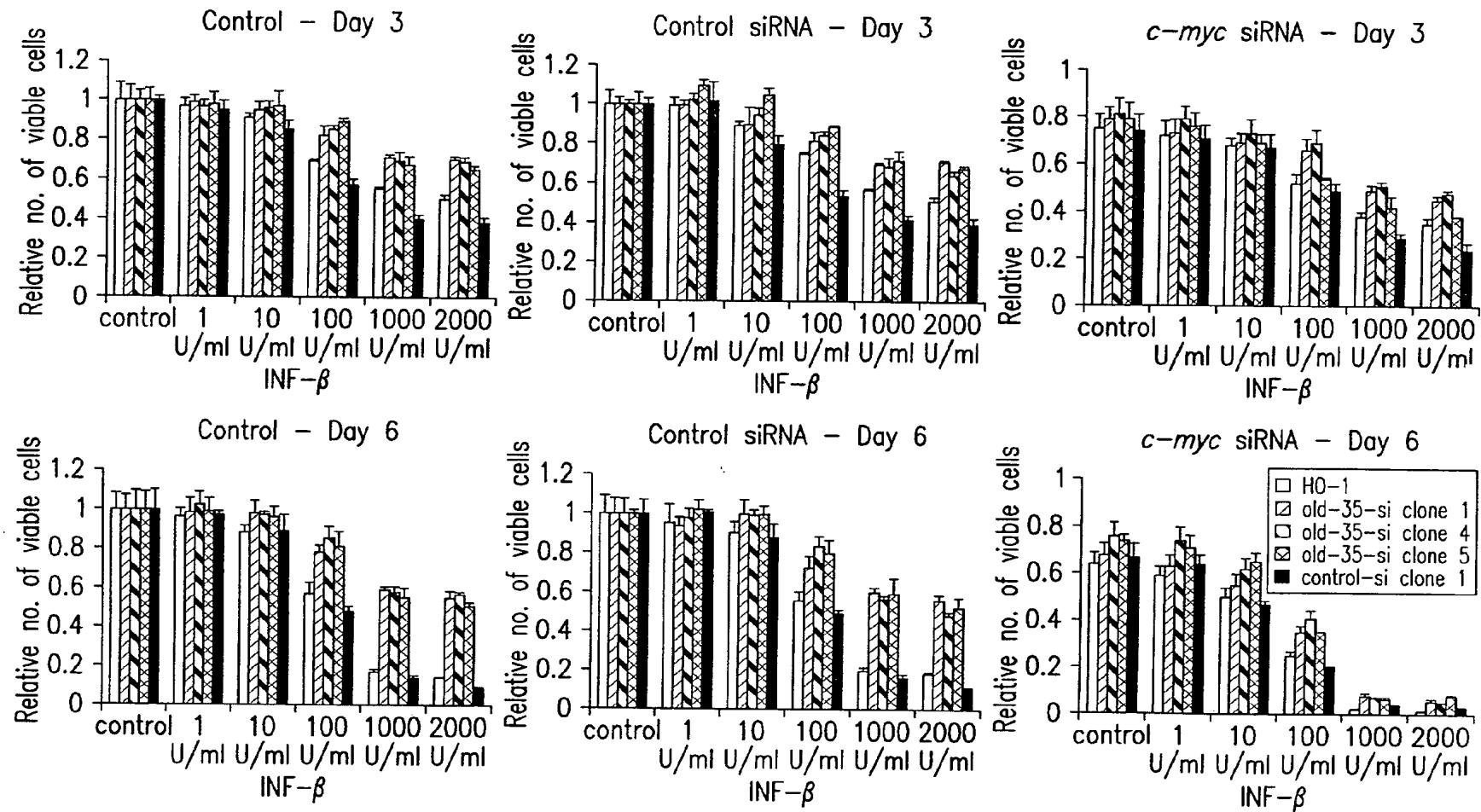


FIG. 22B

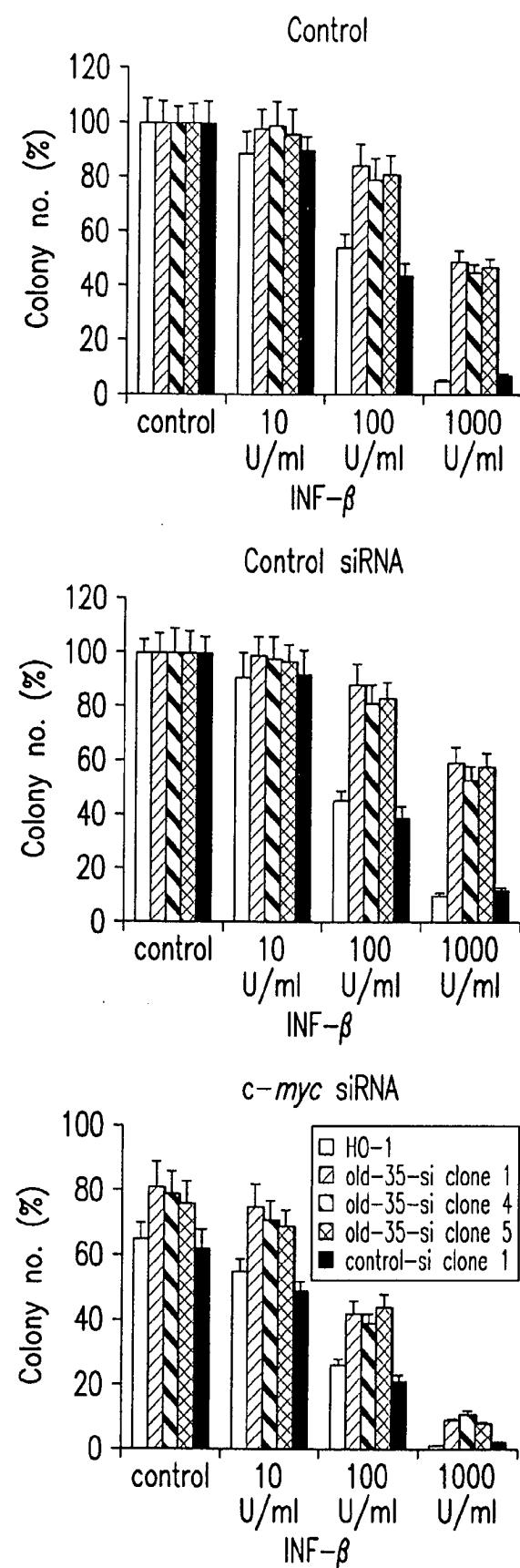


FIG. 23

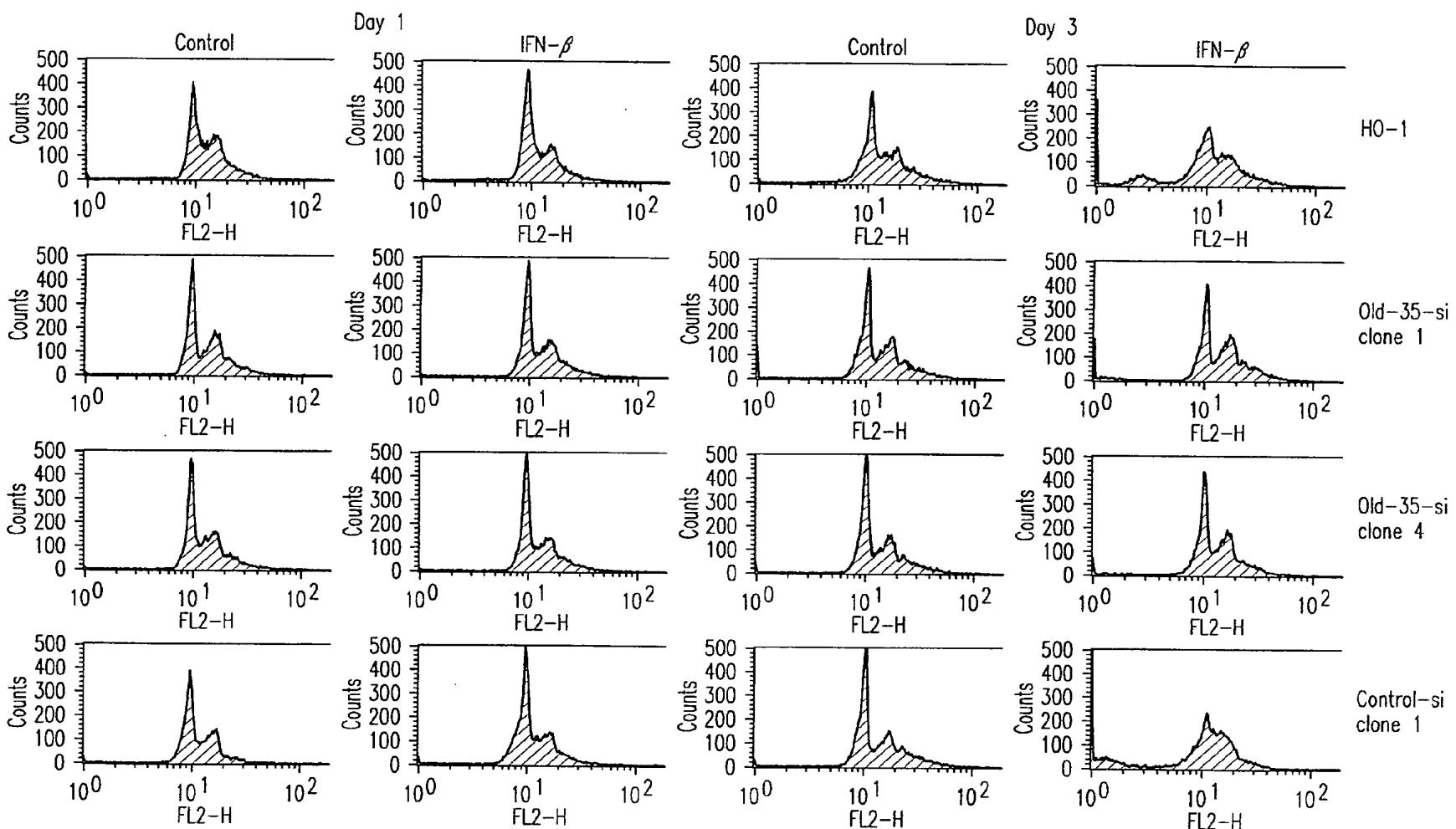


FIG. 24

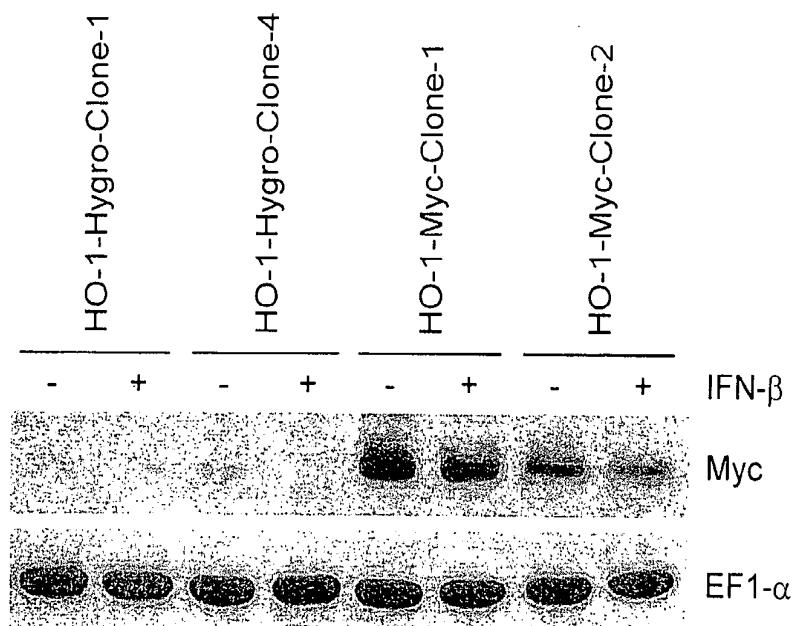


FIG. 25A

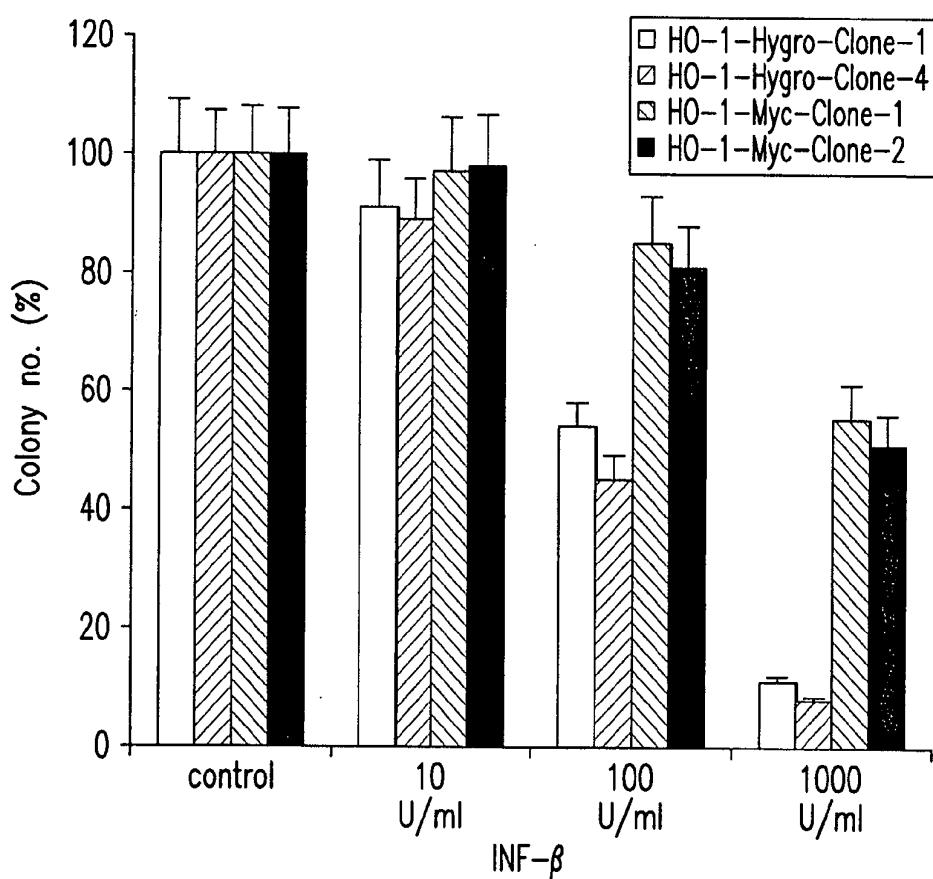


FIG. 25B

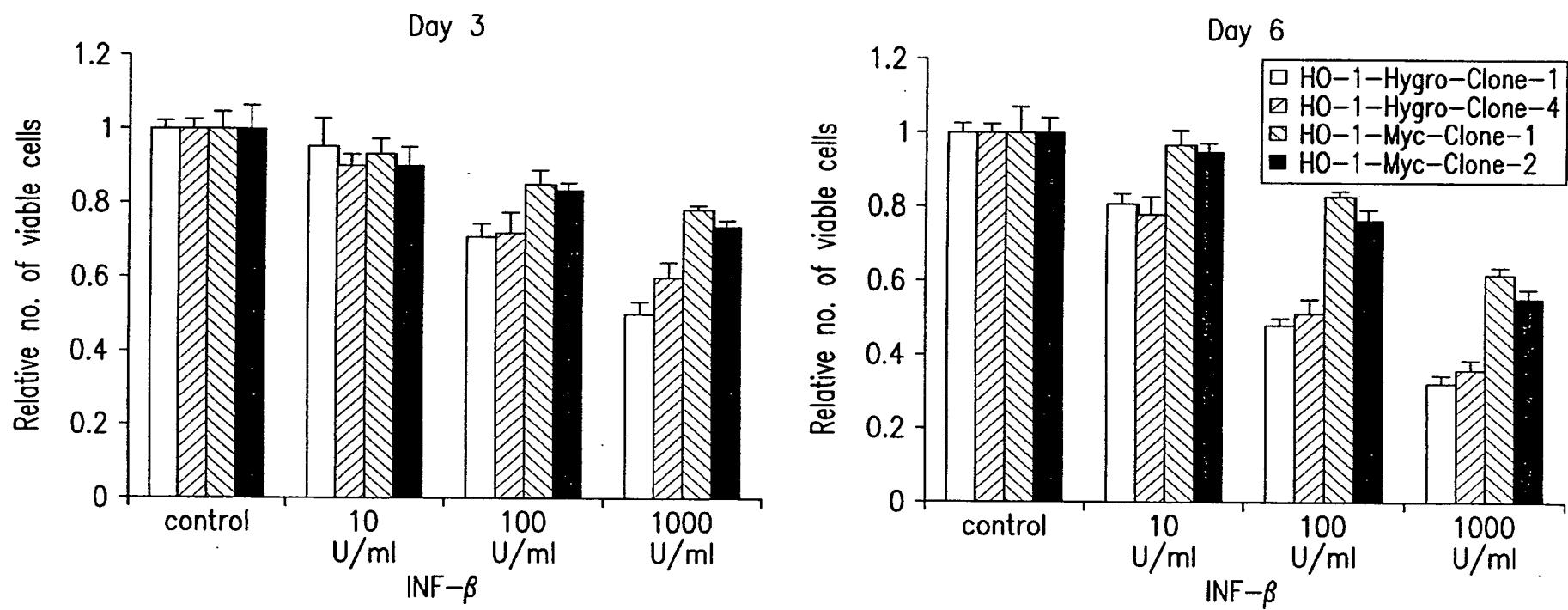


FIG.25C

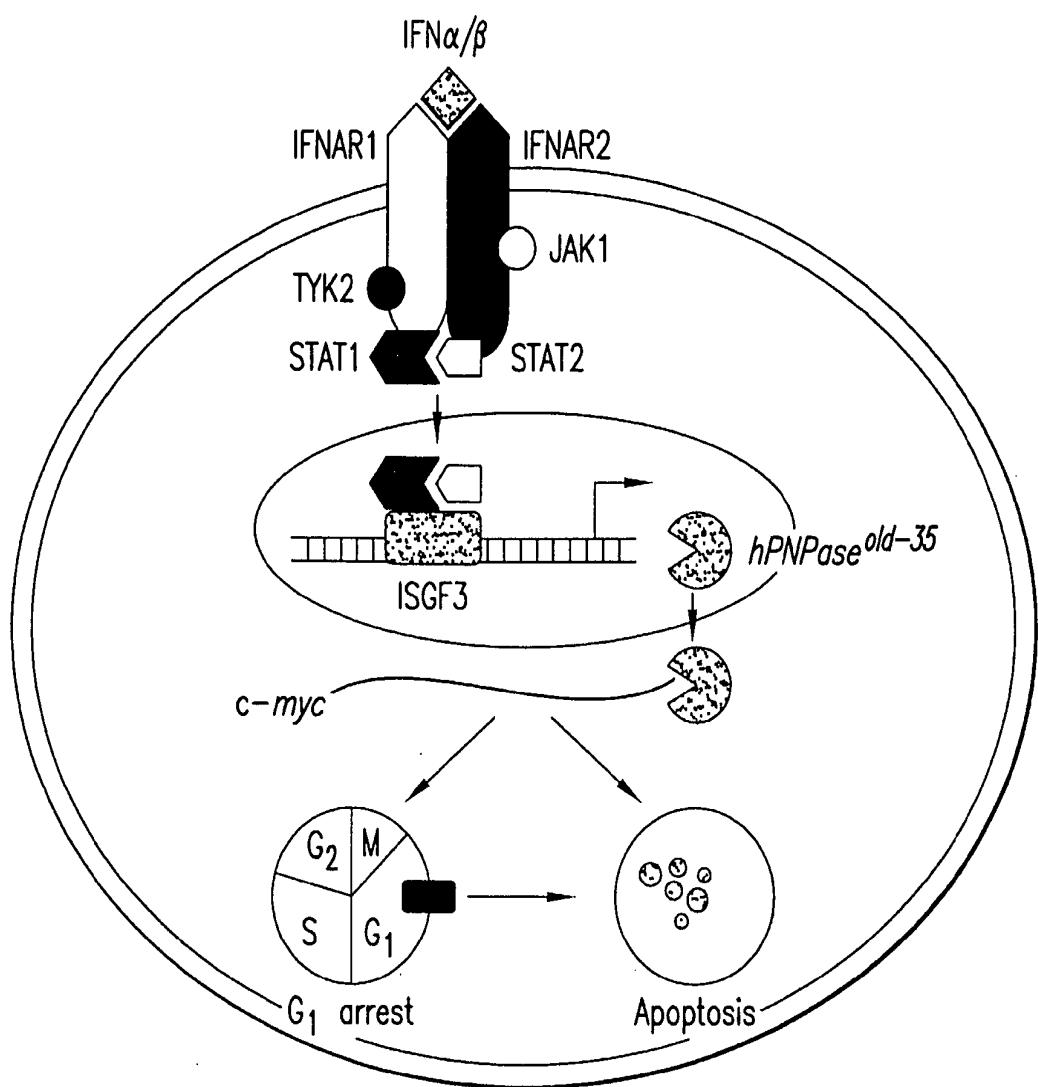


FIG.26

Table 1. IFN- β induces G₁ cell cycle arrest and apoptosis in melanoma cells and normal melanocytes.

G₁

	Day 1	Day 2	Day 3
HO-1 control	50.6	44.4	45.45
HO-1 IFN- β	59.05*	55.8*	43.33
WM-35 control	48.94	47.57	55.59
WM-35 IFN- β	60.8*	43.26	30.91
MeWo control	48.91	53.19	40.7
MeWo IFN- β	56.05*	58.13	35.81
FM-516 control	43	50.59	51.11
FM-516 IFN- β	50.86*	47.12	38.54

S

	Day 1	Day 2	Day 3
HO-1 control	9.28	11.37	11.42
HO-1 IFN- β	6.97*	6.77*	6.73*
WM-35 control	8.21	12.82	8.86
WM-35 IFN- β	6.2*	6.54*	4.65*
MeWo control	9.47	13.58	10.53
MeWo IFN- β	7.89*	5.94*	4.65*
FM-516 control	13.6	11.1	8.42
FM-516 IFN- β	8.6*	6.47*	5.31*

G₂+M

	Day 1	Day 2	Day 3
HO-1 control	37.88	47.09	38.28
HO-1 IFN- β	29.83	31.65	31.12
WM-35 control	38.82	35.56	33.55
WM-35 IFN- β	29.51	38.59	33.9
MeWo control	41.33	30.02	46.25
MeWo IFN- β	33.4	26.2	44.32
FM-516 control	40.83	35.31	37.39
FM-516 IFN- β	37.64	40.48	42.67

A₀

	Day 1	Day 2	Day 3
HO-1 control	1.61	1.14	0.75
HO-1 IFN- β	1.60*	5.78*	13.81*
WM-35 control	1.35	1.95	1.48
WM-35 IFN- β	1.51	8.87*	21.87*
MeWo control	0.71	1.68	2.52
MeWo IFN- β	1.4	5.89*	15.22*
FM-516 control	2.57	2.99	3.08
FM-516 IFN- β	2.9	5.93*	13.48*

FIG. 27

Table 2. HO-1 clones expressing *hPNPase old-35* siRNA are resistant to IFN- β -induced G₁ cell cycle arrest and apoptosis which can be inhibited by c-myc siRNA.

G ₁	Day 1			Day 2			Day 3		
	control	control siRNA	c-myc siRNA	control	control siRNA	c-myc siRNA	control	control siRNA	c-myc siRNA
HO-1 control	51.1	51.2	56.48	43.8	43.58	52.64*	45.09	44.52	46.25
HO-1 IFN- β	58.07*	50.54*	63.25*	55.1*	53.48	57.48*	44.13	41.26	44.65
Old-35-si clone 1 Control	51.85	50.26	55.45	45.22	46.21	50.21	51.56	49.85	50.25
Old-35-si clone 1 IFN- β	53.97	52.84	61.58*	46.48	47.85	58.64*	47.31	44.56	47.23
Old-35-si clone 4 control	51.31	50.28	57.42	48.02	46.54	51.64	49.17	44.62	47.56
Old-35-si clone 4 IFN- β	52.81	51.36	60.58*	48.16	47.87	56.87*	45.59	46.32	48.35
Old-35-si clone 5 control	50.87	52.36	54.23	44.59	45.81	52.34	47.85	46.17	45.63
Old-35-si clone 5 IFN- β	51.23	50.62	59.21*	43.96	44.58	54.49*	45.62	42.89	47.51
Control-si clone 1 control	52.08	50.26	57.48	50.53	49.23	54.65	49.48	47.25	48.62
Control-si clone 1 IFN- β	63.07*	61.56*	64.48*	52.21	47.81	59.42*	41.53	42.68	43.54

S	Day 1			Day 2			Day 3		
	control	control siRNA	c-myc siRNA	control	control siRNA	c-myc siRNA	control	control siRNA	c-myc siRNA
HO-1 control	9.11	9.54	7.19	10.91	10.58	5.31*	11.19	10.64	5.22*
HO-1 IFN- β	6.76*	6.15*	5.05*	6.69*	5.59*	4.28*	5.98*	5.56*	4.11*
Old-35-si clone 1 Control	8.58	9.14	7.24	8.12	9.04	7.09	6.05	7.57	6.87
Old-35-si clone 1 IFN- β	8.1	9.04	6.24*	8.12	8.88	5.98*	6.02	7.48	4.14*
Old-35-si clone 4 control	12	11.54	9.32	11.02	11.47	8.96	7.24	7.95	6.21
Old-35-si clone 4 IFN- β	10.48	10.29	7.43*	9.55	10.01	7.13*	6.55	7.12	4.65*
Old-35-si clone 5 control	9.85	10.02	7.54	9.59	9.69	7.15	7.88	7.61	6.33
Old-35-si clone 5 IFN- β	9.58	9.84	6.21*	9.09	8.99	6.07*	7.54	7.18	4.51*
Control-si clone 1 control	7.74	8.29	5.67	10.84	9.52	5.57	9.22	9.17	5.13
Control-si clone 1 IFN- β	5.9*	5.61*	4.58*	7.07*	5.21*	4.38*	4.25*	4.89*	3.99*

FIG. 28A

Table 2. HO-1 clones expressing *hPNPase old-35* siRNA are resistant to IFN- β -induced G₁ cell cycle arrest and apoptosis which can be inhibited by c-myc siRNA.

	Day 1			Day 2			Day 3		
	control	control siRNA	c-myc siRNA	control	control siRNA	c-myc siRNA	control	control siRNA	c-myc siRNA
HO-1 control	38.21	38.03	34.66	44.18	44.82	37.84	42.74	43.72	41.66
HO-1 IFN- β	33.2	31.95	29.82	32.04	33.62	29.91	35.68	39.31	33.64
Old-35-si clone 1 Control	38.87	39.71	36.29	45.77	43.72	39.46	41.73	41.69	37.04
Old-35-si clone 1 IFN- β	37.26	37.39	30.99	44.48	42.28	28.21	44.43	46.11	32.13
Old-35-si clone 4 control	36.18	37.52	32.27	40.52	41.18	35.91	43.03	46.46	41.65
Old-35-si clone 4 IFN- β	35.99	37.4	31.1	41.06	40.98	29.01	46.27	45.09	32.3
Old-35-si clone 5 control	38.27	36.65	37.09	44.64	43.08	36.54	43.03	44.86	42.93
Old-35-si clone 5 IFN- β	38.06	38.43	33.34	45.97	45.56	32.36	44.74	47.96	31.58
Control-si clone 1 control	39.37	40.44	35.68	37.92	40.11	35.4	40.75	42.71	40.1
Control-si clone 1 IFN- β	30.23	31.79	29.73	35.85	40.81	27.24	37.91	37.68	35.27

A0	Day 1			Day 2			Day 3		
	control	control siRNA	c-myc siRNA	control	control siRNA	c-myc siRNA	control	control siRNA	c-myc siRNA
HO-1 control	1.58	1.23	1.67	1.11	1.02	4.21*	0.98	1.12	6.87*
HO-1 IFN- β	1.97	1.36	1.88	6.17*	6.95*	8.33*	14.21*	13.87*	17.6*
Old-35-si clone 1 Control	0.7	0.89	1.02	0.89	1.03	3.24	0.66	0.89	5.84
Old-35-si clone 1 IFN- β	0.67	0.73	1.19	0.92	0.99	7.17*	2.24	1.85	16.5*
Old-35-si clone 4 control	0.51	0.66	0.99	0.44	0.81	3.49	0.56	0.97	4.58
Old-35-si clone 4 IFN- β	0.72	0.95	0.89	1.23	1.14	6.99*	1.59	1.47	14.7*
Old-35-si clone 5 control	1.01	0.97	1.14	1.18	1.42	3.97	1.24	1.36	5.11
Old-35-si clone 5 IFN- β	1.13	1.11	1.24	0.98	0.87	7.08*	2.10	1.97	16.4*
Control-si clone 1 control	0.81	1.01	1.17	0.71	1.14	4.38	0.55	0.87	6.15*
Control-si clone 1 IFN- β	0.8	1.04	1.21	4.87*	6.17*	8.96*	16.31*	14.75*	17.2*

FIG.28B

Table 3. H0-1 clones overexpressing Myc are resistant to IFN- β -induced G₁ cell cycle arrest and apoptosis

G₁

	Day 1	Day 2	Day 3
H0-1-Hygro-Clone-1 control	59.7	50.3	50.2
H0-1-Hygro-Clone-1 IFN- β	74.84*	61.7*	50.23
H0-1-Hygro-Clone-4 control	57.6	49.2	45.8
H0-1-Hygro-Clone-4 IFN- β	69.1*	58.5*	49.7
H0-1-Myc-Clone-1 control	56.66	51.4	49.1
H0-1-Myc-Clone-1 IFN- β	60.2	54.9	50.6
H0-1-Myc-Clone-2 control	57.8	52.8	44.6
H0-1-Myc-Clone-2 IFN- β	61.9	54.32	48.1

S

	Day 1	Day 2	Day 3
H0-1-Hygro-Clone-1 control	16.3	17.8	18.2
H0-1-Hygro-Clone-1 IFN- β	8.1*	7.5*	6.3*
H0-1-Hygro-Clone-4 control	14.8	16.1	17.2
H0-1-Hygro-Clone-4 IFN- β	7.2*	6.1*	5.8*
H0-1-Myc-Clone-1 control	15.6	16.9	18.4
H0-1-Myc-Clone-1 IFN- β	12.5	11.8	13.6
H0-1-Myc-Clone-2 control	16.9	14.23	17.8
H0-1-Myc-Clone-2 IFN- β	13.8	12.5	12.9

G₂+M

	Day 1	Day 2	Day 3
H0-1-Hygro-Clone-1 control	21.9	29.6	29.8
H0-1-Hygro-Clone-1 IFN- β	14.46	22	28.17
H0-1-Hygro-Clone-4 control	26.2	32.85	35.33
H0-1-Hygro-Clone-4 IFN- β	21.8	28.1	25.7
H0-1-Myc-Clone-1 control	26.04	30.5	30.1
H0-1-Myc-Clone-1 IFN- β	25.2	30.1	28.9
H0-1-Myc-Clone-2 control	23.8	31.17	35.3
H0-1-Myc-Clone-2 IFN- β	22.4	30.37	33.6

A₀

	Day 1	Day 2	Day 3
H0-1-Hygro-Clone-1 control	2.1	2.3	1.8
H0-1-Hygro-Clone-1 IFN- β	2.6	8.8*	15.3*
H0-1-Hygro-Clone-4 control	1.4	1.85	1.67
H0-1-Hygro-Clone-4 IFN- β	1.9	7.3*	18.8*
H0-1-Myc-Clone-1 control	1.7	1.2	2.4
H0-1-Myc-Clone-1 IFN- β	2.1	3.2	6.9
H0-1-Myc-Clone-2 control	1.5	1.8	2.3
H0-1-Myc-Clone-2 IFN- β	1.9	2.81	5.4

FIG. 29

OLD-35 AS AN INFLAMMATORY AGENT**CROSS-REFERENCE TO RELATED APPLICATIONS**

[0001] This application claims priority to U.S. Provisional Patent Application No. 60/616,774 filed Oct. 7, 2004 which is incorporated by reference in its entirety herein.

1. INTRODUCTION

[0002] The present invention relates to methods of modulating inflammation by altering the expression and/or activity of OLD-35, a protein originally identified by its association with senescence and terminal cell differentiation. The invention is based on the discovery that OLD-35, at least in part through the generation of reactive oxygen species, induces a number of inflammatory cytokines and promotes nuclear translocation and binding of the transcriptional activator NF- κ B. The present invention further provides for active portions of OLD-35 which may be used therapeutically.

2. BACKGROUND OF THE INVENTION

[0003] Human polynucleotide phosphorylase (hPNPase^{old-35}, referred to as "OLD-35" herein) was identified as a previously unknown gene, old-35, by an overlapping pathway screening (OPS) approach due to its upregulation during cellular differentiation and senescence (1). Old-35, a 3', 5' exoribonuclease, is a predominantly type I interferon-inducible gene highly evolutionary conserved in plants, prokaryotes and eukaryotes having similar domain structures and functional properties in all species (1-3). Its expression is also augmented in senescent progeroid fibroblasts in comparison to young fibroblasts (1). Overexpression of old-35 via a replication incompetent adenovirus (Ad.hPNPase) in HO-1 human melanoma cells and in normal human melanocytes (NHuMel) produced a senescent phenotype characterized by growth arrest in the G₁ phase, increased Senescence Associated β -galactosidase activity, decreased telomerase activity and defined senescence-associated gene expression changes (4). These profound alterations induced by old-35 suggest an essential role in controlling senescence and differentiation through its property as an exoribonuclease by targeting selective RNA degradation. This hypothesis is supported by the observations that old-35 induces specific degradation of c-myc mRNA and that overexpression of c-myc partially protects HO-1 cells from old-35-induced growth arrest (4).

[0004] Oxidative stress is a potential mediator of in vitro replicative and premature senescence and in vivo aging (5). The free radical theory of aging, as proposed by Harman, states that endogenous reactive oxygen species ("ROS") are generated in cells resulting in a pattern of cumulative damage (6). Oxidative damage can be measured by formation of 8-oxo-2'-deoxyguanosine (oxo⁸dG) in DNA or free 8-oxoguanine base (oxo⁸Gua) release by cells (7). Replicative senescent cells contain approximately 30% more oxo⁸dG in their DNA and produce four times more free oxo⁸Gua bases (8). Tissues from aged individuals or aged experimental animals accumulate oxidative damage in their DNA, protein and lipids (9). Moreover, repeated subcytotoxic oxidative damage can induce premature senescence in multiple cell types such as fibroblasts, keratinocytes, mel-

anocytes or umbilical vascular endothelial cells (10) and treatment with a cell-permeable anti-oxidant or culturing cells in a reduced ambient oxygen content can reverse growth arrest of fibroblasts induced by expression of activated Ras (11). ROS comprise a variety of diverse chemical species including superoxide anions, hydroxyl radicals and hydrogen peroxide (5). Although cytosolic enzymes such as NADPH oxidases contribute to the generation of ROS, the majority of intracellular ROS production generates from mitochondria (5). Additionally, aged animals contain defective mitochondria and can produce higher levels of ROS than their young counterparts (12).

[0005] A prominent mechanism by which ROS modulates diverse intracellular molecular processes is by regulating the activity of transcription factors, most notably nuclear factor (NF)- κ B (13). As a corollary to increased ROS generation during the aging process, increased NF- κ B DNA binding activity has been documented in multiple tissues of aged animals compared with young animals (14-21). In resting cells, NF- κ B resides in the cytoplasm in an inactive form bound to an inhibitory protein known as I κ B (22). Upon receiving a stimulus, such as ROS, I κ B kinase (IKK) is activated which in turn phosphorylates I κ B proteins making them susceptible to ubiquitin-proteosome-mediated degradation (23, 24). The destruction of I κ B unmasks the nuclear localization signal of NF- κ B, leading to nuclear translocation and regulation of gene transcription by binding to the decameric motif, "GGGRNNYYCC" in the promoters of target genes (25). Presently, five mammalian NF- κ B family members, NF- κ B1 (p50/p105), NF- κ B2 (p52/p100), p65 (RelA), RelB and c-Rel, have been identified and cloned (26). The most abundant activated form of NF- κ B is a heterodimer composed of a p50 and a p65 subunit that functions predominantly as a transcriptional activator.

3. SUMMARY OF THE INVENTION

[0006] The present invention relates to the discovery that OLD-35, at least in part through the generation of reactive oxygen species, induces a number of inflammatory cytokines and promotes nuclear translocation and binding of the transcriptional activator NF- κ B. Accordingly, the present invention provides for assay systems (which either utilize the old-35 promoter or the old-35 gene) that may be used to identify new anti-inflammatory agents; model systems of inflammation based on over-expression of the old-35 gene in cells and tissues (including specific model systems for arthritis, atherosclerosis and Alzheimer's disease); methods and kits for diagnosing old-35 associated inflammatory conditions, and methods of treatment and anti-inflammatory compositions that utilize agents that antagonize OLD-35 activity. The present invention further relates to portions of OLD-35 which exhibit PNPase activity. Such portions may be used therapeutically to inhibit cell proliferation (for example, in the context of malignancy) or to act as "vaccines" to inhibit inflammation.

4. BRIEF DESCRIPTION OF THE FIGURES

[0007] FIG. 1A-D. OLD-35 is localized in the mitochondria and Ad.hPNPase infection results in the generation of ROS. (A.) HeLa cells were either uninfected or infected with Ad.vec or Ad.hPNPase at a m.o.i. of 50 pfu/cell. The expression of OLD-35 was analyzed 2 days post-infection by Western blot analysis. (B.) HeLa cells were infected with

Ad.hPNPase at a m.o.i. of 50 pfu/cell. Subcellular localization of OLD-35 was analyzed by staining with MitoTracker and anti-OLD-35 antibody. (C.) HeLa cells were infected with Ad.vec or Ad.hPNPase at a m.o.i. of 50 pfu/cell and untreated or treated with 20 mM NAC 2 hr post-infection. The generation of ROS was measured by flow cytometry as described in Materials and Methods (below). The panels represent flow cytometry histograms at 24 h post-infection. (D.) Graphical representation of quantification of ROS containing cells at indicated time periods after infection. The data represent the mean \pm S.D. of three independent experiments.

[0008] FIG. 2A-B. OLD-35 augments NF- κ B reporter gene activity by generating ROS. (A.) HeLa cells were infected with the indicated adenoviruses, transfected with the indicated plasmids and luciferase activity was measured as described in materials and methods. (B.) HeLa cells were infected with either Ad.vec or Ad.hPNPase at a m.o.i. of 50 pfu/cell and 12 hr after infection transfected with the plasmid 3 KB-Luc. The cells were pretreated with NAC or Tiron 2 hr before transfection. Luciferase assay was carried out 48 hr post-transfection. Lanes 1-3: Ad.vec; 2: +NAC (20 mM); 3: +Tiron (4.5 mM). 4-10: Ad.hPNPase; 5: +NAC (5 mM); 6: +NAC (10 mM); 7: +NAC (20 mM); 8: +Tiron (0.9 mM) 9: +Tiron (2.25 mM); 10: +Tiron (4.5 mM). The luciferase activity was normalized by β -galactosidase activity. The data represent mean \pm S.D. of three independent experiments each performed in triplicates.

[0009] FIG. 3A-D. Ad.hPNPase infection increases NF- κ B DNA binding by generating ROS. (A.) HeLa cells were infected as in FIG. 1A or with Ad.vec at 50 m.o.i. or with Ad.hPNPase at 1, 5, 10 or 50 m.o.i. and NF- κ B DNA binding was analyzed in the nuclear extracts of the cells by EMSA at the indicated time points. (B.) HeLa cells were infected with Ad.vec or Ad.hPNPase at 50 m.o.i. and NF- κ B DNA binding was analyzed 2 days post-infection. Cold WT: unlabeled consensus NF- κ B probe; Cold MUT: unlabeled mutated NF- κ B probe. Supershift analysis was carried out with the indicated antibodies. *: supershifted band by anti-p50 antibody; ** supershifted band by anti-p65 antibody. (C.) HeLa cells were infected with the indicated adenoviruses at 50 m.o.i. and NF- κ B DNA binding was analyzed 2 days post-infection. D. HeLa cells were infected with Ad.vec or Ad.hPNPase at 50 m.o.i. and treated or not with 20 mM NAC. NF- κ B DNA binding was analyzed 2 days post-infection.

[0010] FIG. 4A-C. Ad.hPNPase infection results in I κ B κ c degradation and nuclear translocation of p65 by generating ROS. HeLa cells were infected as in FIG. 1A and the expressions of the indicated proteins were analyzed in (A.) cytoplasmic extract and (B.) nuclear extract by Western blot analysis at the indicated time points. (C.) HeLa cells were infected as in FIG. 1A and treated with 20 mM NAC. The expressions of the indicated proteins were analyzed by Western blot analysis 2 days post-infection.

[0011] FIG. 5A-C. Ad.hPNPase infection induces the expression of IL-6 and IL-8 by generating ROS. (A.) HeLa cells were infected as in FIG. 1A and RT-PCR was performed for the indicated genes. Lanes 1, 3 and 5: Ad.vec; 2, 4 and 6: Ad.hPNPases; lanes 1 and 2 are 1 day, 3 and 4 are 2 days and 5 and 6 are 3 days post-infection. (B) and (C.) HeLa cells were infected as in FIG. 1A and treated with 20

mM NAC. The levels of secreted IL-6 (B) and IL-8 (C) in culture supernatants were analyzed by ELISA. The data represent mean \pm S.D. of two independent experiments each performed in triplicates.

[0012] FIG. 6A-C. Ad.hPNPase infection induces pro-inflammatory cytokines. HeLa cells were infected as in FIG. 1A and the levels of secreted cytokines in culture supernatants were analyzed by TranSignal™ human cytokine antibody array. (A.) Schematic representation of the layout of the blotted membranes. The grids marked by asterisks indicate the cytokines which were induced by OLD-35. (B.) Membrane incubated with culture supernatants from Ad.vec-infected cells. (C.) Membrane incubated with culture supernatants from Ad.hPNPases-infected cells.

[0013] FIG. 7A-B. Nucleic acid sequence of old-35 promoter (SEQ ID NO:1). The end of the promoter and the beginning of the transcribed gene is indicated by underlining of the gene sequence. The portion of the promoter incorporated in p2000 is set forth in boldface print. Parts (A) and (B) of the figure contain consecutive sequence.

[0014] FIG. 8. Graphical representation of various deletions in the old-35 promoter. The construct sizes are not drawn to scale. Numbers on the right describe the approximate construct sizes.

[0015] FIG. 9. Graphical representation of the cloned 2-kb old-35 promoter. The immediate 400-bp sequence of the old-35 promoter (SEQ ID NO:2) including the transcription initiation site is shown (the arrow). Additional sites that may be related to IFN signaling or to the constitutive activity of the old-35 promoter are underlined and bolded. The numbers below the elements indicate the initial bp at which the element is located.

[0016] FIG. 10. Sequences of the consensus ISRE (SEQ ID NO: 3), the old-35 ISRE (SEQ ID NO:4).

[0017] FIG. 11A-B. Sequence of (A) old-35 cDNA (SEQ ID NO:5) and (B) OLD-35 protein (SEQ ID NO:6).

[0018] FIG. 12A-E. The RPH domain of hPNPase^{old-35} is required for induction of senescence in HO-1 cells. A. Schematic representation of the domain structure of the hPNPase^{old-35} protein and the different deletion mutants. The numbers represent the amino acid numbers. The grey box is the mitochondrial localization signal, white boxes are the RPH domains and the hatched boxes are the RNA binding domains. B. HO-1 cells were either infected with the indicated Ad at an m.o.i. of 50 pfu/cell or treated with interferon- β at a dose of 1000 units/ml and the levels of the indicated proteins were analyzed by Western blot analysis 48 h later. C. HO-1 cells were either uninfected or infected with Ad.vec at an m.o.i. of 50 pfu/cell or with the indicated Ad at an m.o.i. of 10, 20 or 50 pfu/cell and colony formation assays were determined as described in experimental procedures. The data represents mean \pm S.D. and is a representation of three independent experiments each performed in triplicates. D. Microphotograph of HO-1 cells infected with the indicated Ad at an m.o.i. of 50 pfu/cell 4 days post-infection. The white arrows indicate the large, flattened cells that stain for SA- β -gal. E. Quantification of SA- β -gal-positive cells. At least 1,000 cells were counted for each group. The data represents mean \pm S.D. of three independent experiments.

[0019] FIG. 13A-D. hPNPase^{old-35} and its RPH-containing deletion mutants inhibit cell cycle at the G₁ phase. HO-1 cells were infected with the indicated Ad at an m.o.i. of 50 pfu/cell and cell cycle was analyzed on day 1, 2 and 3 post-infection. A. Flow cytometry histogram of cells infected with the indicated Ad 3 days post-infection. B-D. Graphical representation of the percentage of cells in the G₁ (B), S(C) and G₂+M (D) phases of the cell cycle. The data represents mean ± S.D. of three independent experiments.

[0020] FIG. 14A-C. Regulation of expression of cell cycle regulatory proteins by hPNPase^{old-35} and its deletion mutants. A. HO-1 cells were infected with the indicated Ad at an m.o.i. of 50 pfu/cell for 3 days and the expression of the indicated proteins in the cell lysates were analyzed by Western blot analysis. B. HO-1 cells were treated as in A and CDK2 activity was assayed using Histone H1 as substrate as described in experimental procedures. The expression level of CDK2 in 5% of the input used for the CDK2 activity assay was determined by Western blot analysis. C. HO-1 cells were infected with Ad.hPNPase^{old-35} at an m.o.i. of 50 pfu/cell for 2 days and immunofluorescence studies were performed to analyze the expression of Ad.hPNPase^{old-35} and p27^{KIP-1} as described in materials and methods.

[0021] FIG. 15A-D. The RPH domain of hPNPase^{old-35} is required for c-myc downregulation. A. HO-1 cells were treated as in FIG. 3A and the expression of the indicated mRNAs were analyzed by Northern blot analysis. B. Graphical representation of the c-myc/GAPDH mRNA level in the indicated treatment groups. The data represents mean ± S.D. of three independent experiments. C. HO-1 cells were treated as in FIG. 14A and the expression of the indicated proteins were analyzed by Western blot analysis. D. HO-1 cells were either transfected with an empty vector or with a c-myc expression vector and 36 h later infected with the indicated Ad at an m.o.i. of 50 pfu/cell and colony formation assays were performed. The data represents the mean ± S.D. and is a representation of three independent experiments each performed in triplicates.

[0022] FIG. 16A-C. The RPH domain of hPNPase^{old-35} is required for in vitro degradation of c-myc mRNA. A. Representation of the in vitro translated products from the indicated plasmids documenting their authenticity. B. In vitro degradation assays were performed as described in experimental procedures. The expression of c-myc, GADD34 and GAPDH mRNAs were detected by Northern blot analysis. C. Quantification of c-myc/GAPDH mRNA levels in the different groups analyzed in panel B. The data represents the mean ± S.D. of three independent experiments.

[0023] FIG. 17. hPNPase^{old-35} downregulates Myc in cells in the G₁ phase of the cell cycle. HO-1 cells were transfected with either empty vector or a c-myc expression plasmid and then infected with either Ad.vec or Ad.hPNPase^{old-35} at an m.o.i. of 50 pfu/cell for 2 days. The cells were sorted as described in materials and methods and the expression levels of the indicated proteins in cells of different phases of cell cycle were analyzed by Western blot analysis. G₂ represents cells in G₂+M phases.

[0024] FIG. 18A-B. Distribution of hPNPase^{OLD-35} and its deletion mutants in mitochondria and cytoplasmic compartments. A. HO-1 cells were infected with the indicated Ad and cell fractionation was performed 36 h later. The expres-

sions of the indicated proteins were detected by Western blot analyses. The asterisks indicate the specific bands in the corresponding lanes. B. HO-1 cells were infected with the indicated Ad and 36 h later loaded with MitoTracker and stained with anti-HA antibody and visualized using a confocal laser scanning microscope.

[0025] FIG. 19A-C. Treatment with IFN-β upregulates hPNPase^{old-35} and downregulates c-myc mRNAs and proteins. A. The various cell lines were treated with IFN-β(1000 U/ml) for the indicated periods of time and the expression of hPNPase^{old-35}, c-myc and GAPDH mRNAs were analyzed by Northern blot analysis. B. The indicated cells were treated with IFN-β (1000 U/ml) for 1 and 2 days or with 1, 10, 100 or 1000 U/ml of IFN-β for 2 days and the expression of hPNPase^{OLD-35}, Myc and EF1-α proteins were analyzed by Western blot analysis. C. 2fTGH human fibrosarcoma cells and its four variants, U1A (Tyk2-), U3A (STAT1-), U4A (JAK1-) and U5A (IFN2AR-), were treated with IFN-β (1000 U/ml) for 2 days and the expression of the indicated proteins were analyzed by Western blot analysis.

[0026] FIG. 20A-B. IFN-β treatment inhibits growth and colony formation of human melanoma cells and immortalized human melanocytes. A. The different cell types were treated with the indicated concentrations of IFN-β and cell viability was assessed by standard MTT assay on day 3 and 6 post-treatment. The data represents the mean ± S.D. of three independent experiments each performed in octaplates. B. The different cells were plated in 6-cm dish at a density of 1,000 cells/dish and then treated with the indicated concentrations of IFN-β. Colonies were counted after 2 weeks. At least 4 dishes were used for each data point in each experiment. The data represents the mean ± S.D. of two independent experiments.

[0027] FIG. 21A-B. HO-1 clones expressing hPNPase^{old-35} siRNA are resistant to IFN-β-mediated c-myc downregulation. A. Parental HO-1 cells, HO-1 clones expressing hPNPase^{old-35} siRNA (old-35-si clone 1, clone 4 and clone 5) and HO-1 clones expressing control siRNA (control-si clone 1) were treated with IFN-β (1000 U/ml) for 2 days and the expression of hPNPase^{OLD-35}, Myc, MDA-5 and EF1-α proteins were analyzed by Western blot analysis. B. For analysis of half-life of c-myc mRNA, cells were either untreated or treated with IFN-β (1000 U/ml) for 24 h and then exposed to Actinomycin D (5 µg/ml) for 0.5, 1, 2, 4 and 8 h after which the cells were harvested for total RNA extraction and Northern blot analysis using the indicated probes.

[0028] FIG. 22A-B. HO-1 clones expressing hPNPase^{old-35} siRNA are resistant to IFN-β-mediated growth inhibition that can be reversed by c-myc siRNA. A. HO-1 cells were transfected with either control siRNA or c-myc siRNA and the expression of Myc and EF1-α proteins were analyzed by Western blot analysis. B. HO-1 cells, HO-1 clones expressing hPNPase^{old-35} siRNA (old-35-si clone 1, clone 4 and clone 5) and HO-1 clones expressing control siRNA (control-si clone 1) were either mock-transfected (control) or transfected with control siRNA or c-myc siRNA and then treated with the indicated concentrations of IFN-β and cell viability was assessed by standard MTT assay on day 3 and 6 post-treatment. The data represents the mean ± S.D. of three independent experiments each performed in octaplates.

[0029] FIG. 23. HO-1 clones expressing hPNPase^{old-35} siRNA are resistant to IFN- β -mediated colony formation inhibition that can be reversed by c-myc siRNA. HO-1 cells, HO-1 clones expressing hPNPase^{old-35} siRNA (old-35-si clone 1, clone 4 and clone 5) and HO-1 clones expressing control siRNA (control-si clone 1) were either mock-transfected (control) or transfected with control siRNA or c-myc siRNA and then treated with the indicated concentrations of IFN- β and colony formation assay was performed. Colonies were counted after 2 weeks. At least 4 dishes were used for each data point in each experiment. The data represents the mean \pm S.D. of two independent experiments.

[0030] FIG. 24. HO-1 clones expressing hPNPase^{old-35} siRNA are resistant to IFN- β -induced G₁ cell cycle arrest and apoptosis. HO-1 cells, HO-1 clones expressing hPNPase^{old-35} siRNA (old-35-si clone 1 and clone 4) and HO-1 clones expressing control siRNA (control-si clone 1) were treated with IFN- β (1000 U/ml) and cell cycle was analyzed by flow cytometry on day 1 and 3 post-treatment.

[0031] FIG. 25A-C. HO-1 clones overexpressing Myc are resistant to IFN- β -mediated growth and colony formation inhibition. A. Myc and EF1- α expressions were analyzed by Western blot analysis in control HO-1 clones (HO-1-Hygro-clone-1 and HO-1-Hygro-clone-4) and Myc overexpressing clones (HO-1-Myc-clone-1 and HO-1-Myc-clone-2) treated or not with IFN- β (1000 U/ml) for 2 days. B. Control HO-1 clones (HO-1-Hygro-clone-1 and HO-1-Hygro-clone-4) and Myc overexpressing clones (HO-1-Myc-clone-1 and HO-1-Myc-clone-2) were treated with the indicated concentrations of IFN- β and colony formation assays were performed. Colonies were counted after 2 weeks. At least 4 dishes were used for each data point in each experiment. The data represents the mean \pm S.D. of two independent experiments. C. Control HO-1 clones (HO-1-Hygro-clone-1 and HO-1-Hygro-clone-4) and Myc overexpressing clones (HO-1-Myc-clone-1 and HO-1-Myc-clone-2) were treated with the indicated concentrations of IFN- β and cell viability was assessed by standard MTT assay on day 3 and 6 post-treatment. The data represents the mean \pm S.D. of three independent experiments each performed in octuplicates.

[0032] FIG. 26. A schematic model of regulation of hPNPase^{old-35} and c-myc by IFN- β . Binding of IFN- α/β to the cognate receptors IFNAR1 and IFNAR2 results in cross-phosphorylation and activation of TYK2 and JAK1 with subsequent phosphorylation of STAT1 and STAT2. Phosphorylated STAT1/STAT2 heterodimer translocates to the nucleus, associates with p48 to form the ISGF3 complex that binds to the promoter of hPNPase^{old-35} and upregulates its transcription. hPNPase^{OLD-35} protein enters into cytoplasm and binds and degrades c-myc mRNA by its 3', 5' exoribonuclease activity. Downregulation of c-myc results in cell cycle arrest in G₁ phase with subsequent apoptosis.

[0033] FIG. 27. Table 1. HO-1, WM-35, MeWo and FM-516 cells were treated with IFN- β (1000 U/ml) and cell cycle was analyzed by flow cytometry on day 1, 2 and 3 post-treatment. Bold IFN- β -treated data points marked with asterisks indicate significant differences (p<0.01) from the control data points.

[0034] FIG. 28. Table 2. HO-1 cells, HO-1 clones expressing hPNPase^{old-35} siRNA (old-35-si clone 1, clone 4 and clone 5) and HO-1 clones expressing control siRNA (control-si clone 1) were either mock-transfected (control) or

transfected with control siRNA or c-myc siRNA and then treated with IFN- β (1000 U/ml) and cell cycle was analyzed by flow cytometry on day 1, 2 and 3 post-treatment. Bold data points marked with asterisks indicate significant differences (p<0.01) from the control data points.

[0035] FIG. 29. Table 3. Control HO-1 clones (HO-1-Hygro-clone-1 and HO-1-Hygro-clone-4) and Myc overexpressing clones (HO-1-Myc-clone-1 and HO-1-Myc-clone-2) were treated with IFN- β (1000 U/ml) and cell cycle was analyzed by flow cytometry on day 1, 2 and 3 post-treatment. Bold IFN- β -treated data points marked with asterisks indicate significant differences (p<0.01) from the control data points.

5. DETAILED DESCRIPTION OF THE INVENTION

[0036] For clarity and not by way of limitation, the detailed description of the invention is divided into the following subsections:

- [0037] (i) assay systems (which either utilize the old-35 promoter or the old-35 gene);
- [0038] (ii) model systems of inflammation;
- [0039] (iii) diagnostic methods and kits;
- [0040] (iv) methods of treatment and anti-inflammatory compositions;
- [0041] (v) active subregions of OLD-35; and
- [0042] (vi) use of OLD-35 variants.

[0043] Note that the old-35 gene and corresponding nucleic acids (genomic DNA, cDNA, mRNA, antisense RNA, RNA-i, etc.) is designated by all lower case letters, the protein is designated by all capital letters (i.e., OLD-35) and the gene and protein are collectively designated by a capitalized first letter only (i.e., Old-35).

[0044] The term “antibody,” as used herein, refers to a complete immunoglobulin molecule as well as fragments and derivatives thereof, single-chain antibodies, and any other functional equivalents. The antibody may be polyclonal or monoclonal, chimeric, and biologically or chemically produced.

5.1. Assay Systems

[0046] The present invention provides for assay methods and systems that may be used to identify agents that modulate the transcription of old-35, its translation into protein and/or its biological activity. The agents may be molecules that occur in nature (e.g., a protein that binds to the old-35 promoter, an enzyme that specifically inhibits OLD-35 activity), or, alternatively, synthetic molecules such as (but not limited to) small molecules generated in a combinatorial chemical library. In sections 5.1.1 and 5.1.2, below, the assays identify agents that antagonize Old-35, but the skilled artisan would be able, given the instant disclosure, to use methods that look for an opposite result (e.g., an increase in promoter activity, an increase in reactive oxygen species, increased NF- κ B binding or translocation into the nucleus, or increased cytokine levels) to identify agents that enhance rather than decrease the inflammatory activity of Old-35.

[0047] 5.1.1 Assay Systems that Use the old-35 Promoter

[0048] The present invention provides for a method for identifying an agent that inhibits inflammation, comprising exposing a test agent to a system comprising an old-35 promoter element operatively linked to a reporter gene and determining whether the exposure to the test agent increases transcription of the reporter gene, wherein a decrease in transcription of the reporter gene indicates that the test agent inhibits inflammation.

[0049] As set forth above, the test agent may be a naturally occurring molecule or substance or may be synthetic.

[0050] An old-35 promoter element is a promoter element that is found in nature operatively linked to an old-35 gene. The human old-35 promoter element has been cloned and characterized and is set forth in FIG. 7, as SEQ ID NO:1. In addition, the old-35 genes of mouse (GenBank Acc. No. AF465249) has been cloned and a person of ordinary skill in the art would be able to obtain the promoter element operatively linked to these non-human old-35 genes using standard laboratory techniques.

[0051] In preferred embodiments of the invention, the promoter element used is the human old-35 promoter (SEQ ID NO:1) or a variant thereof. The term "variant" includes fragments, deletion mutants, insertional mutants, point mutants, substitution mutants, nucleic acid molecules comprising one or more modified nucleic acid, etc. The wild-type old-35 promoter and variants thereof are collectively referred to as "old-35 promoters." Preferably variants are at least 85 percent, preferably at least 90 percent homologous to a nucleic acid molecule having a sequence set forth in SEQ ID NO:1 (FIG. 7) and/or hybridize to a nucleic acid molecule having a sequence set forth in SEQ ID NO:1 (FIG. 7), or its complementary strand, under stringent conditions for detecting hybridization of nucleic acid molecules as set forth in "Current Protocols in Molecular Biology", Volume I, Ausubel et al., eds. John Wiley: New York N.Y., pp. 2.10.1-2.10.16, first published in 1989 but with annual updating, wherein maximum hybridization specificity for DNA samples immobilized on nitrocellulose filters may be achieved through the use of repeated washings in a solution comprising 0.1-2×SSC (15-30 mM NaCl, 1.5-3 mM sodium citrate, pH 7.0) and 0.1% SDS (sodium dodecylsulfate) at temperatures of 65-68° C. or greater. For DNA samples immobilized on nylon filters, a stringent hybridization washing solution may be comprised of 40 mM NaPO₄, pH 7.2, 1-2% SDS and 1 mM EDTA. Again, a washing temperature of at least 65-68° C. is recommended, but the optimal temperature will depend on the length of the nucleic acid probe, its GC content, the concentration of monovalent cations and the percentage of formamide, if any, that was contained in the hybridization solution.

[0052] Deletion mutants of the old-35 promoter preferably hybridize to a nucleic acid molecule having a sequence as set forth in SEQ ID NO:1 under stringent conditions.

[0053] In one non-limiting embodiment of the invention, a human old-35 promoter variant is contained in p2000, as depicted in FIG. 8, and the sequence of which is set forth as bold face text in FIG. 7. Additional non-limiting examples of old-35 promoter variants include p1000, p400, p2000/-400, p400/-60 and p400-mlSRE, also as depicted in FIG. 8 (with reference to FIG. 7 and SEQ ID NO:1). The nucleic acid sequence of p400 is depicted in FIG. 9 and SEQ ID NO:2.

[0054] The present invention further provides for isolated nucleic acid molecules comprising subregions of an old-35 promoter, including but not limited to an old-35 Interferon-Stimulated Response Element ("ISRE") having a sequence as set forth in SEQ ID NO:4 and depicted in FIG. 10.

[0055] Data demonstrating that IFN-β was more effective in upregulating p400 than the p2000 construct indicate that one or more repressor element(s) is present in the p2000 construct. It may be desirable to omit this one or more repressor element from constructs intended to optimize promoter activity. One non-limiting example of an old-35 promoter variant lacking the repressor is the p400 variant.

[0056] The present invention provides for an old-35 promoter operatively linked to a gene of interest which, when introduced into a suitable host cell, results in the transcription of the gene of interest and preferably in the expression of a protein encoded by the gene of interest. The gene of interest may be an old-35 gene or may be another gene (a "heterologous") gene. Examples of non-old-35 genes of interest include but are not limited to reporter genes, such as the genes encoding green fluorescent protein (or another naturally occurring fluorescent protein or engineered variant thereof), β-glucuronidase, β-galactosidase, luciferase, and dihydrofolate reductase.

[0057] The transcriptional activity of the old-35 promoter/reporter gene construct may be evaluated in vitro (e.g., nuclear run-off assays) or in vivo. The construct may be introduced into a cell using standard techniques, including transformation, transduction, transfection, electroporation, etc. The construct, for propagation purposes or for introduction into a cell, may be incorporated into a suitable vector molecule, such as a plasmid, a phage, a phagemid or a virus. Where the vector is an expression vector, suitable expression vectors include virus-based vectors and non-virus based DNA or RNA delivery systems. Examples of appropriate virus-based gene transfer vectors include, but are not limited to, those derived from retroviruses, for example Moloney murine leukemia-virus based vectors such as LX, LNSX, LNCX or LXSN (Miller and Rosman, 1989, Biotechniques 7:980-989); lentiviruses, for example human immunodeficiency virus ("HIV"), feline leukemia virus ("FIV") or equine infectious anemia virus ("EIAV")-based vectors (Case et al., 1999, Proc. Natl. Acad. Sci. U.S.A. 96: 22988-2993; Curran et al., 2000, Molecular Ther. 1:31-38; Olsen, 1998, Gene Ther. 5:1481-1487; U.S. Pat. Nos. 6,255,071 and 6,025,192); adenoviruses (Zhang, 1999, Cancer Gene Ther. 6(2):113-138; Connnelly, 1999, Curr. Opin. Mol. Ther. 1(5):565-572; Stratford-Perricaudet, 1990, Human Gene Ther. 1:241-256; Rosenfeld, 1991, Science 252:431-434; Wang et al., 1991, Adv. Exp. Med. Biol. 309:61-66; Jaffe et al., 1992, Nat. Gen. 1:372-378; Quantin et al., 1992, Proc. Natl. Acad. Sci. U.S.A. 89:2581-2584; Rosenfeld et al., 1992, Cell 68:143-155; Mastrangeli et al., 1993, J. Clin. Invest. 91:225-234; Ragot et al., 1993, Nature 361:647-650; Hayashi et al., 1994, J. Biol. Chem. 269:23872-23875; Bett et al., 1994, Proc. Natl. Acad. Sci. U.S.A. 91:8802-8806), for example Ad5/CMV-based E1-deleted vectors (Li et al., 1993, Human Gene Ther. 4:403-409); adeno-associated viruses, for example pSub201-based AAV2-derived vectors (Walsh et al., 1992, Proc. Natl. Acad. Sci. U.S.A. 89:7257-7261); herpes simplex viruses, for example vectors based on HSV-1 (Geller and Freese, 1990, Proc. Natl. Acad. Sci. U.S.A. 87:1149-1153); baculoviruses, for example AcM-

NPV-based vectors (Boyce and Bucher, 1996, Proc. Natl. Acad. Sci. U.S.A. 93:2348-2352); SV40, for example SVluc (Strayer and Milano, 1996, Gene Ther. 3:581-587); Epstein-Barr viruses, for example EBV-based replicon vectors (Hambor et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:4010-4014); alphaviruses, for example Semliki Forest virus- or Sindbis virus-based vectors (Polo et al., 1999, Proc. Natl. Acad. Sci. U.S.A. 96:4598-4603); vaccinia viruses, for example modified vaccinia virus (MVA)-based vectors (Sutter and Moss, 1992, Proc. Natl. Acad. Sci. U.S.A. 89:10847-10851) or any other class of viruses that can efficiently transduce human tumor cells and that can accommodate the nucleic acid sequences required for therapeutic efficacy.

[0058] A decrease in transcription of the reporter gene may be detected by measuring reporter gene mRNA, the protein product of the reporter gene, or a property of the reporter gene product (e.g., enzyme activity, fluorescence, etc.).

[0059] The decrease in transcription is preferably measured relative to a control in which the old-35 promoter/reporter gene construct is not exposed to test agent.

[0060] Preferably, the decrease in transcription is at least about 10, 30, 50, 70 or 90 percent.

[0061] 5.1.2 Assay Systems that Use the Old-35 Gene

[0062] In a first set of non-limiting embodiments, the present invention provides for a method for identifying an agent that inhibits inflammation, comprising administering a test agent (that is a putative anti-inflammatory agent) to a cell comprising an old-35 gene operatively linked to a promoter element, wherein the old-35 gene is transcribed and expressed as OLD-35 protein, and determining whether the exposure to the test agent decreases the amount of reactive oxygen species in the cell. In preferred, non-limiting related embodiments of the invention, the cell is a test cell into which an old-35 gene operatively linked to a promoter element has been introduced, and the amount of reactive oxygen species in the test cell exposed to the test agent is decreased relative to the amount of reactive oxygen species in a first control cell into which the old-35 gene operatively linked to the promoter has been introduced, but which is not exposed to the test agent. Further, the amount of reactive oxygen species in the first control cell is greater than that in a second control cell which does not contain the old-35 gene operatively linked to the promoter element and which is not senescent or terminally differentiated.

[0063] Reactive oxygen species that may be measured include, but are not limited to, superoxide anions, hydroxyl radicals and hydrogen peroxide. Reactive oxygen species may be detected by methods known in the art, for example, but not by way of limitation, by staining with hydroethidine and dichlorofluorescein diacetate, followed by flow cytometry, as set forth in example section 6, below.

[0064] In a second set of non-limiting embodiments, the present invention provides for a method for identifying an agent that inhibits inflammation, comprising administering a test agent to a cell comprising an old-35 gene operatively linked to a promoter element, wherein the old-35 gene is transcribed and expressed as OLD-35 protein, and determining whether the exposure to the test agent decreases the amount of binding between NF- κ B and its target sequence in the cell. In preferred non-limiting related embodiments,

the cell is a test cell into which an old-35 gene operatively linked to a promoter element has been introduced, and the amount of binding between NF- κ B and its target sequence in the test cell exposed to the test agent is decreased relative to the amount of binding between NF- κ B and its target sequence in a first control cell into which the old-35 gene operatively linked to the promoter has been introduced, but which is not exposed to the test agent. Further, the amount of binding between NF- κ B and its target sequence in the first control cell is greater than that in a second control cell which does not contain the old-35 gene operatively linked to the promoter element and which is not senescent or terminally differentiated.

[0065] Binding of NF- κ B to its target sequence may be demonstrated by any method known in the art. For example, a cell, test cell or control cell may contain a transgene comprising a reporter gene, such as luciferase, operably linked to a promoter containing one or more NF- κ B binding site, and the amount of reporter gene produced in the presence and absence of the test agent may be monitored. Alternatively, electrophoretic mobility shift assays may be performed using nuclear extracts. See, for example, section 6 below.

[0066] In a third set of non-limiting embodiments, the present invention provides for a method for identifying an agent that inhibits inflammation, comprising administering a test agent to a cell comprising an old-35 gene operatively linked to a promoter element, wherein the old-35 gene is transcribed and expressed as OLD-35 protein, and determining whether the exposure to the test agent decreases the amount of translocation of a NF- κ B protein from the cytoplasm into the nucleus of the cell. In preferred non-limiting related embodiments, the cell is a test cell into which an old-35 gene operatively linked to a promoter element has been introduced, and the amount of translocation of a NF- κ B protein from the cytoplasm into the nucleus in the test cell exposed to the test agent is decreased relative to the amount of translocation of a NF- κ B protein from the cytoplasm into the nucleus in a first control cell into which the old-35 gene operatively linked to the promoter has been introduced, but which is not exposed to the test agent. Further, the amount of translocation of a NF- κ B protein from the cytoplasm into the nucleus in the first control cell is greater than that in a second control cell which does not contain the old-35 gene operatively linked to the promoter element and which is not senescent or terminally differentiated.

[0067] The translocation of a NF- κ B protein from the cytoplasm into the nucleus may be detected and measured using standard laboratory techniques, for example, by cell fractionation into nuclear and cytoplasmic fractions followed by Western blot analysis using NF- κ B specific antibodies (see example section 6, below).

[0068] In a fourth set of non-limiting embodiments, the present invention provides for a method for identifying an agent that inhibits inflammation, comprising administering a test agent to a cell comprising an old-35 gene operatively linked to a promoter element, wherein the old-35 gene is transcribed and expressed as OLD-35 protein, and determining whether the exposure to the test agent decreases the amount of a cytokine selected from the group consisting of interleukin-6, interleukin-8, TNFR1, RANTES and MMP-3 in the cell. In preferred related, non-limiting embodiments,

the cell is a test cell into which an old-35 gene operatively linked to a promoter element has been introduced, and the amount of a cytokine selected from the group consisting of interleukin-6, interleukin-8, TNFR1, RANTES and MMP-3 in the test cell exposed to the test agent is decreased relative to the amount of a cytokine selected from the group consisting of interleukin-6, interleukin-8, TNFR1, RANTES and MMP-3 in a first control cell into which the old-35 gene operatively linked to the promoter has been introduced, but which is not exposed to the test agent. Further, the amount of a cytokine selected from the group consisting of interleukin-6, interleukin-8, TNFR1, RANTES and MMP-3 in the first control cell is greater than that in a second control cell which does not contain the old-35 gene operatively linked to the promoter element and which is not senescent or terminally differentiated. Cytokines may be measured using methods known in the art, including but not limited to ELISA analysis, Western blot, or antibody array using anti-cytokine antibodies (see example section 6, below) or Northern analysis of cellular RNA using cytokine specific nucleic acid probes.

[0069] In the foregoing four sets of embodiments, where a promoter/old-35 construct is not introduced, the cell may naturally express the old-35 gene, and may be identified as expressing the old-35 gene at a level that is at least equal to the level expressed in neonatal human fibroblasts. Examples of such cells include senescent cells, including cells of progeria patients, and terminally differentiated cells, such as melanoma cells exposed to interferon beta and mezerein (see United States Patent Application Publication No. 20030099660). The cell or test cell may be any cell that permits expression of OLD-35. Where a promoter/old-35 construct is introduced into the test cell, the test cell absent the construct preferably (but not necessarily) produces undetectable or low levels of OLD-35, to minimize background.

[0070] Any promoter that is active or inducible in the cell, test cell and/or first control cell may be used. Non-limiting examples of such promoters include the cytomegalovirus immediate early promoter, the Rous sarcoma virus long terminal repeat promoter, the human elongation factor lea promoter, the human ubiquitin c promoter, etc. Non-limiting examples of inducible promoters include the murine mammary tumor virus promoter (inducible with dexamethasone); commercially available tetracycline-responsive or ecdysone-inducible promoters, etc.

[0071] Old-35 genes which may be used according to the invention include the human old-35 gene, having a nucleic acid sequence as deposited in GenBank Accession No. AY027528 SEQ ID NO:5, or a nucleic acid that hybridizes thereto under stringent conditions (see section 5.1.1, above). In addition, a nucleic acid comprising an old-35 gene may encode human OLD-35 protein having a sequence as set forth in FIG. 11, GenBank Accession No. AY027528 and SEQ ID NO: 6. The present invention further comprises OLD-35 fusion proteins and nucleic acids encoding the same.

[0072] In addition, non-human forms of old-35 may also be used according to the invention. For example, where murine assay systems are used, murine old-35 homolog, GenBank Acc. No. AF465249, may optionally be used.

[0073] The promoter/old-35 construct may be introduced into a cell using standard techniques, including transforma-

tion, transfection, transduction, electroporation, etc. For propagation or for introduction into a host cell, the construct may be comprised in a vector molecule, such as a plasmid, phage, phagemid, or virus (see section 5.1.1, above). In a preferred embodiment, the construct may be comprised in a recombinant adenovirus vector wherein transcription of the old-35 cDNA is driven by the cytomegalovirus immediate early (CMV) promoter.

[0074] 5.2 Model Systems of Inflammation

[0075] The present invention provides for cells and animals engineered to overexpress (in the case of animals, in at least some cells and tissues) an old-35 gene, and thereby serve as model systems for the study of inflammation and for the evaluation of agents that modify inflammation (for example, for the discovery of novel anti-inflammatory compounds).

[0076] Suitable transgenic animals include, but are not limited to, mice, rats, goats, sheep, pigs, cows, etc.

[0077] Old-35 may be over expressed in some or all cells of said animals. Overexpression may be limited to certain tissues to provide model systems of particular inflammatory conditions. A number of non-limiting examples of such systems follow.

[0078] In one set of non-limiting embodiments, the present invention provides for a model system of arthritis, comprising a non-human animal carrying a transgene comprising an old-35 gene operatively linked to a promoter element that is selectively active in cells comprised in a joint of the animal. Examples of such promoters include but are not limited to the chondromodulin-1 (ChM-I) gene promoter (Aoyama et al., J Biol Chem. 2004, 279:28789-28797), chicken collagen X regulatory sequences (Campbell et al., Am J Pathol. 2004, 164:487-499), connective tissue growth factor (CTGF/Hcs24) gene promoter (Kubota et al., Bone. 2003; 33:694-702), the 4-kb murine Col10a1 promoter of the alpha1 (X) collagen gene (Zheng et al., J. Cell Biol. 2003; 162:833-842), cartilage oligomeric matrix protein gene (COMP) promoter (Issack et al., J Orthop Res. 2004, 22:751-758).

[0079] In another set of non-limiting embodiments, the present invention provides for a model system for atherosclerosis, comprising a non-human animal carrying a transgene comprising an old-35 gene operatively linked to a promoter element that is selectively active in cells of the vascular system. Examples of such promoters include but are not limited to the Angiopoietin-2 (Ang-2) gene promoter (Hegen et al., Arterioscler Thromb Vasc Biol. 2004 Jul. 29 [Epub ahead of print]), endothelial nitric-oxide synthase (eNOS) gene promoter (Chan et al., J Biol Chem. 2004, 279:35087-35100), Cysteine-rich protein (CRP)2 gene promoter (Chang et al., Am J Physiol Heart Circ Physiol. 2003 285:H1675-1683), intercellular adhesion molecule 2 (ICAM-2), platelet endothelial cell adhesion molecule 1 (PECAM-1) and endoglin, gene promoters (Cowan et al., Xenotransplantation. 2003; 10:223-231).

[0080] In yet another set of non-limiting embodiments, the present invention provides for a model system for Alzheimer's disease, comprising a non-human animal carrying a transgene comprising an old-35 gene operatively linked to a promoter element that is selectively active in cells of the central nervous system. Examples of such promoters include

the neuron-specific enolase gene promoter (Tanaka et al., 2001, Anticancer Res. 21:291-294) and the excitatory amino acid transporter-2 promoter, as set forth in GenBank Acc. No. AF510107.

[0081] To study or monitor inflammation in said cells or animals, the present invention provides for a method for evaluating inflammation in a transgenic non-human animal carrying a transgene comprising an old-35 gene operatively linked to a promoter element, comprising determining, in a cell, tissue, or fluid of the animal, whether the amount of reactive oxygen species is increased, whether the amount of binding of a NF- κ B protein to its target sequence is increased, whether the amount of a NF- κ B protein translocated into the nucleus is increased, or whether the amount of a cytokine selected from the group consisting of interleukin-6, interleukin-8, TNFR1, RANTES and MMP-3 is increased. Such determinations may be made using methods known in the art (see section 5.1.2 above and example section 6 below).

[0082] The present invention also provides for administering a test agent to a transgenic animal carrying a transgene comprising an old-35 gene, and then determining whether inflammation is increased or decreased in said animal. Such transgenic animals may be used to discover new anti-inflammatory agents or to demonstrate anti-inflammatory activity of a putative or known anti-inflammatory agent.

[0083] Such transgenic animals may provide the advantage of creating a model system of inflammation as it occurs with aging.

[0084] 5.3 Diagnostic Methods and Kits

[0085] The present invention provides for a method of detecting inflammation in a subject, comprising determining whether there is an increase in the expression of an old-35 gene in a cell of the subject relative to a control cell. Such an increase may be determined by showing an increase in old-35 mRNA (for example by Northern blot or dot-blot analysis) using a suitable old-35 nucleic acid probe, or by showing an increase in OLD-35 protein using, for example, an antibody directed toward OLD-35. The method may be practiced in vivo or preferably in vitro.

[0086] Inflammation may be detected so as to demonstrate acute or chronic inflammation. Detection of increased expression of Old-35 (nucleic acid and/or protein) may support a diagnosis of a chronic inflammatory condition such as, but not limited to, arthritis, atherosclerosis, periodontal disease, Alzheimer's disease or chronic obstructive pulmonary disease.

[0087] In related embodiments, the present invention provides for a kit for detecting inflammation in a subject, comprising a probe that binds to an old-35 gene product selected from the group consisting of old-35 mRNA (in which case the probe is a nucleic acid) and OLD-35 protein (in which case the probe is an antibody) and an antibody that binds to a cytokine selected from the group consisting of interleukin-6, interleukin-8, TNFR1, RANTES and MMP-3. Alternatively, the kit may comprise a nucleic acid probe that may be used to detect cytokine mRNA.

[0088] 5.4 Methods of Treatment and Anti-Inflammatory Compositions

[0089] The present invention provides for a method of inhibiting inflammation in a subject in need of such treatment, comprising administering, to the subject, an effective amount of an agent that antagonizes the expression and/or activity of OLD-35, including, but not limited to, an effective amount of an antibody that binds to OLD-35 protein, an old-35 RNA-i, or an antisense old-35 nucleic acid.

[0090] As one non-limiting example, one of the foregoing Old-35 antagonist agents may be administered into the synovial fluid of an inflamed joint.

[0091] In non-limiting embodiments, the amount of antagonist may be administered to achieve a concentration of, for an OLD-35 antibody (10 μ g-1 mg/ml), for an old-35 RNA-i or antisense RNA (25-100 nM).

[0092] The present invention further provides for a method of inhibiting inflammation in a subject in need of such treatment, comprising introducing, into cells of the subject, a nucleic acid comprising an old-35 promoter element operatively linked to a gene that inhibits inflammation. Non-limiting examples of such genes include antisense NFkB p65 subunit, dominant negative versions of IkB or NFkB p65 subunit etc.

[0093] The present invention still further provides for an anti-inflammatory composition, comprising an agent that antagonizes old-35 activity selected from the group consisting of an old-35 RNA-i, an old-35 antisense RNA, and an antibody directed toward OLD-35, and a second anti-inflammatory agent. Non-limiting examples of second anti-inflammatory agents include a steroid compound or a non-steroidal anti-inflammatory agent such as aspirin, ibuprofen, naprosyn, celecoxib, valdecoxib, diclofenac, and anti-inflammatory antibodies (or their fragments) such as etanercept, infliximab or anakinra.

[0094] 5.5 Active Subregions of OLD-35

[0095] 5.5.1 Domains of OLD-35

[0096] The present invention provides for a group of proteins collectively referred to as "OLD-35 variants" comprising active subregions of the OLD-35 protein. "Active" refers to anti-proliferative activity, inflammatory activity, PNPase activity, RNA degradation activity, cell-cycle arresting/slowing activity, and/or senescence inducing activity. Old-35 belongs to an RNA processing enzyme family comprising the polynucleotide phosphorylases (PNPases). PNPases typically contain RNase PH (RPH) domains and RNA binding domains. Native full length OLD-35 protein contains 783 amino acid residues (SEQ ID NO:6, FIG. 11B) comprising the following domains: (i) a mitochondrial localization signal at amino acids 1-45 (SEQ ID NO:20, FIG. 12); (ii) two RPH domains, involved in RNA degradation, one at amino acids 52-183 (SEQ ID NO:15, FIG. 12), the other at amino acids 366-501 (SEQ ID NO:16, FIG. 12); (iii) an α -helix PNPase domain at amino acids 289-363 (SEQ ID NO:21, FIG. 12) involved in RNA binding; (iv) a RNA binding KR domain at amino acids 605-667 (SEQ ID NO:22, FIG. 12); and (v) a RNA binding S1 domain at amino acids 676-750 (SEQ ID NO:23, FIG. 12).

[0097] In one embodiment of the invention an OLD-35 variant is a protein that is not the amino acid sequence set forth as SEQ ID NO:6, which encodes the full length OLD-35 protein consisting of 783 amino acids, but that

comprises a RPH domain as set forth in SEQ ID NOS: 15-19 (FIG. 12), or that is at least about 85, at least about 90, or at least about 95 percent homologous thereto (where “homology” as that term is used herein, may be determined using standard homology search software such as BLAST or FASTA). The invention also provides for a nucleic acid encoding an OLD-35 variant protein as set forth in SEQ ID NOS:15-19, comprising coding regions in the sequence set forth in SEQ ID NOS:24-28, or another sequence which, when translated, produces a protein having essentially the same amino acid sequence as SEQ ID NOS: 15-19. Preferably nucleic acids encoding Old-35 variants are at least 85 percent, preferably at least 90 to 95 and most preferably 100 percent homologous to the nucleic acid molecules having a sequence set forth in SEQ ID NOS:24-28 and/or hybridize to a nucleic acid molecule having a sequence set forth in SEQ ID NOS:24-28, or its complementary strand, under stringent conditions for detecting hybridization of nucleic acid molecules as set forth in “*Current Protocols in Molecular Biology*”, Volume I, Ausubel et al., eds. John Wiley: New York N.Y., pp. 2.10.1-2.10.16, first published in 1989 but with annual updating, wherein maximum hybridization specificity for DNA samples immobilized on nitrocellulose filters may be achieved through the use of repeated washings in a solution comprising 0.1-2×SSC (15-30 mM NaCl, 1.5-3 mM sodium citrate, pH 7.0) and 0.1% SDS (sodium dodecylsulfate) at temperatures of 65-68° C. or greater. For DNA samples immobilized on nylon filters, a stringent hybridization washing solution may be comprised of 40 mM NaPO₄, pH 7.2, 1-2% SDS and 1 mM EDTA. Again, a washing temperature of at least 65-68° C. is recommended, but the optimal temperature will depend on the length of the nucleic acid probe, its GC content, the concentration of monovalent cations and the percentage of formamide, if any, that was contained in the hybridization solution.

[0098] As set forth in SEQ ID NOS: 17-19, an Old-35 variant may comprise of either one or both RPH domains and any additional domain such as the mitochondrial localization signal (SEQ ID NO:20), the PNPase domain (SEQ ID NO:21), the KH domain (SEQ ID NO:23) or the S1 domain (SEQ ID NO:24) or that is at least about 85, at least about 90, or at least about 95 percent homologous thereto. In an additional non-limiting embodiment, an Old-35 variant need not have a contiguous arrangement of various its domains in the same linear order as present in the native OLD-35 protein.

[0099] In particular, non-limiting embodiments, the present invention provides for an OLD-35 variant protein comprising one, but not two, RPH domain and having an activity selected from the group consisting of anti-proliferative activity, PNPase activity, RNA degradation activity, cell-cycle arrest/slowing activity, senescence-inducing activity and a combination thereof. In an alternative non-limiting embodiment, an OLD-35 variant protein may comprise one, but not two, RPH domain which is immunogenic in a mammal. The invention also provides for an OLD-35 variant possessing anti-proliferative activity, PNPase activity, RNA degradation activity, cell-cycle slowing activity, senescence-inducing activity, a combination thereof and which is immunogenic in mammals, comprising amino acid residues 52-183 (SEQUENCE ID NO:15) or amino acid residues 366-501 (SEQUENCE ID NO:16) of native OLD-35 protein, or a sequence that is at least 90 percent, preferably at least 95 percent homologous to residues 52-183 or

residues 366-501 respectively. The invention also provides for an OLD-35 variant possessing anti-proliferative activity, PNPase activity, RNA degradation activity, cell-cycle slowing activity, senescence-inducing activity and a combination thereof and which is immunogenic in mammals, comprising amino acid residues 289-363 (SEQUENCE ID NO:21) of native OLD-35 protein, or a sequence that is at least 90 percent, preferably at least 95 percent homologous to residues 289-363.

[0100] Accordingly, in specific, nonlimiting embodiments, the present invention provides for Old-35 variants as follows, where the residues identified are as set forth in SEQ ID NOS:

- (i) lacking residues 676-750, and comprising residues 52-183 and residues 299-363, operably joined, optionally by a linker sequence, and having PNPase activity;
- (ii) lacking residues 676-750, comprising residues 366-501 and residues 289-363, operably joined, optionally by a linker sequence, and having PNPase activity;
- (iii) lacking residues 1-45, and comprising residues 52-183 and having anti-proliferative activity;
- (iv) lacking residues 1-45, and comprising residues 366-501, and having antiproliferative activity;
- (v) not the complete native OLD-35 sequence, comprising residues 289-363, and immunogenic in a mammal;
- (vi) not the complete native OLD-35 sequence, comprising residues 366-501, and immunogenic in a mammal.
- (vii) not the complete native OLD-35 sequence, comprising residues 607-667, and immunogenic in a mammal;
- (viii) not the complete native OLD-35 sequence, comprising residues 676-750, and immunogenic in a mammal;

[0101] 5.5.2 Expression Systems

[0102] The Old-35 variants of the invention may be produced by any method known in the art. Such methods include but are not limited to chemical synthesis and recombinant DNA techniques.

[0103] With regard to production of Old-35 variants using recombinant DNA techniques, the present invention provides for nucleic acids encoding said variants. Such nucleic acids may either be nucleic acid fragments of the aforesaid Old-35 nucleic acids derived from SEQ ID NO:5- and include SEQ ID NOS:24-28, encoding the variants, or may be nucleic acids designed, using the genetic code, to encode such variants, wherein an alternate or optimized codon usage provides the basis for conservative codon or amino acid substitutions.

[0104] A nucleic acid encoding a Old-35 variant of the invention may be comprised in a suitable vector molecule, and may optionally be operatively linked to a suitable promoter element, for example, but not limited to, the cytomegalovirus immediate early promoter, the Rous sarcoma virus long terminal repeat promoter, the human elongation factor 1α promoter, the human ubiquitin c promoter, etc. It may be desirable, in certain embodiments of the invention, to use an inducible promoter. Non-limiting examples of inducible promoters include the murine mammary tumor virus promoter (inducible with dexamethasone); commercially available tetracycline-responsive or ecdys-

one-inducible promoters, etc. In specific non-limiting embodiments of the invention, the promoter may be selectively active in cancer cells; one example of such a promoter is the PEG-3 promoter, as described in International Patent Application No. PCT/US99/07199, Publication No. WO 99/49898 by Fisher et al., published on Oct. 7, 1999; other non-limiting examples include the prostate specific antigen gene promoter (O'Keefe et al., 2000, Prostate 45:149-157), the kallikrein 2 gene promoter (Xie et al., 2001, Human Gene Ther. 12:549-561), the human alpha-fetoprotein gene promoter (Ido et al., 1995, Cancer Res. 55:3105-3109), the c-erbB-2 gene promoter (Takakuwa et al., 1997, Jpn. J. Cancer Res. 88:166-175), the human carcinoembryonic antigen gene promoter (Lan et al., 1996, Gastroenterol. 111:1241-1251), the gastrin-releasing peptide gene promoter (Inase et al., 2000, Int. J. Cancer 85:716-719), the human telomerase reverse transcriptase gene promoter (Pan and Koenman, 1999, Med. Hypotheses 53:130-135), the hexokinase II gene promoter (Katabi et al., 1999, Human Gene Ther. 10:155-164), the L-plastin gene promoter (Peng et al., 2001, Cancer Res. 61:4405-4413), the neuron-specific enolase gene promoter (Tanaka et al., 2001, Anticancer Res. 21:291-294), the midkine gene promoter (Adachi et al., 2000, Cancer Res. 60:4305-4310), the human mucin gene MUC1 promoter (Stackhouse et al., 1999, Cancer Gene Ther. 6:209-219), and the human mucin gene MUC4 promoter (Genbank Accession No. AF241535), which is particularly active in pancreatic cancer cells (Perrais et al., J Biol Chem. 2001, 276:30923-30933).

[0105] Suitable expression vectors include virus-based vectors and non-virus based DNA or RNA delivery systems. Examples of appropriate virus-based gene transfer vectors include, but are not limited to, pCEP4 and pREP4 vectors from Invitrogen, and, more generally, those derived from retroviruses, for example Moloney murine leukemia-virus based vectors such as LX, LNSX, LNCX or LXSN (Miller and Rosman, 1989, Biotechniques 7:980-989); lentiviruses, for example human immunodeficiency virus ("HIV"), feline leukemia virus ("FIV") or equine infectious anemia virus ("EIAV")-based vectors (Case et al., 1999, Proc. Natl. Acad. Sci. U.S.A. 96: 22988-2993; Curran et al., 2000, Molecular Ther. 1:31-38; Olsen, 1998, Gene Ther. 5:1481-1487; U.S. Pat. Nos. 6,255,071 and 6,025,192); adenoviruses (Zhang, 1999, Cancer Gene Ther. 6:113-138; Connelly, 1999, Curr. Opin. Mol. Ther. 1:565-572; Stratford-Perricaudet, 1990, Human Gene Ther. 1:241-256; Rosenfeld, 1991, Science 252:431-434; Wang et al., 1991, Adv. Exp. Med. Biol. 309:61-66; Jaffe et al., 1992, Nat. Gen. 1:372-378; Quantin et al., 1992, Proc. Natl. Acad. Sci. U.S.A. 89:2581-2584; Rosenfeld et al., 1992, Cell 68:143-155; Mastrangeli et al., 1993, J. Clin. Invest. 91:225-234; Ragot et al., 1993, Nature 361:647-650; Hayaski et al., 1994, J. Biol. Chem. 269:23872-23875; Bett et al., 1994, Proc. Natl. Acad. Sci. U.S.A. 91:8802-8806), for example Ad5/CMV-based E1-deleted vectors (Li et al., 1993, Human Gene Ther. 4:403-409); adeno-associated viruses, for example pSub201-based AAV2-derived vectors (Walsh et al., 1992, Proc. Natl. Acad. Sci. U.S.A. 89:7257-7261); herpes simplex viruses, for example vectors based on HSV-1 (Geller and Freese, 1990, Proc. Natl. Acad. Sci. U.S.A. 87:1149-1153); baculoviruses, for example AcMNPV-based vectors (Boyce and Bucher, 1996, Proc. Natl. Acad. Sci. U.S.A. 93:2348-2352); SV40, for example SVLc (Strayer and Milano, 1996, Gene Ther. 3:581-587); Epstein-Barr viruses, for example EBV-based

replicon vectors (Hambor et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:4010-4014); alphaviruses, for example Semliki Forest virus- or Sindbis virus-based vectors (Polo et al., 1999, Proc. Natl. Acad. Sci. U.S.A. 96:4598-4603); vaccinia viruses, for example modified vaccinia virus (MVA)-based vectors (Sutter and Moss, 1992, Proc. Natl. Acad. Sci. U.S.A. 89:10847-10851) or any other class of viruses that can efficiently transduce human tumor cells and that can accommodate the nucleic acid sequences required for therapeutic efficacy.

[0106] Non-limiting examples of non-virus-based delivery systems which may be used according to the invention include, but are not limited to, so-called naked nucleic acids (Wolff et al., 1990, Science 247:1465-1468), nucleic acids encapsulated in liposomes (Nicolau et al., 1987, Methods in Enzymology 198:157-176), nucleic acid/lipid complexes (Legendre and Szoka, 1992, Pharmaceutical Research 9:1235-1242), and nucleic acid/protein complexes (Wu and Wu, 1991, Biother. 3:87-95).

[0107] Old-35 variant protein may also be produced by yeast or bacterial expression systems. For example, bacterial expression may be achieved using plasmids such as pGEX expression system (Amersham Biosciences, Piscataway, N.J.), pQE His-tagged expression system (Qiagen, Valencia, Calif.), pET His-tagged expression system (EMD Biosciences, Inc., La Jolla, Calif.), or IMPACT expression system (New England Biolabs, Beverly, Mass.).

[0108] In a specific, non-limiting embodiment of the invention, a nucleic acid encoding an Old-35 variant, in expressible form, may be *in vitro* translated to produce OLD-35 variant protein. *In vitro* translation may be performed using an appropriate system such as the TNT® coupled Reticulocyte Lysate Systems (Promega) or TNT® Coupled Wheat Germ Extract Systems. The expressed protein may include an affinity tag so that an Old-35 variant protein may be purified and recovered from the extracts for further use.

[0109] 5.5.3 Delivery Systems

[0110] Depending on the expression system used, nucleic acid may be introduced by any standard technique, including transfection, transduction, electroporation, bioballistics, microinjection, etc.

[0111] In preferred, non-limiting embodiments of the invention, the expression vector is an E1-deleted human adenovirus vector of serotype 5. To prepare such a vector, an expression cassette comprising a transcriptional promoter element operatively linked to a Old-35 variant coding region and a polyadenylation signal sequence may be inserted into the multiple cloning region of an adenovirus vector shuttle plasmid, for example pXCJL.1 (Berkner, 1988, Biotechniques 6:616-624). In the context of this plasmid, the expression cassette may be inserted into the DNA sequence homologous to the 5' end of the genome of the human serotype 5 adenovirus, disrupting the adenovirus E1 gene region. Transfection of this shuttle plasmid into the E1-transcomplementing 293 cell line (Graham et al., 1977, J. General Virology 36:59-74), or another suitable cell line known in the art, in combination with either an adenovirus vector helper plasmid such as pJM17 (Berkner, 1988, Biotechniques 6:616-624; McGrory et al., 1988, Virology 163:614-617) or pBHG10 (Bett et al., 1994, Proc. Natl. Acad. Sci. U.S.A. 91: 8802-8806) or a Clal-digested fragment isolated from the adenovirus 5 genome (Berkner, 1988,

Biotechniques 6:616-624), allows recombination to occur between homologous adenovirus sequences contained in the adenovirus shuttle plasmid and either the helper plasmid or the adenovirus genomic fragment. This recombination event gives rise to a recombinant adenovirus genome in which the cassette for the expression of the foreign gene has been inserted in place of a functional E1 gene. When transcomplemented by the protein products of the human adenovirus type 5 E1 gene (for example, as expressed in 293 cells), these recombinant adenovirus vector genomes can replicate and be packaged into fully-infectious adenovirus particles. The recombinant vector can then be isolated from contaminating virus particles by one or more rounds of plaque purification (Berkner, 1988, Biotechniques 6:616-624), and the vector can be further purified and concentrated by density ultracentrifugation.

[0112] In a specific, non-limiting embodiment of the invention, a nucleic acid encoding an Old-35 variant, in expressible form, may be inserted into the modified Ad expression vector pAd.CMV (Falck-Pedersen et al., 1994, Mol. Pharmacol. 45:684-689).

[0113] This vector contains, in order, the first 355 base pairs from the left end of the adenovirus genome, the cytomegalovirus immediate early promoter, DNA encoding splice donor and acceptor sites, a cloning site for the Old-35 variant gene, DNA encoding a polyadenylation signal sequence from the globin gene, and approximately three kilobase pairs of adenovirus sequence extending from within the EIB coding region. This construct may then be introduced into 293 cells (Graham et al., 1977, J. Gen. Virol. 36:59-72) together with plasmid JM17 (above), such that, as explained above, homologous recombination can generate a replication defective adenovirus containing Old-35 variant encoding nucleic acid.

[0114] 5.6 Use of OLD-35 Variants

[0115] 5.6.1 Use of OLD-35 Variants as Antiproliferative Agents

[0116] Old-35 variants are as effective as full length OLD-35 protein in inducing senescence or differentiation (FIG. 12), inhibiting colony formation (FIG. 12), causing cell cycle arrest (FIG. 13) and inhibiting the expression of c-myc protein by degrading c-myc mRNA (FIG. 15-17). In specific non-limiting embodiments an OLD-35 variant may be used to modulate cell proliferation in a subject, wherein a nucleic acid encoding the variant, in expressible form, may be introduced into a cell of the subject.

[0117] In preferred, non-limiting embodiments, the nucleic acid encoding the OLD-35 variant may be contained in a viral vector, operably linked to a promoter element that is inducible or constitutively active in the target cell. In preferred, non-limiting embodiments, the viral vector is a replication-defective adenovirus (as described in section 5.5.3 above).

[0118] In a specific, non-limiting embodiment of the invention, a viral vector containing a nucleic acid encoding a OLD-35 variant, such as a single RPH or two RPH domain containing proteins operably linked to a suitable promoter element, may be administered to a population of target cells at a multiplicity of infection (MOI) ranging from 10-100 MOI.

[0119] In another specific, non-limiting embodiment, the amount of a viral vector administered to a subject may be 1×10^9 pfu to 1×10^{12} pfu.

[0120] In specific, non-limiting embodiments, a nucleic acid encoding an OLD-35 variant comprised in a vector or otherwise, may be introduced into a cell ex vivo and then the cell may be introduced into a subject. For example, a nucleic acid encoding a OLD-35 variant may be introduced into a cell of a subject (for example; an irradiated tumor cell, glial cell or fibroblast) ex vivo and then the cell containing the nucleic acid may be optionally propagated and then (with its progeny) introduced into the subject.

[0121] 5.6.2 Use of OLD-35 Variants as an Anti-Inflammatory Vaccine

[0122] The present invention also provides for administering an OLD-35 variant in protein or peptide form in a subject in need of treatment for an acute or chronic inflammatory condition. Enhanced levels and activity of full length OLD-35 protein is associated with enhancing the levels of reactive oxygen species, inducing activation of the NF- κ B pathway and enhancing the level of proinflammatory cytokines (FIGS. 4-6). Thus, in a specific non-limiting embodiment the present invention provides for delivery of an OLD-35 variant peptide to a subject so as to elicit an immune reaction against the injected peptide. Antibodies raised in a subject against an OLD-35 variant will also be active in binding to an endogenously expressed wild type OLD-35 protein. Thus, injection of an OLD-35 variant will act as a vaccine against an inflammatory response by reducing effective amounts of inflammation causing OLD-35 protein in a subject.

[0123] As such, the OLD-35 variant of the invention may be prepared by chemical synthesis or recombinant DNA techniques, purified by methods known in the art, and then administered to a subject in need of such treatment. An OLD-35 variant may be comprised, for example, in solution, in suspension, and/or in a carrier particle such as microparticles, liposomes, or other protein-stabilizing formulations known in the art. In a non-limiting specific example, formulations of OLD-35 variant peptides may stabilized by addition of zinc and/or protamine stabilizers as in the case of certain types of insulin formulations. Alternatively, in specific non-limiting embodiments, an OLD-35 variant may be linked covalently or non-covalently, to a carrier protein which is preferably non-immunogenic.

[0124] In preferred, non-limiting embodiments, an OLD-35 variant protein/peptide is administered in an amount which achieves a local concentration in the range of 18 to 50 ng per microliter. For example, a subject may be administered a range of 50-100 mg per kilogram. For a human subject, the dose range may be between 1000-2500 mg/day.

6. EXAMPLE

OLD-35 is an Agent of Inflammation

[0125] 6.1 Materials and Methods

[0126] Cell Lines, Reagents and Virus Infection protocol. The human cervical carcinoma cell line HeLa was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, penicillin (50 units/ml) and streptomycin (50 μ g/ml) at 37° C. in 5% CO₂ and 100%

relative humidity. N-acetyl-L-cysteine and Tiron were obtained from Sigma (St. Louis, Mo.). The recombinant replication-incompetent adenovirus expressing old-35 (Ad.hPNPase) was created in two steps as described previously and plaque purified by standard procedures (1, 28). Cells were infected with a multiplicity of infection (m.o.i.) of 1 to 50 plaque forming units (pfu)/cell of Ad.vec (control replication-incompetent adenovirus) or Ad.hPNPase as described (29).

[0127] Transient Transfection and Luciferase Assay. Cells (5×10^3 /well in 12-well plates) were either uninfected or infected with either Ad.vec or Ad. hPNPase at an m.o.i. of 50 p.f.u./cell. Transient transfection was conducted 12 hr post-infection using Lipofectamine-2000 transfection reagent (Invitrogen, Carlsbad, Calif.) and 1.2 μ g of plasmid DNA per well that included 1 μ g of pGL3Basic, 3 KB-Luc or 3 kBmut-Luc plasmids (30) and 0.2 μ g of β -galactosidase-expression plasmid (pSV- β -gal; Promega, Madison, Wis.). For inhibition experiments, the cells were pre-treated with different inhibitors for 2 hr before transfection. Luciferase assays were performed 48 hr post-transfection using a Luciferase Reporter Gene Assay kit (Promega) according to the manufacturer's protocol. The β -galactosidase activity was determined using the Galacto-Light Plus kit (Tropix). Luciferase activity was normalized by β -galactosidase activity and the data from triplicate determinations were expressed as mean \pm S.D.

[0128] Generation of Anti-OLD-35 Antibody. A C-terminal His-tagged old-35 protein was produced in a baculovirus expression system (Pharmingen, San Diego, Calif.) according to the manufacturer's instructions. The protein was purified by Ni-NTA agarose column and subsequently ion exchange chromatography. The purified protein was used to immunize chickens to generate anti-OLD-35 antibody (Genetel Laboratories, Madison, Wis.).

[0129] Cell Fractionation. Cells were harvested and the cytoplasm and nucleus were fractionated by the modified Schreiber's method as described (31). Briefly, the cells were washed with PBS and lysed for 10 min in Buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet P-40) containing protease inhibitor cocktail (Roche, Mannheim, Germany). After centrifugation, the supernatant was saved as the cytoplasmic fraction and the pellet containing nuclei was lysed in Buffer B (20 mM HEPES pH 7.9, 400 mM KCl, 20% glycerol, 1 mM EDTA, 1 mM DTT) containing protease inhibitor cocktail (Roche) with one freeze-thaw cycle. After centrifugation the supernatant was collected as the nuclear fraction. Protein concentration was determined by Bio-Rad protein assay kit (Bio-Rad laboratories, Hercules, Calif.).

[0130] Electrophoretic Mobility Shift Assay (EMSA). EMSA was performed as described (31). The sequences of the consensus and mutated NF- κ B probes are 5'-AGT-TGAGGGACTTCCCAGGC-3' (SEQ ID NO:7) and 5'-AGTTGAGGCGACTTCCCAGGC-3' (SEQ ID NO:8), respectively (Santa Cruz Biotechnology, Santa Cruz, Calif.). The probes were labeled using T4 polynucleotide kinase (Promega) and [γ ³²P]ATP. Ten (10) μ g of nuclear extract was incubated in a final volume of 10 μ l containing gel shift binding buffer (Promega) for 10 min at room temperature and 20,000 cpm of labeled probe was added and incubated for another 20 min at room temperature. For competition

studies, unlabeled probes were added 15 min before the labeled probe. For supershift analysis, the reaction mixture was incubated for 1 hr with 1 μ l of antibody before the addition of the radiolabeled probe. The antibodies used were anti-p50, anti-p65, anti-p52 and anti-cRel (Santa Cruz; rabbit polyclonal). Free and bound DNA was separated on a 4% non-denaturing polyacrylamide gel in 0.5% Tris-borate-EDTA at a constant voltage of 165v. The gel was dried on filter paper and autoradiographed.

[0131] Preparation of Whole Cell Lysates and Western Blot Analysis. Whole cell lysates were prepared and Western blotting was performed as described (4). Briefly, cells were harvested in RIPA buffer containing protease inhibitor cocktail (Roche, Mannheim, Germany), 1 mM Na₃VO₄ and 50 mM NaF and centrifuged at 12,000 rpm for 10 min at 4° C. The supernatant was used as total cell lysate. Thirty μ g of total cell lysate were used for SDS-PAGE and transferred to a nitrocellulose membrane. The primary antibodies used were anti-p65, anti-p50, I κ B- α (Santa Cruz; rabbit polyclonal; 1:250), anti-EF1 α (Upstate Biotechnology; mouse monoclonal; 1:1000), and anti-OLD-35^s (chicken; 1:10,000).

[0132] RNA Extraction and RT-PCR. Total RNA was extracted from the cells using Qiagen RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Two μ g of total RNA was used for RT-PCR according to standard methods. The primers used were: IL-6 sense: 5' CCACACAGACAGCCACTCACC 3'; (SEQ ID NO:9) IL-6 antisense: 5' TGGCATTGTTGGTGGGTCA 3'; (SEQ ID NO:10) IL-8 sense: 5' GGTGCAGAGGGTTGTGGAGAA 3'; (SEQ ID NO:11) IL-8 antisense: 5' GCAGACTAGGGTTGCCAGATT 3'; (SEQ ID NO:12) GAPDH sense: 5' ATGGGAAGGTGAAGGTCGGAGTC 3'; (SEQ ID NO:13) GAPDH antisense: 5' GCTGATGATCTTGAGGCTGTTGTC 3' (SEQ ID NO:14).

[0133] Staining for Mitochondria and OLD-35. Live cells were loaded with 200 nM MitoTracker (Molecular Probes, Eugene, Oreg.) for 30 min according to the manufacturer's protocol, fixed in 3.7% formaldehyde in PBS for 15 min and permeabilized in 0.1% Triton X-100 in PBS. Immunocytochemistry was then performed by standard methods using chicken anti-old-35 antibody (1:1000) and FITC-conjugated anti-chicken secondary antibody (Genetel). The cells were visualized and the images were analyzed using a Zeiss confocal laser scanning microscope (LSM510) with a \times 100 objective.

[0134] Monitoring ROS Production. Cells were stained with 2.5 μ M hydroethidine (HE) and 5 μ M 5,6-carboxy-2',7'-dichlorofluorescein diacetate (DCFH-DA) in PBS for 30 min in the dark (32). Immediately after staining the cells were analyzed by flow cytometry (FACScan; Becton Dickinson, Mountain View, Calif.) and data were analyzed using CellQuest software, version 3.1 (Becton Dickinson). The cells were gated to exclude cell debris. For inhibition experiments, NAC was added 2 hr post-infection.

[0135] Human IL-6 and IL-8 ELISA. The levels of IL-6 and IL-8 were quantified by ELISA in culture supernatants using human IL-6 ELISA kit (Pierce Biotechnology, Rockford, Ill.) and human IL-8 ELISA kit (Pierce), respectively, according to the manufacturer's protocol.

[0136] Human Cytokine Arrays. The expression levels of 36 cytokines following Ad.vec and Ad. hPNPase^{old}-35 infec-

tion were analyzed in culture supernatants using the Tran-Signal™ human cytokine antibody array (Pannomics, Redwood city, CA) according to the manufacturer's protocol.

[0137] Statistical analysis. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Fisher's protected least significant difference analysis. A p value of <0.05 was considered as significant.

[0138] 6.2 Results

[0139] Ad.hPNPase^{old-35} Infection Generates OLD-35 Protein that Localizes to Mitochondria. The expression of OLD-35 in HeLa cells following Ad.hPNPase^{old-35} infection was analyzed two days post-infection by Western blot analysis. As shown in FIG. 1A, OLD-35 expression was detected only in Ad.hPNPase^{old-35}-infected cells indicating that Ad.hPNPase^{old-35} infection generates functional protein.

[0140] To confirm the subcellular localization of Ad.hPNPase^{old-35}-generated protein, cells were loaded with the mitochondria-specific dye MitoTracker and immunostained for OLD-35. Image analysis revealed that merging the signals generated from OLD-35 (green) and mitochondria (red) resulted in intense yellow color indicating overlapping distribution of the two signals (FIG. 1B). These findings strongly suggest that Ad.hPNPase^{old-35}-generated protein also localizes to mitochondria.

[0141] Infection with Ad.hPNPase^{old-35} Induces ROS. The levels of intracellular ROS following Ad.hPNPase^{old-35} infection were determined using two dyes, DCFH-DA and HE. Nonfluorescent DCFH-DA diffuses into cells where it is deacetylated to DCF that fluoresces on reaction with hydrogen peroxide or nitrous oxide (32). HE enters the cells and can be oxidized by superoxide or free hydroxyl radicals to yield fluorescent ethidium (32). Infection with Ad.hPNPase^{old-35} induced both DCFH-DA and HE fluorescence. Flow cytometry analysis of cellular fluorescence revealed a time-dependent increase in ROS production following Ad.hPNPase^{old-35} infection in comparison to Ad.vec-infected cells (FIGS. 1C and 1D). The generation of ROS by Ad.hPNPase^{old-35} was inhibited by treatment with a non-cytotoxic dose (20 mM) of a general antioxidant NAC which reduced the percentage of high ROS containing cells from ~9% and ~15% to ~2% and ~3% at 24 and 36 hr, respectively (FIG. 1D).

[0142] ROS Mediates Activation of the NF-κB Pathway following Ad.hPNPase^{old-35} Infection. To test whether Ad.hPNPase^{old-35} infection activates the NF-κB pathway a luciferase-based reporter gene assay was employed. HeLa cells were either uninfected or infected with Ad.vec or Ad.hPNPase^{old-35} and then transfected with either empty vector (pGL3Basic), 3kB-Luc containing 3 tandem NF-κB binding sites upstream of the luciferase gene or 3kBmut-Luc containing mutated NF-κB binding sites. As shown in FIG. 2A, cells transfected with either pGL3Basic or 3kBmut-Luc showed only basal luciferase activity under any experimental condition. In control and Ad.vec-infected cells transfection of 3kB-Luc increased basal activity over transfection of either pGL3Basic or 3kBmut-Luc, which is most likely a consequence of constitutive NF-κB DNA binding activity in HeLa cells. However, infection with Ad.hPNPase^{old-35} resulted in a 10-12-fold induction in relative luciferase activity in comparison to control or Ad.vec-infected cells indicating that Ad.hPNPase^{old-35} infection results in induc-

ible activation of the NF-κB pathway (FIG. 2A). To evaluate the role of ROS in Ad.hPNPase^{old-35}-mediated NF-κB activation, HeLa cells were infected with Ad.hPNPase^{old-35}, then transfected with 3kB-Luc and treated with various doses of either NAC or Tiron. Luciferase activity was measured 48 hr post-transfection. As shown in FIG. 2B, treatment with increasing doses of either NAC or Tiron significantly decreased Ad.hPNPase^{old-35}-mediated NF-κB luciferase activity. NAC was much more potent than Tiron and in further studies NAC was used. These findings indicate that the induction of NF-κB upon Ad.hPNPase^{old-35} infection is mediated by the generation of ROS.

[0143] The activation of NF-κB upon Ad.hPNPase^{old-35} infection was further analyzed by EMSA using radiolabeled consensus NF-κB binding site as a probe and nuclear extracts from HeLa cells. As shown in FIG. 3A, in uninfected and Ad.vec-infected cells two shifted bands were observed at all time points. Following Ad.hPNPase^{old-35} infection, the fast migrating band started to disappear with a significant increase in the intensity of the slow migrating band. By 2 and 3 days post-Ad.hPNPase^{old-35} infection only the slow migrating band was detected and the intensity of the band was markedly higher in comparison to that in control and Ad.vec-infected cells. This change in DNA-binding pattern could be observed with as little as 5 m.o.i. of Ad.hPNPase^{old-35} infection and at 50 m.o.i., the intensity of the shifted band was significantly enhanced.

[0144] To characterize the shifted bands, competition assays using excess amounts of cold wild type and mutated probes and supershift assays using antibodies against different subunits of NF-κB were employed. As shown in FIG. 3B, all the shifted bands were completely eliminated by a 100-fold excess of cold wild type probe but not by cold mutated probe indicating that these shifted bands are specific for NF-κB. The anti-p50 antibody supershifted both the slow and fast migrating bands while anti-p65 antibody supershifted only the fast migrating band in Ad.vec-infected cells. The intense slow migrating band in Ad.hPNPase^{old-35}-infected samples was supershifted by both anti-p50 and anti-p65 antibodies, but not by anti-p52 or anti-cRel antibodies. These findings demonstrate that under basal condition, both p50/p50 homodimers and p50/p65 heterodimers bind to the NF-κB probe. However, upon Ad.hPNPase^{old-35} infection, the binding pattern changed with the p50/p50 homodimer disappearing and the binding of the p50/p65 heterodimer increasing markedly. These findings also indicate that OLD-35 is a potent activator of NF-κB, since it promotes increased binding of the potent transcriptional activator p50/p65.

[0145] To confirm that the induction of NF-κB by Ad.hPNPase^{old-35} is specific and not mediated by non-specific events such as protein overload or additional adenoviral proteins, that might activate NF-κB, HeLa cells were infected with three additional replication incompetent adenovirus constructs expressing functionally different genes and NF-κB binding was analyzed by EMSA. The constructs included Ad.mda-5 that expresses mda-5, an interferon-inducible putative RNA helicase (33), Ad.mda-7 that expresses the apoptosis-inducing cytokine mda-7/IL-24 (34) and Ad.PEG-3, that express PEG-3 which is involved in tumor progression (35). As shown in FIG. 3C, infection with only Ad.hPNPase^{old-35} and not with any other adenoviral

construct resulted in a significant increase in the binding of NF- κ B confirming the specificity of this induction.

[0146] The involvement of ROS in mediating increased NF- κ B DNA binding by Ad.hPNPase^{old-35} was also evaluated by EMSA. As shown in FIG. 3D, treatment with NAC significantly reduced the intensity of the shifted band induced by Ad.hPNPase^{old-35} infection, which further strengthens the conclusion that ROS acts as the second messenger to activate NF- κ B following Ad.hPNPase^{old-35} infection.

[0147] The levels of p50 and p65 subunits of NF- κ B and its inhibitor I κ B α were analyzed in cytoplasmic and nuclear extracts following Ad.hPNPase^{old-35} infection. As shown in FIG. 4A, the levels of both p65 and p50 proteins began decreasing in the cytoplasmic extract of cells 2 days post-Ad.hPNPase^{old-31} infection, whereas the level of p65 protein started increasing in the nuclear extract of Ad.hPNPase^{old-35}-infected cells 2 days post-infection. This effect was not apparent in control or Ad.vec-infected cells (FIG. 4B), indicating that Ad.hPNPase^{old-35} infection resulted in translocation of p65 from the cytoplasm to the nucleus. The basal p50 protein level in the nucleus was quite high and this level was not modulated significantly following Ad.hPNPase^{old-35} infection. The level of I κ B α significantly decreased in the cytoplasmic extract with Ad.hPNPase^{old-35} infection, but not in control or after Ad.vec infection (FIG. 4A). Both the degradation of I κ B α and nuclear translocation of p65 by Ad.hPNPase^{old-35} infection were inhibited by treatment with NAC (FIG. 4C) indicating that hPNPase^{old-35}-induced generation of ROS plays a pivotal role in activating NF- κ B.

[0148] IL-6 and IL-8 are Induced by Ad.hPNPase^{old-35} Infection. Expressions of mRNAs of IL-6 and IL-8, two NF- κ B target genes, were analyzed by RT-PCR following Ad.hPNPase^{old-35} infection. A time-dependent increase in the expressions of both IL-6 and IL-8 mRNAs occurred from two days post-infection with Ad.hPNPase^{old-35}, but not with Ad.vec infection (FIG. 5A). The expression of the mRNA of the housekeeping gene GAPDH did not change under any experimental condition.

[0149] ELISA assays quantified secretion of IL-6 and IL-8 protein following Ad.hPNPase^{old-35} infection (FIG. 5B, 5C). There was a significant time-dependent increase in secreted IL-6 and IL-8, the latter markedly more robust, in the culture supernatant from two days post-infection with Ad.hPNPase^{old-35} as compared to that in control or Ad.vec-infected cells. Treatment with NAC markedly inhibited Ad.hPNPase^{old-35}-induced secretion of both IL-6 and IL-8 (FIG. 5B, 5C).

[0150] Analysis of Cytokine Expression Profiles following Ad.hPNPase^{old-35} Infection. Since Ad.hPNPase^{old-35} infection induced two potently pro-inflammatory cytokines, IL-6 and IL-8, the induction of other cytokines in culture supernatants two days post-infection were also tested using a human cytokine antibody array that analyzes the expression levels of 36 cytokines. Ad.hPNPase^{old-35} infection had a very specific cytokine induction profile resulting in marked upregulation of IL-8, moderate elevation of IL-6 and TNFR1 and upregulation of RANTES and MMP-3 to a lesser extent (FIG. 6).

[0151] 6.3 Discussion

[0152] The foregoing demonstrates that OLD-35 activates the NF- κ B pathway via the generation of ROS in HeLa cells.

ROS is involved in the induction of a senescent phenotype characterized by irreversible growth arrest (10). Overexpression of Old-35 induces a senescence-like growth arrest and also generates ROS. Moreover, inhibition of ROS impedes the induction of NF- κ B-responsive genes.

[0153] Contrasting results have been obtained regarding NF- κ B binding activity during aging. No difference was observed in NF- κ B binding between senescent and pre-senescent human fibroblasts as a function of in vitro replicative senescence (36). A reduced NF- κ B activation in T cells from aged humans and mice has been reported (37). On the other hand, an increase in constitutive NF- κ B DNA binding in older animals over young animals has been demonstrated in multiple studies (14-21). A gradual rise in ROS was evident in kidneys from Fischer rats from 6 to 24 months of age and this increase correlated with an age-dependent augmentation in binding of p50/p65 NF- κ B, I κ B α degradation, p65 nuclear translocation and elevated expression of cycloxygenase-2 (COX-2), an NF- κ B-responsive enzyme involved in pro-inflammatory prostanoïd synthesis (16). Vascular smooth muscle cells from 18-month old rats showed considerably higher p50/p65 NF- κ B DNA binding than that from new-born rats which correlated with increased expression of inducible nitric oxide synthase and intracellular adhesion molecule-1, two pro-inflammatory molecules, in old smooth muscle cells upon inflammatory stimulation (17). A similar age-dependent elevation in NF- κ B DNA binding has been reported in mouse and rat liver and heart and in rat brain (14, 19). In these tissues an age-dependent rise in the levels of NF- κ B subunits, such as p50, p52 and p65, could be observed. However, no change in the level of I κ B α was detected. From these studies it might be inferred that tissue-specific regulatory mechanisms may be involved in NF- κ B activation during senescence. Although NF- κ B activation requires degradation of I κ B α , I κ B α itself is an NF- κ B-responsive gene (22, 38). During acute activation of NF- κ B by TNF α or related stimuli, there is an initial decrease in the cytoplasmic I κ B α level followed by gradual restoration because of NF- κ B-mediated transcription (38). Infection with Ad.hPNPase^{old-35} resulted in a persistent decrease in the cytoplasmic I κ B α level, indicating that even though NF- κ B is activated by OLD-35 there might be an additional regulatory mechanism of I κ B α transcription during senescence as compared to acute stimuli. A recent study has shown the lack of involvement of ROS in NF- κ B activation by acute stimuli such as TNF α . (39). It is possible that in a state of chronic oxidative stress, such as senescence, ROS plays a role in activating NF- κ B. Another intriguing observation is the selective induction of NF- κ B-responsive genes by OLD-35 (FIG. 6) indicating that in addition to the primary transactivation of NF- κ B by OLD-35 there might be a secondary level of regulation that targets the transactivation of specific NF- κ B-target genes.

[0154] What is the significance of induction of NF- κ B-responsive genes by OLD-35 in the context of senescence? Ad.hPNPase^{old-35} infection results in the upregulation of pro-inflammatory cytokines via activation of NF- κ B. By turning on pro-inflammatory cytokines, NF- κ B functions as a central transcription factor for the development of chronic inflammatory diseases (40). Gene expression analysis by microarray in human hepatic stellate cells confirms that replicative senescence in these cells is associated with a pronounced inflammatory phenotype characterized by upregulation of pro-inflammatory cytokines, including IL-6

and IL-8 (41). An aging-induced pro-inflammatory shift in cytokine expression profile has been observed in rat coronary arteries (42). Several studies have documented increased blood level of pro-inflammatory cytokines such as IL-1, IL-6, TNF α and IL-8 in aged individuals as compared to young individuals (43). The onset and course of a spectrum of age-associated diseases, such as cardiovascular disease, osteoporosis, arthritis, type 2 diabetes, Alzheimer's disease, certain cancers, periodontal disease, frailty and functional decline, might be associated with the production of pro-inflammatory cytokines (44, 45). Multiple studies have established an association between elevated levels of IL-6 and diseases of old age. IL-6 induces the production of C-reactive protein (CRP), an important risk factor for myocardial infarction (45). High concentrations of CRP predict the risk of future cardiovascular disease in apparently healthy men (45). IL-8 plays a crucial role in initiating atherosclerosis by recruiting monocytes/macrophages to the vessel wall, which promotes atherosclerotic lesions and plaque vulnerability (46). Elevated levels of IL-6 and CRP predict the development of type 2 diabetes in healthy women (47). In another study, elevated serum IL-6 levels predicted future disability in older adults especially by inducing muscle atrophy (48). IL-6 and CRP also play a pathogenic role in several diseases such as osteoporosis, arthritis and congestive heart failure all of which have increasing incidence with age (48). Moreover, increased serum levels of IL-6 and IL-8 have been detected in patients with chronic obstructive pulmonary diseases and chemokines such as IL-8 and RANTES play important roles in the pathogenesis of these diseases (49, 50). Various inflammatory mediators, such as IL-1, TNF- α , IL-6, IL-8, RANTES, MMP-3 are responsible for chronic inflammatory rheumatoid diseases, such as osteoarthritis and rheumatoid arthritis both of which occur during aging (51). The observation that the senescence-associated molecule OLD-35 induces pro-inflammatory cytokines which are intimately involved in the development of aging-associated diseases are consistent with involvement of OLD-35 in these pathological processes.

7. EXAMPLE

Defining the Domains of OLD-35

[0155] 7.1 Materials and Methods

[0156] Cell lines and culture conditions: The human metastatic melanoma cell line HO-1 and the human embryonic kidney cell line HEK-293 were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 50 units/ml penicillin G and 50 μ g/ml streptomycin at 37° C. in 5% CO₂ and 100% relative humidity. HO-1 cells were cultured with IFN- β at a dose of 1000 OLD-35 units/ml for 2 days to detect hPNPase protein.

[0157] Plasmid Construction: All plasmids were constructed in a backbone of pcDNA3.1+(Hygro) (Invitrogen, Carlsbad, Calif.) into the NheI and BamHI sites and all the constructs contained a COOH-terminal Hemagglutinin (HA)-tag. hPNPase^{old-35}, expressing the full-length hPNPase^{OLD-35} protein, was amplified by PCR using the cloned hPNPase^{old-35} cDNA as a template (75) and primers sense (1) 5'-GCTAGCATGGCGGCCTGCAGGTAC-3' (SEQ ID NO:33) and anti-sense (1) 5'-GGATCCTAAGCG-TAATCTGGAACATCGTATGGGTACT-GAGAATTAGATG ATGA-3' (SEQ ID NO:34). p Δ RPH1

was created by PCR using primers sense (2) 5'-GCTAG-CATGCCTTGGAAATGGACCTGTTGGG-3' (SEQ ID NO:35) and antisense (1). p Δ RPH2 was cloned in two steps. First, a 3' PCR fragment was amplified by PCR using primers sense (3) 5'-GTTAACATGGATTCAAGGGTTCC-AATT-3' (SEQ ID NO:36) and antisense (1) and ligated to pGEMT-easy vector (Promega, Madison, Wis.) by TA-cloning. This fragment was digested with HpaI and BamHI and ligated into HpaI and BamHI-digested pHNPase^{old-35}. p Δ RPH1+2 was generated by PCR using primer sense (4) 5'-GCTAGCATGGATTCAAGGGTTCCAATT-3' (SEQ ID NO:37) and antisense (1). p Δ C-term was generated by PCR using primers sense (1) and antisense (2) 5'-GGATCCT-CAAGCGTAATCTGGAACATCGTATGGG-TACTGCAACAGCAGAT GAAATTGG-3' (SEQ ID NO:38). The authenticity of all the constructs was confirmed by sequencing. The PCR fragments were first cloned into the vector pGEMT-easy by TA-cloning and then transferred to pcDNA3.1+(Hygro). The c-myc expression plasmid [p290-myc(2,3)] was provided by Dr. Riccardo Dalla-Favera (Columbia University Medical Center, N.Y.).

[0158] Virus Construction and Infection Protocol: The construction of hPNPase^{old-35} expressing replication-defective Ad.hPNPase^{old-35} was performed by cloning the transgene into a shuttle vector (p0TgCMV) and then performing homologous recombination of the shuttle vector with E1 and E3 region deleted parental adenoviral vector in *E. coli* as described previously (69, 75). A similar method was employed to generate Ad. Δ RPH1, Ad. Δ RPH2, Ad. Δ RPH1+2 and Ad. Δ C-term. The transgene was digested from the pGEMT-easy vector with NotI and ligated into the NotI site of p0TgCMV. The direction of the cloning was confirmed by restriction enzyme digestion and sequencing. The empty adenoviral vector (Ad.vec) was used as a control. The Ad was propagated in HEK293 cells (69), purified by BD AdenoX™ Virus Purification Kit (BD Biosciences, Palo Alto, Calif.) and viral titer was determined by measuring O.D. at 260 nm and using BD AdenoX™ rapid titer kit (BD Biosciences). Ad infection was performed 24 h after cell plating in one-fifth the volume of the original culture medium in a serum-free condition for 2 h with rocking the plates several times (69).

[0159] Colony formation assays: HO-1 cells were plated at a density of 3 \times 10³ cells per 6-cm dish and 24 h later were infected with different Ad at an m.o.i. of 10, 20 and 50 pfu/cell. For colony formation assays with c-myc overexpression, HO-1 cells were plated at a density of 3 \times 10³ cells per 6-cm dish and 24 h later were transfected with 5 μ g of either empty vector or p290-myc(2,3) using Superfect® (Qiagen, Hilden, Germany) transfection reagent according to the manufacturer's protocol. After 36 h, the cells were infected with different Ad at an m.o.i. of 50 pfu/cell. Six h after infection, the cells were trypsinized, counted and 1 cells were plated in 6-cm dishes. Colonies>50 cells were counted after 2 weeks.

[0160] Western Blot Analysis. Western blotting was performed as previously described (84). Briefly, cells were harvested in RIPA buffer (1xPBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Roche, Mannheim, Germany), 1 mM Na₃VO₄ and 50 mM NaF and centrifuged at 12,000 rpm for 10 min at 4° C. The supernatant was used as total cell lysate. Thirty μ g of total cell lysate were used for SDS-PAGE and transferred to

a nitrocellulose membrane. The primary antibodies included: from Santa Cruz Biotechnology, Santa Cruz, Calif.: Myc (1:200; mouse monoclonal), Max (1:200; rabbit polyclonal), Mad1 (1:200; rabbit polyclonal), p21 (1:200; rabbit polyclonal), p27 (1:200; rabbit polyclonal), CDK2 (1:250; rabbit polyclonal); from BD Biosciences: p16 (1:500; mouse monoclonal), Rb (1:500, mouse monoclonal), actin (1:1000, mouse monoclonal), cytochrome c (1:1000, mouse monoclonal), cyclin B1 (1:5000; mouse monoclonal); anti-hPNPase^{OLD-35} (1:10,000; chicken polyclonal) (82); anti-HA (1:1000; mouse monoclonal; Covance Research Products, Inc, Berkeley, Calif.); and EF1 α (1:1000; mouse monoclonal; Upstate Biotechnology, Waltham, Mass.).

[0161] Immunofluorescence analysis: HO-1 cells were plated on chamber slides (Falcon 4102; Becton Dickinson; Franklin Lakes, N.J.) and infected with different Ad. After 36 h, the cells were loaded with 250 mM MitoTracker (Molecular Probes, Eugene, Oreg.) for 30 min at 37° C., fixed with 3.7% formaldehyde for 15 min at 37° C. and permeabilized with 0.1% Triton-X-100 in PBS for 5 min at room temperature (RT). The cells were blocked with PBS containing 10% normal rabbit serum for 2 h at RT and incubated in the blocking solution containing anti-HA antibody (1:200) overnight at 4° C. After washing in PBS, the cells were incubated in the blocking solution containing anti-mouse-FITC (1:200) for 2 h at RT, washed again in PBS, mounted and visualized using a Zeiss confocal laser scanning microscope (LSM510) and a 40 \times objective. In case of double immunofluorescence studies for hPNPase^{OLD-35} and p27^{KIP-1}, HO-1 cells were plated on chamber slides, infected with Ad.hPNPase^{old-35} and 48 h later they were fixed, permeabilized and blocked with PBS containing 5% normal goat serum. The cells were incubated first with anti-p27 antibody and Alexa Fluor 594 (red) goat anti-rabbit IgG (Molecular probes) followed by anti-hPNPase^{OLD-35} antibody and rabbit anti-chicken-FITC (Genetel Laboratories, Madison, Wis.). Image analysis was performed using a Zeiss confocal laser scanning microscope (LSM510) and a 40 \times objective.

[0162] Cell Fractionation: 2 \times 10⁷ cells were harvested by trypsinization and mitochondrial and cytoplasmic fractions were separated using a Mitochondria Isolation kit (Pierce, Rockford, Ill.) according to the manufacturer's protocol.

[0163] Assay for Senescence Associated β -galactosidase (SA- β -gal) activity: SA- β -gal activity was assayed 4 days after infection with different Ad at an m.o.i. of 50 pfu/cell (61). The cells were fixed with 2% formaldehyde+0.2% glutaraldehyde and then stained with X-gal (1 mg/ml) in 40 mM citric acid/Na phosphate buffer (pH 6.0) containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl and 2 mM MgCl₂. After the development of color the cells were washed with PBS and methanol, air-dried and micro-photographed. Quantification of SA- β -gal positive cells was determined by counting at least 1,000 cells for each group.

[0164] Cell cycle analysis: Cell cycle was analyzed at 1, 2 and 3 days post-infection. Cells were harvested, washed in PBS and fixed overnight at -20° C. in 70% ethanol. Cells were treated with RNase A (1 mg/ml) at 37° C. for 30 min and then with propidium iodide (50 μ g/ml). Cell cycle was

analyzed using a FACS Calibur flow cytometer, and data was analyzed using CellQuest software (Becton Dickinson, San Jose, Calif.).

[0165] Cell sorting analysis: HO-1 cells were transfected with either empty vector or c-myc expression plasmid and infected the next day with either Ad.vec or Ad.hPNPaseold-35 at an m.o.i. of 50 pfu/cell. Two days after infection, live cells were incubated with 5 μ g/ml of Hoechst 33342 (Molecular Probes) for 1 h in the dark. After trypsinization, cells were resuspended at a concentration of 1 \times 10⁷ cells/ml for sorting. Cells were sorted based on the amount of DNA by defining three regions for sorting: one for G₁, one for S and one for G₂+M using BD FACSAria (BD Biosciences) equipped with a UV laser required for Hoechst 33342 excitation. The separated cells (at least 1 \times 10⁶ cells from each sorted population) were collected, protein was extracted and Western blot analysis was performed.

[0166] RNA Isolation and Northern Blot Analysis: Total RNA was extracted from the cells using Qiagen RNeasy mini kit (Qiagen) according to the manufacturer's protocol and Northern blotting was performed as described (84). The cDNA probes used were a 500-bp fragment from human c-myc, full-length human GADD34 and full-length human GAPDH.

[0167] In vitro Translation and in vitro mRNA Degradation Assays: In vitro translation was performed using the TNT coupled Reticulocyte Lysate Systems (Promega) using the plasmids pcDNA3.1+(Hygro) as a control, GADD153 expression plasmid, phPNPase^{old-35}, p Δ RPH1, p Δ RPH2, p Δ RPH1+2 and p Δ C-term according to the manufacturer's protocol. Five μ g of total RNA from HO-1 cells were incubated with 5 μ l of each in vitro translated protein at 37° C. from 0.5 to 2 h. The RNA was repurified using the Qiagen RNeasy mini kit (Qiagen) and Northern blotting was performed.

[0168] CDK Activity Assay: Cells were harvested in RIPA buffer and 500 μ g of protein were incubated overnight at 4° C. with anti-CDK2 antibody and then with protein A agarose for 1 h at 4° C. The agarose beads were spun down at 10,000 g for 5 min, washed three times in RIPA buffer and once in kinase buffer. The immunoprecipitated material was employed for kinase assays using Histone H1 (Upstate Biotechnology) as substrate and [γ -³²P]ATP (Amersham, Piscataway, N.J.) in a kinase buffer containing 25 mM Tris-HCl pH 7.5, 150 mM NaCl, 18 mM MgCl₂ and 1 mM DTT for 30 min at 30° C. Following the reaction the samples were subjected to 15% SDS-PAGE, the gel was dried, exposed to x-ray film and densitometric analysis was performed.

[0169] Statistical analysis: Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Fisher's protected least significant difference analysis.

[0170] 7.3 Results

[0171] Polynucleotide phosphorylases are highly conserved across species ranging from bacteria, plants to mammals (74). They have a conventional structure containing RNase PH domains and RNA binding domains (88, 89). FIG. 12A (top panel) shows the different domains of hPNPase^{OLD-35}. The hPNPase^{OLD-35} protein contains 783 amino acid residues. The first 45 a.a. contains a mitochondrial

localization signal. Interestingly, the bacterial PNPase does not contain such a signal, whereas the plant PNPase contains a chloroplast localization signal suggesting possible evolutionary divergence of this gene in eukaryotes. hPNPase^{OLD-35} contains two RNase PH(RPH) domains, involved in RNA degradation, one at a.a. 52-183, the other at a.a. 366-501. Between the two RPH domains there is an α -helix at a.a. 289-363 that is unique for PNPase and is involved in RNA binding. There are two RNA binding domains at the COOH-terminal of the molecule, the KH domain is at a.a. 605-667 and the S1 domain is at a.a. 676-750.

[0172] In order to comprehend the involvement of these domains in mediating the hPNPase^{old-35}-induced senescent phenotype, a number of deletion mutants were created and replication-incompetent adenoviruses (Ad) expressing these deletion mutants were generated (FIG. 12A). All the constructs were tagged with a C-terminal Hemagglutinin (HA)-epitope (YPYDVPDYA) (SEQ ID NO:39) for monitoring the site and level of expression of the proteins. Ad.hPNPase^{old-35} contains the complete ORF of hPNPase^{old-35}, Ad. Δ RPH1 contains a.a. 183-783 lacking the mitochondrial localization signal and RPH1 domain, Ad. Δ RPH2 contains a.a. 1-202 and 496-783 lacking the PNPase and RPH2 domains, Ad. Δ RPH1+2 contains a.a. 496-783 lacking the mitochondrial localization signal, both RPH domains and PNPase domain and Ad. Δ C-term contains a.a. 1-507 lacking the KH and S1 RNA binding domains. The predicted molecular weight of the proteins generated from these constructs are 86-, 67-, 54-, 31- and 55-kDa for hPNPase^{old-35}, Δ RPH1, Δ RPH2, Δ RPH1+2 and Δ C-term, respectively, which was confirmed by Western blotting.

[0173] It was considered important to initially determine if the level of expression of hPNPase^{old-35} resulting from adenoviral delivery of this gene was comparable to the level of endogenous protein induced following IFN- β treatment. To address this issue, HO-1 cells were infected with either Ad.vec (control empty Ad) or with Ad.hPNPase^{old-35} at an m.o.i. of 50 pfu/cell or treated with 1000 units/ml of IFN- β and the expression of hPNPase^{old-35} was analyzed two days later by Western blot analysis using anti-hPNPase^{old-35} antibody (82). Treatment with IFN- β resulted in marked induction of hPNPase^{old-35} and the level of the protein generated upon Ad.hPNPase^{old-35} infection was ~2-fold more than that with IFN- β treatment (FIG. 12B). These findings indicate that Ad.hPNPase^{old-35} generates hPNPase^{old-35} protein that is within a physiological range and the effects observed with Ad.hPNPase^{old-35} infection represent potentially physiologically relevant events.

[0174] To analyze the involvement of the different domains in hPNPase^{old-35}-induced growth inhibition and senescence, HO-1 human melanoma cells were infected with the different Ad at an m.o.i. of 10, 20 and 50 pfu/cell and colony formation assays were performed. As a control, cells were either uninfected or infected with Ad.vec at an m.o.i. of 50 pfu/cell. As shown in FIG. 12C, infection with Ad.hPNPase^{old-35}, Ad. Δ RPH1, Ad. Δ RPH2 and Ad. Δ C-term resulted in a dose-dependent inhibition in growth. At an m.o.i. of 50 pfu/cell colony formation was reduced by 60%, 54%, 51% and 51% upon infection with Ad.hPNPase^{old-35}, Ad. Δ RPH1, Ad. Δ RPH2 and Ad. Δ C-term, respectively. On the other hand, infection with Ad.vec or Ad. Δ RPH1+2 did not result in any significant growth inhibition. These findings confirm that retention of only the C-terminal RNA

binding domains are not adequate, whereas either of the RPH domains is sufficient for mediating growth inhibition. The observation that Ad. Δ C-term also has potent growth inhibiting properties indicates that the PNPase RNA binding domain might be sufficient for RNA binding and subsequent RPH activation.

[0175] To determine the senescence-inducing properties of these deletion mutants, HO-1 cells were infected with the different Ads and monitored for changes in cell morphology and senescence-associated β -galactosidase (SA- β -Gal) activity, a characteristic marker of senescence (61). Infection with Ad.hPNPase^{old-35}, Ad. Δ RPH1, Ad. Δ RPH2 and Ad. Δ C-term, but not Ad.vec and Ad. Δ RPH1+2, resulted in typical morphological changes in HO-1 cells (FIG. 12D). Large, flattened cells that stained for SA- β -Gal were observed (white arrows). In Ad.vec and Ad. Δ RPH1+2-infected cells 7 and 6% of the cells, respectively, stained positive for SA- β -Gal while in Ad.hPNPase^{old-35}, Ad. Δ RPH1, Ad. Δ RPH2 and Ad. Δ C-term-infected cells 35, 28, 45 and 29% of the cells, respectively, displayed SA- β -Gal-positivity (FIG. 12E). These findings indicate that similar to growth inhibition the morphological and biochemical changes, characteristic of senescence and induced by hPNPase^{old-35}, also require at least one of the two RPH domains.

[0176] Senescence is associated with arrest of cell cycle especially at the G₁ phase, with a decrease in the S phase indicative of inhibition of DNA synthesis (86). Cell cycle analysis was performed in HO-1 cells infected with the different Ads. A time-dependent change in the cell cycle pattern was observed (FIG. 13A-D). Three days after infection, 51% of the cells were in the G₁ phase following Ad.vec and Ad. Δ RPH1+2 infection, respectively. At the same time point, 73%, 71%, 72% and 69% of the cells were in the G₁ phase following Ad.hPNPase^{old-35}, Ad. Δ RPH1, Ad. Δ RPH2 and Ad. Δ C-term infection, respectively. Similarly, while 17% and 15% of the cells were in the S phase following Ad.vec and Ad. Δ RPH1+2 infection, respectively, 7%, 9%, 8% and 8% of the cells were in the S phase following Ad.hPNPase^{old-35}, Ad. Δ RPH1, Ad. Δ RPH2 and Ad. Δ C-term infection, respectively. These findings corroborate the findings of SA- β -gal staining that growth arrest in the G₁ phase and inhibition of DNA synthesis induced by hPNPase^{old-35} also requires the RPH domain.

[0177] We next investigated the expressions of proteins that regulate the progression of the cell cycle beyond the G₁ phase by Western blot analysis. There was a significant increase in p27^{KIP1} and a decrease in p21^{CIP1/WAF-1/MDA-6} upon infection with Ad.hPNPase^{old-35}, Ad. Δ RPH1, Ad. Δ RPH2 and Ad. Δ C-term, but not with Ad.vec and Ad. Δ RPH1+2 (FIG. 14A). No p16^{INK4A} protein was detected in HO-1 cells, which is due to the fact that a majority of melanomas have genomic abnormalities in the p16^{INK4A} gene (56). The level of phosphorylated Rb decreased significantly upon infection with Ad.hPNPase^{old-35}, Ad. Δ RPH1, Ad. Δ RPH2 and Ad. Δ C-term. These findings show that the increase in the level of p27^{KIP1} inhibits cyclin dependent kinase activity resulting in hypophosphorylation of Rb and arrest of cell cycle in the G₁ phase. This possibility was confirmed by assaying for CDK2 activity by *in vitro* kinase assays using Histone H1 as a substrate. Cell lysates obtained from Ad.vec and Ad. Δ RPH1+2 had high CDK2 kinase activity that was markedly reduced in cell lysates obtained from Ad.hPNPase^{old-35}, Ad. Δ RPH1, Ad. Δ RPH2

and Ad. Δ C-term-infected cells (FIG. 14B). The level of CDK2 itself was similar in all the samples indicating that the decrease in the CDK2 activity is not because of a decrease in CDK2 itself, but a consequence of upregulation of the CDKI p27^{KIP1} (FIG. 14B). The relationship between Ad.hPNPase^{old-35} infection and p27^{KIP1} upregulation was further documented by double immunofluorescence analysis. The cells expressing no hPNPase^{OLD-35} showed basal level of p27^{KIP1} expression (FIG. 14C, arrow) while hPNPase^{OLD-35}-expressing cells displayed higher levels of p27^{KIP1} (FIG. 14C).

[0178] It was documented previously that downregulation of c-myc plays a significant role in mediating hPNPase^{old-35}-induced growth inhibition (83). Considering this possibility, the involvement of the domains of hPNPase^{old-35} in regulating c-myc expression was analyzed. Infection with Ad.hPNPase^{old-35}, Ad. Δ RPH1, Ad. Δ RPH2 and Ad. Δ C-term, but not with Ad.vec and Ad. Δ RPH1+2, resulted in ~50% reduction in c-myc mRNA level 3 days post-infection (FIG. 15A-B). This reduction was also reflected at the level of the MYC protein (FIG. 15C). The expression of MYC protein decreased while that of MAD-1 protein increased significantly following Ad.hPNPase^{old-35}, Ad. Δ RPH1, Ad. Δ RPH2 and Ad. Δ C-term, but not with Ad.vec and Ad. Δ RPH1+2 infection. The level of MAX and EF1 α remained unchanged under all treatment protocols. These findings indicate that the RPH domains of hPNPase^{old-35} are required to downregulate c-myc expression, which results in a switch from a MYC-MAX transcriptional activator to MAD-1-MAX transcriptional repressor.

[0179] To support the observations of these expression studies, c-myc was overexpressed in HO-1 cells and these cells were infected with the different Ads and colony-forming ability was determined. Infection with Ad.hPNPase^{old-35}, Ad. Δ RPH1, Ad. Δ RPH2 and Ad. Δ C-term decreased colony formation by 75%, 73%, 79% and 74%, respectively in comparison to infection with Ad.vec and Ad. Δ RPH1+2 (FIG. 15D). Overexpression of c-myc provided partial but significant protection so that colony formation was decreased by 46%, 44%, 53% and 46% upon infection with Ad.hPNPase^{old-35}, Ad. Δ RPH1, Ad. Δ RPH2 and Ad. Δ C-term, respectively. These findings suggest that downregulation of c-myc plays a prominent role in hPNPase^{old-35}-mediated growth inhibition.

[0180] hPNPase^{old-35} is a 3', 5' exoribonuclease (75), so we investigated whether the downregulation of c-myc is a consequence of direct degradation of c-myc mRNA by hPNPase^{old-35}. hPNPase^{OLD-35} and its different deletion mutants were in vitro translated and the proteins were used for RNA degradation assays. As shown in FIG. 16A, the plasmid constructs phPNPase^{old-35}, p Δ RPH1, p Δ RPH2, p Δ RPH1+2 and p Δ C-term give rise to proteins of the expected molecular weight of 86-, 67-, 54-, 31- and 55-kDa, respectively, indicating the authenticity of the constructs. The in vitro translated proteins were incubated with total RNA for 0.5 and 2 h. The RNA was purified and the expression of c-myc, GADD34 and GAPDH mRNAs were analyzed by Northern blot analyses. Incubation with phPNPase^{old-35}, p Δ RPH1, p Δ RPH2 and p Δ C-term resulted in significant degradation of c-myc mRNA (FIG. 16B, 16C). No degradation was observed upon incubation with the control plasmid pcDNA3.1, p Δ RPH1+2 and an unrelated plasmid pGADD153 that expresses a transcription factor.

The expressions of GADD34 and GAPDH mRNAs remained unchanged under all experimental conditions. These findings indicate that the presence of either RPH domain is necessary and sufficient to degrade c-myc mRNA and degradation is specific for c-myc mRNA.

[0181] To establish a direct correlation between cell cycle arrest in the G₁ phase and c-myc downregulation by hPNPase^{OLD-35} HO-1 cells were sorted following Ad.hPNPase^{old-35} infection and the expressions of hPNPase^{OLD-35} and Myc were analyzed in the different phases of the cell cycle (FIG. 17). As a control for authenticity of the sorting procedure the expression of cyclin B1 was analyzed. Cyclin B1 starts being synthesized in late G₁ and its expression is maximum in G₂+M phase, which was confirmed by our cyclin B1 expression analysis in different phases of the cell cycle, indicating the effectiveness of the sorting procedure (FIG. 17, fourth panel). The equal expression of EF1- α in all the samples served as a loading control (FIG. 17, third panel). Although hPNPase^{OLD-35} protein could be detected in the cells in S and G₂+M phases, significantly higher levels of hPNPase^{old-35} were detected in cells in the G₁ phase of the cell cycle following Ad.hPNPase^{old-35} infection (FIG. 17, first panel, lanes 4 and 10 versus lanes 5, 6, 11 and 12). The level of hPNPase^{OLD-35} in different phases of the cell cycle inversely correlated with the level of Myc, which was markedly downregulated in cells in the G₁ phase (FIG. 17, second panel, lane 1 versus lane 4) and moderately downregulated in the cells in S and G₂+M phases (FIG. 17, second panel, lane 2, 3 versus lane 5, 6) following Ad.hPNPase^{old-35} infection. When Myc was overexpressed in cells, there was a slight reduction of Myc in cells in the G₁ phase upon Ad.hPNPase^{old-35} infection (FIG. 17, second panel, lane 7 versus lane 10) but this level of Myc was still markedly higher than that in Ad.hPNPase^{old-35}-infected G₁ phase cells without Myc overexpression (FIG. 17, second panel, lane 4 versus lane 10). The decrease of Myc in the Myc-overexpressed G₁ phase cells is probably because of downregulation of endogenous Myc. These findings indicate that hPNPase^{OLD-35} decreases endogenous but not exogenous Myc thereby explaining the potential protection against hPNPase^{OLD-35}-induced growth inhibition by overexpressing Myc. The c-myc expression plasmid does not contain the 3' untranslated region (UTR) of the endogenous mRNA indicating that the sequence in the 3' UTR of c-myc mRNA might contain a potential binding site for hPNPase^{OLD-35}.

[0182] The presence of the mitochondrial localization signal indicates that hPNPase^{OLD-35} is a predominantly mitochondrial protein (79, 82). The question naturally arises as to how a mitochondrial protein might degrade a cytoplasmic mRNA, like c-myc. To address this question, cells infected with different Ad were fractionated into mitochondrial and cytoplasmic fractions and the expressions of the proteins were analyzed by Western blotting using anti-HA antibody. As a control for the quality of the purification, membranes were reprobed with anti-actin antibody (for cytoplasmic fraction) and anti-cytochrome C antibody (for mitochondria). hPNPase^{OLD-35}, Δ RPH2 and Δ C-term showed predominantly mitochondrial expression resulting from the presence of the mitochondrial localization signal in these constructs (FIG. 18A) while ARPH1 and Δ RPH1+2 was located predominantly in the cytoplasmic fraction (FIG. 18A) because of lack of the mitochondrial localization signal. However, high level expression of hPNPase^{OLD-35} and low level expression of Δ RPH2 and Δ C-term was also

detected in the cytoplasmic fraction and very low level expression of Δ RPH1 was detected in the mitochondrial fraction. In the case of hPNPase^{OLD-35}, a smear was detected above the major band, which might result from incomplete denaturation of the homotrimer formed by hPNPase^{OLD-35}. These findings indicate that the proteins are localized both in cytoplasm and mitochondrial compartments and the bands that are detected are not a consequence of cross-contamination during purification, which is strongly supported by the observation that no cross staining for actin in mitochondria or for cytochrome c in cytoplasm was detected.

[0183] The fractionation results were confirmed by immunofluorescence studies using anti-HA antibody (green) and MitoTracker (red) to determine the localization of hPNPase^{OLD-35} and its deletion mutants (FIG. 18B). hPNPase^{OLD-35}, ARPH2 and Δ C-term showed a speckled expression pattern that co-localized with mitochondrial staining as evidenced by the presence of yellow color in the merged image. However, in addition to the yellow staining, there were also isolated green stainings observed in the merged images indicating the presence of hPNPase^{OLD-35}, Δ RPH2 and Δ C-term in cytoplasmic compartments. Δ RPH1 and Δ RPH1+2 showed a diffuse expression pattern throughout the cytoplasm that did not co-localize with the mitochondrial staining indicating the presence of Δ RPH 1 and Δ RPH 1+2 in the cytoplasm.

[0184] 7.4 Discussion

[0185] In this study, the importance of the RPH domains in mediating the characteristic phenotypic changes induced by hPNPase^{old-35} is firmly established. We observed that presence of at least one RPH domain is required for the functional activity of the protein and either of the domains is as potent as the full-length molecule. This contrasts with bacterial PNPase in which mutation in the key residues in either of the RPH domains inhibits catalytic activity (70). However, our result is comparable to chloroplast PNPase in which the first RPH domain alone (comparable to our ARPH2 construct) is highly active enzymatically (91). Although the second RPH domain (our Δ RPH1) of chloroplast has low RNA degradation activity of non-polyadenylated RNA it has high activity for polyadenylated RNA (91) which explains the efficiency of the Δ RPH1 construct in degrading human polyadenylated mRNAs. The RPH domains themselves can bind to RNA and the PNPase domain is also involved in RNA binding which explains the preservation of the RNA degradation activity of the AC-term construct that lacks the KH and S1 RNA binding domains. Current studies are determining the effects of mutation in specific important and evolutionary conserved amino acid residues of hPNPase^{old-35}, especially those surrounding the tungsten binding region implicated in the enzymatic activity of the bacterial protein (70).

[0186] A unique and potentially significant finding is that hPNPase^{OLD-35} displays specific degradation activity for c-myc mRNA. The 3' stem-loop structure of a RNA species determines the enzymatic activity of PNPases (91). It is of significant interest to see whether there exists a difference between the 3' secondary structure of c-myc and other mRNAs, such as GAPDH and GADD34, that facilitates its degradation by hPNPase^{old-35}. PNPases function as a homotrimer (53) and our findings suggest that hPNPase^{OLD-35} also multimerizes. However, the deletion mutants, that do

not multimerize, still retain their specific mRNA degradation activity. We can rule out the involvement of a c-myc specific RNA binding protein regulating the activity of hPNPase^{OLD-35}, since our in vitro RNA degradation assays, which contained a single protein, could effectively achieve RNA degradation. Elucidation and comparison of three-dimensional models of c-myc and other mRNAs might provide insights into the specific RNA degradation activity of hPNPase^{old-35}.

[0187] Structure analysis and localization studies reveal that hPNPase^{OLD-35} is a predominantly mitochondrial protein (79, 82), thereby provoking the obvious question as to how hPNPase^{OLD-35} might degrade a cytoplasmic target mRNA, such as c-myc. By fractionation and immunofluorescence analyses, we demonstrate that although the primary site of localization of hPNPase OLD-3 is the mitochondria, a considerable amount of the protein is also present in the cytoplasmic fraction of the cell. Similarly, Δ RPH2 and Δ C-term, that retain the mitochondrial localization signal, are also mainly localized in mitochondria, although low but detectable levels are still found in the cytoplasm. Δ RPH1 and Δ RPH1+2, lacking the mitochondrial localization signal, were located in the cytoplasm although a very low level of Δ RPH1 was also evident in the mitochondria. This is not because of cross-contamination which was confirmed by mutually exclusive expression of cytochrome c and actin in mitochondria and cytoplasmic fractions, respectively. These findings suggest the existence of a potential cytoplasmic-mitochondrial shuttling of the hPNPase^{OLD-35} protein that might be regulated by chaperone proteins. Further studies designed to identify and define interacting partners of hPNPase^{OLD-35} will help address this important question.

[0188] Adenovirus-mediated delivery usually results in robust transgene expression that raises the important question of whether the senescence-inducing effect of Ad.hPNPase^{old-35} is a genuine physiological phenomenon. We have observed that there is a comparable level of hPNPase^{OLD-35} protein upon IFN- β treatment and with Ad.hPNPase^{old-35} infection suggesting that the senescent phenotype observed with hPNPase^{OLD-35} is indeed a physiologically relevant event. Bacterial PNPase autocontrols its expression post-transcriptionally by degrading its own mRNA (71). Although not yet confirmed for hPNPase^{old-35}, this post-transcriptional control might explain the restricted expression level of hPNPase^{OLD-35} even with adenovirus-mediated delivery of this enzyme.

[0189] CDKIs, especially p16^{INK4A} and p21^{CIP1/WAF-1/MDA-6} are intimately involved in the process of cellular senescence (52, 87). hPNPase^{old-35}-induced senescence is associated with an increase in p27^{KIP1} and a decrease in p21^{CIP1/WAF-1/MDA-6}, a phenomenon that is observed in two other models of senescence-like growth arrest, one resulting from iron chelation in hepatocytes, and the other a consequence of inhibition of the phosphoinositide-3-kinase pathway in mouse embryo fibroblasts (57, 92). The involvement of p16^{INK4A} is ruled out as an essential component of this process because HO-1 cells do not express the p16^{INK4A} protein. The increase in p27^{KIP1} is most likely secondary to the decrease in c-myc that controls p27^{KIP1} expression at multiple levels, including repression of transcription and facilitation of ubiquitination and subsequent degradation (77, 78, 90). Myc plays an important role in controlling cell cycle (65). It promotes entry into the S phase and shortens the G₁ phase.

We observed that downregulation of c-myc by hPNPase^{old-35} resulted in a corresponding increase in Mad1 and this particular shift from MYC-MAX transcriptional activator to MAD1-MAX transcriptional repressor might be important for mediating the plethora of effects promulgated by hPNPase^{old-35}. However, since overexpression of Myc could not provide complete protection against hPNPase^{old-35}-mediated growth inhibition, additional targets of hPNPase^{old-35} are likely to exist that might be involved in mediating its effects. While the effect of hPNPase^{old-35} on c-myc is direct, its effect on MAD1 is probably indirect via additional targets, downregulation of which might lead to derepression of MAD1 expression.

[0190] In summary, we now confirm the importance of the RPH domains in hPNPase in inducing senescence, an event that is unique in its molecular mechanism and is critical for regulating organismal homeostasis. These studies provide unique perspectives on the molecular mechanism of senescence and the structure-function relationship of the hPNPase^{OLD-35} protein. Crystallization of the hPNPase^{old-35} protein and comparison of its crystal structure to that of the PNPase proteins of other species will directly facilitate identification of important residues mediating catalytic and senescence-inducing activity. Of added significance, the development of an animal model conditionally overexpressing hPNPase^{old-35} will provide valuable insights into the involvement of hPNPase^{old-35} in *in vivo* senescence.

8. EXAMPLE

Defining the Mechanism by which IFN- β Downregulates c-myc Expression in Human Melanoma Cells: Pivotal Role for Human Polynucleotide Phosphorylase (hPNPase^{old-35})

[0191] 8.1 Materials and Methods

[0192] Cell lines and cell viability assays: FM516-SV (referred to as FM516) normal immortal human melanocyte, WM35 early radial growth phase (RGP) primary human melanomas, HO-1 and MeWo metastatic melanomas, 2fTGH human fibrosarcoma and its derivates U1A, U3A, U4A and U5A, HeLa human cervical carcinoma and 293 adenovirus transformed human embryonic kidney (HEK 293) cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and penicillin (100 U/ml) and streptomycin (100 μ g/ml). 2fTGH cells are wild type in IFN signaling while its derivates U1A, U3A, U4A and U5A have defects in IFN signaling that could be complemented by expression of TYK2, STAT1, JAK1 or IFNAR2, respectively (112, 113). Cell growth and viable cell numbers were monitored by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) staining as described (150).

[0193] Generation of lentivirus expressing siRNA for hPNPase^{old-35}: Using the software siRNA Target Finder (Ambion, Austin, Tex.) four potential siRNAs for hPNPase^{old-35} were designed and the siRNAs were constructed by *in vitro* transcription using the Silencer siRNA construction kit (Ambion) according to the manufacturer's protocol. These siRNAs were transfected into HeLa cells using Lipofectamine 2000 (Invitrogen, Carlsbad, Calif.) according to the manufacturer's protocol and the next day the cells were treated with 1000 U/ml IFN- β for 24 h. The expression of

hPNPase^{OLD-35} in the lysates of these cells was analyzed by Western blot analysis. The siRNA demonstrating the maximum inhibition of hPNPase^{OLD-35} induction by IFN- β was selected for construction of the lentivirus. The hPNPase^{old-35} and control siRNA sequences were 5' AACAAAACCTTC-CCCTTCCCC 3' (SEQ ID NO:40) and 5' AAGGGTCGTC-TATAGGGATCGAT 3' (SEQ ID NO:41), respectively. Lentiviruses expressing either control siRNA or hPNPase^{old-35} siRNA were constructed using BLOCK-iT Lentiviral RNAi Expression System (Invitrogen) according to the manufacturer's protocol. The siRNA was first ligated into BLOCK-iT U6 RNAi Entry Vector that drives expression of the siRNA under control of the human U6 promoter. The siRNA expression cassette was transferred to pLenti6 BLOCK-iT-DEST lentiviral vector by the LR recombination reaction. The resultant construct was transfected into HEK293FT cells with Lipofectamine 2000 along with ViraPower lentiviral packaging mix that expresses the proteins required for lentivirus replication. The lentivirus was amplified and titered by standard plaque assay.

[0194] Generation of stable cell clones: Stable clones of HO-1 cells expressing either control siRNA or hPNPase^{old-35} siRNA were generated by transducing the cells with lentiviruses expressing the corresponding siRNA and selecting clones for 2 weeks using 4 μ g/ml blasticidin. Stable HO-1 clones expressing c-myc were generated by transfecting HO-1 cells with a c-myc expression vector and selecting the cells for 2 weeks with 100 μ g/ml hygromycin. Transfecting the cells with empty vector and selecting with hygromycin generated corresponding control clones.

[0195] Cell Cycle Analysis: Cells were harvested, washed in PBS and fixed overnight at -20° C. in 70% ethanol. The cells were treated with RNase A (1 mg/ml) at 37° C. for 30 min and then with propidium iodide (50 μ g/ml). Cell cycle was analyzed using a FACScan flow cytometer and data were analyzed using CellQuest software (Becton Dickinson, San Jose, Calif.).

[0196] Colony formation assays: One-thousand cells were plated in 6-cm dishes and then treated with different doses of IFN- β for 2 weeks at which point the colonies were fixed, stained with Giemsa and colonies \geq 50 cells were counted.

[0197] Transfection of siRNA: Cells (5×10^5) were plated in a 6 cm dish and the next day were transfected with 25 nM of either control siRNA or c-myc siRNA (Ambion; catalogue# 4250) using Lipofectamine 2000 (Invitrogen) according to the Manufacturer's protocol. After 24 h, the cells were trypsinized and seeded into 96-well plates for cell viability assays and 6-cm dishes for colony formation assays and cell cycle analyses as described above.

[0198] RNA Isolation and Northern Blot Analysis: Total RNA was extracted from cells using Qiagen RNeasy mini kit (Qiagen) according to the manufacturer's protocol and Northern blotting was performed as described (102). The cDNA probes used were a 400-bp fragment from human c-myc, a 500-bp fragment from hPNPase^{old-35} and full-length human GAPDH. For analysis of half-life of c-myc mRNA, cells were either untreated or treated with IFN- β (1000 U/ml) for 24 h and then treated with Actinomycin D (5 μ g/ml) for 0.5, 1, 2, 4 and 8 h following which the cells were harvested for total RNA extraction and Northern blot analysis.

[0199] Western Blot Analysis: Western blotting was performed as previously described (102). Briefly, cells were

harvested in RIPA buffer containing protease inhibitor cocktail (Roche, Mannheim, Germany), 1 mM Na₃VO₄ and 50 mM NaF and centrifuged at 12,000 rpm for 10 min at 4° C. The supernatant was used as total cell lysate. Thirty µg of total cell lysate were used for SDS-PAGE and transferred to a nitrocellulose membrane. The primary antibodies included: Myc (1:200; mouse monoclonal; Santa Cruz biotechnology, Santa Cruz, Calif.), hPNPase^{OLD-35} (1:10000; chicken polyclonal), MDA-5 (1:5000; rabbit polyclonal) and EF1 α (1:1000; mouse monoclonal; Upstate Biotechnology, Waltham, Mass.).

[0200] Statistical analysis: Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Fisher's protected least significant difference analysis.

[0201] 8.2 Results

[0202] Regulation of hPNPase^{old-35} and c-myc mRNA expression by IFN-β: To define a potential correlation between the expression regulation of hPNPase^{old-35} and c-myc by IFN-β, HO-1, WM35 and MeWo human melanoma cells and SV40 T/t Ag-immortalized human melanocytes (FM-516-SV, here forth indicated as FM-516) were treated with 1000 U/ml of IFN-β for different times ranging from 12 to 48 h and the expression of hPNPase^{old-35} and c-myc mRNA was determined by Northern blot analysis (FIG. 19A). Under basal condition, there was little to barely detectable hPNPase^{old-35} mRNA expression in the different cell types. Upon IFN-β-treatment a marked increase in hPNPase^{old-35} mRNA expression was detected 12 h post-treatment. In HO-1, WM-35 and FM-516 cells hPNPase^{old-35} mRNA expression gradually decreased with time and by 48 h post-IFN-β-treatment returned to the basal level. However, in MeWo cells hPNPase^{old-35} mRNA expression persisted even 48 h after IFN-β-treatment. While hPNPase^{old-35} mRNA expression was increased by IFN-β, c-myc mRNA expression decreased with the same treatment and there was a temporal correlation in the expression regulation of these two mRNAs by IFN-β. A significant time-dependent IFN-β-mediated decrease in c-myc mRNA expression was also evident in all four cell lines. In MeWo cells, with persistence of hPNPaseold-35 mRNA expression, c-myc mRNA expression disappeared completely at 48 h post IFN-β-treatment.

[0203] The mRNA expression results were confirmed on a protein level by Western blot analysis (FIG. 19B). Under basal condition, hPNPase^{OLD-35} protein was undetectable in all four cell lines. With IFN-β-treatment, hPNPase^{OLD-35} protein expression was markedly induced and persisted even 2 days after treatment. With the exception of MeWo cells, the corresponding mRNA levels decreased at 48 h in HO-1, WM-35 and FM-516 cells. The Myc protein levels also showed a temporal decrease following IFN-β-treatment.

[0204] A direct correlation in IFN-β-induced dose-dependent changes in hPNPase^{OLD-35} and Myc proteins was also evident. In HO-1 and WM-35 cells, hPNPase^{OLD-35} induction and Myc downregulation were detected with 100 and 1000 U/ml of IFN-β, but not with 1 or 10 U/ml (FIG. 19B). In MeWo and FM-516 cells, changes in protein levels could be detected with as little as 1 U/ml or 10 U/ml of IFN-β, respectively. In IFN-β-treated FM-516 cells in addition to the Myc band, a faster migrating band was detected which might represent a degradation product of the Myc protein. These findings confirm that the concentration of IFN-β

required to upregulate hPNPase^{OLD-35} is also required to downregulate Myc protein indicating a potential cooperative regulation in the expression of these two genes.

[0205] The regulation of expression of hPNPase^{OLD-35} and Myc by IFN-β was confirmed in 2fTGH human fibrosarcoma cells and in its four variants. U1A (Tyk2-), U3A (STAT1-), U4A (JAK1-) and U5A (IFNAR2-), that have mutations in different molecules involved in the IFN-signaling pathway (112, 113). As shown in FIG. 19C, the upregulation of hPNPase^{OLD-35} and downregulation of Myc by IFN-β were observed only in parental 2fTGH cells but not in its mutant clones, which are non-responsive to type I IFN.

[0206] IFN-β its growth of melanoma cells and melanocytes: The effect of IFN-β on the growth of HO-1, WM35, MeWo and FM-516 cells were analyzed by standard MTT cell survival assays (FIG. 20A). Cells were treated with 1, 10, 100, 1000 and 2000 U/ml of IFN-β for up to 6 days. HO-1, WM-35 and FM-516 cells did not respond to 1 or 10 U/ml of IFN-β. With 100 U/ml, there was a significant inhibition in cell growth and with 1000 U/ml and 2000 U/ml, there was ~90% inhibition in growth 6 days after IFN-β-treatment. The growth of MeWo cells was significantly inhibited even with 1 U/ml of IFN-β, which became marked with 100 or more U/ml of IFN-β. These studies document a direct correlation between gene expression changes and the levels of IFN-β required to evoke growth inhibition in specific target cells. This finding is particularly relevant in the case of MeWo cells in which corresponding changes could be observed even with 1 U/ml of IFN-β.

[0207] The results obtained using cell viability assays were confirmed by colony formation assays (FIG. 20B). In HO-1, WM-35 and FM-516 cells colony formation was significantly inhibited with 100 U/ml of IFN-D and with 1000 U/ml, the colony formation was inhibited by >90%. In the case of MeWo cells, 10 U/ml of IFN-β significantly inhibited colony formation and with 1000 U/ml of IFN-β colony formation was inhibited by >95%.

[0208] Cell cycle analysis was performed to characterize growth inhibition. IFN-β treatment (1000 U/ml) in HO-1, WM-35, MeWo and FM-516 cells resulted in initial (day 1) cell cycle arrest in the G₁ phase of the cell cycle with a concomitant decrease in the DNA synthesis phase as substantiated by the reduction in S phase (FIG. 27-Table 1). With longer exposure to IFN-β the cells gradually became apoptotic as evidenced by a steady increase in the sub-G₁ (A₀) cell population.

[0209] hPNPase^{OLD-35} regulates IFN-β-mediated downregulation of Myc: Since hPNPase^{OLD-35} is a 3', 5' exoribonuclease and one of its substrates is c-myc mRNA we tested whether hPNPase^{OLD-35}, induced by IFN-β, promotes Myc downregulation. We have identified siRNA active in downregulating hPNPase^{old-35} and created a lentivirus expressing this siRNA. Stable clones in an HO-1 background expressing either control siRNA or hPNPase^{old-35} siRNA were generated by selection with blasticidin. As shown in FIG. 21A, three clones, clone 1, 4 and 5, which express hPNPase^{old-35}-siRNA, were identified that significantly inhibited IFN-β-induction of hPNPase^{OLD-35}, with clone 1 being the most efficient producing almost 100% inhibition in hPNPase^{OLD-35} induction. The clone expressing control siRNA retained its ability to induce hPNPase^{OLD-}

35 following IFN- β treatment. Remarkably, while the parental HO-1 cells and control-siRNA expressing clone could downregulate Myc in response to IFN- β treatment, all three hPNPase^{old-35} siRNA expressing clones lost this ability. However, these clones retained their ability to respond to IFN- β as evidenced by the induction of another IFN-inducible gene mda-5 (114). These findings indicate that hPNPase^{OLD-35} specifically mediates downregulation of Myc but not the modulation of other genes by IFN- β . The observation of similar responses in multiple clones rules out the possibility that the observed effects are simply a consequence of clonal variability in response to IFN- β .

[0210] The half-life of c-myc mRNA with or without IFN- β treatment was analyzed in HO-1 cells and control siRNA and hPNPase^{old-35} siRNA expressing clones (FIG. 21B). The cells were treated with IFN- β (1000 U/ml) for 24 h and then exposed to Actinomycin D (Act D; 5 μ g/ml) for 0.5 to 8 h (FIG. 21B). In the untreated cells, the half-life of c-myc mRNA was ~1 h. In HO-1 cells and control siRNA expressing clones, IFN- β treatment resulted in significant downregulation of c-myc mRNA so that by 0.5 h of Act D treatment no c-myc mRNA could be detected in these cells. This downregulation correlated with upregulation of hPNPase^{old-35} mRNA that had a half-life of ~4 h. In contrast, IFN- β treatment did not induce hPNPase^{old-35} mRNA expression in hPNPase^{old-35}-siRNA expressing clones and the half-life of c-myc mRNA remained unchanged when compared to control untreated cells (FIG. 21B). These findings indicate that under basal condition hPNPase^{old-35} is not expressed and therefore it does not affect the turnover of c-myc mRNA. However, upon IFN- β treatment this enzyme is induced and it degrades c-myc mRNA.

[0211] Resistance of hPNPase^{old-35}-siRNA clones to IFN- β -mediated growth inhibition: Overexpression of hPNPase^{old-35} induces growth inhibition and apoptosis in melanoma cells and c-myc is a positive regulator of cell growth, allowing cells to traverse the G₁ phase of the cell cycle. Based on these considerations, we tested whether the lack of these two events in hPNPase^{old-35}-siRNA expressing clones would render them resistant to IFN- β -mediated growth inhibition. As shown in FIG. 22B, while the parental HO-1 cells and control siRNA expressing clones were sensitive to IFN- β -treatment, as monitored by standard MTT assays, the hPNPase^{old-35}-siRNA expressing clones showed significant resistance to IFN- β , which became more pronounced after 6 days of IFN- β treatment. These findings were confirmed by colony formation assays, which also demonstrated significant resistance of hPNPase^{old-35}-siRNA expressing clones to IFN- β -induced inhibition of colony formation (FIG. 23).

[0212] The results obtained from cell survival and colony formation assays were confirmed by cell cycle analysis using flow cytometry. As shown in FIG. 24 and FIG. 28-Table 2, the parental HO-1 cells and control siRNA expressing clones showed an initial G₁ arrest and eventually cells underwent apoptosis. However, hPNPase^{old-35}-siRNA expressing clones showed remarkable resistance to growth inhibition by IFN- β with no statistically significant increase in the G₁ phase or the number of A₀ cells. In these contexts, blocking hPNPase^{old-35} expression prevents cell cycle arrest and apoptosis induced by IFN- β .

[0213] To confirm that the mechanism underlying the resistance of hPNPase^{old-35}-siRNA expressing clones to

IFN- β is mediated by their inability to downregulate c-myc, HO-1 cells and control siRNA and hPNPase^{old-35}-siRNA expressing clones were transfected with either control or c-myc siRNA and treated with IFN- β and cell viability, colony formation and cell cycle analyses were performed. Transfection of c-myc siRNA resulted in marked downregulation of Myc protein (FIG. 22A) indicating the authenticity of its function. Cell viability and colony formation ability was similar between control untransfected cells and control siRNA-transfected cells (FIGS. 22B and 23) with hPNPase^{old-35}-siRNA expressing clones showing resistance to IFN- β and HO-1 cells and control siRNA expressing clones showing sensitivity to IFN- β . Transfection of c-myc siRNA alone reduced cell viability and colony formation ability of all the cell lines and together with IFN- β markedly inhibited cell viability and colony formation ability in all of the cell lines, including hPNPase^{old-35}-siRNA expressing clones (FIGS. 22B and 23). Cell cycle analysis also revealed that transfection of c-myc siRNA rendered hPNPase^{old-35}-siRNA expressing clones susceptible to IFN- β mediated cell cycle arrest and apoptosis (FIG. 28-Table 2). In total, these findings indicate that inhibition of c-myc downregulation in hPNPase^{old-35}-siRNA expressing clones confers their resistance to growth inhibition by IFN- β .

[0214] Resistance of c-myc overexpressing clones to IFN- β -mediated growth inhibition: We next evaluated the involvement of c-myc downregulation in IFN- β -mediated growth inhibition. For this purpose, stable Myc overexpressing HO-1 clones (HO-1-Myc) were developed by transfection with a c-myc expression vector and selection with hygromycin. Control hygromycin-resistant clones (HO-1-Hygro) were similarly generated. FIG. 25A provides data from two representative Myc-overexpressing HO-1 clones. IFN- β treatment for 3 days resulted in marked downregulation of endogenous Myc protein in HO-1-Hygro clones (FIG. 25A). However, the exogenous Myc protein in HO-1-Myc clones was not significantly downregulated by IFN- β . The c-myc expression construct contains only the open reading frame and not the 3' or 5' untranslated regions (UTR) of the cDNA. The inability of IFN- β to downregulate exogenous c-myc indicates that the 3'-UTR of the endogenous c-myc sequence might confer its sensitivity to hPNPase since hPNPase^{OLD-35} is a 3', 5' exoribonuclease. The growth of the HO-1-Hygro clones (clones 1 and 4) was significantly inhibited by IFN- β (1000 U/ml) treatment as documented by cell viability assays (FIG. 7C). HO-1-Myc clones overexpressing Myc provided partial but significant protection from IFN- β -mediated growth inhibition (FIG. 25C). These findings were also confirmed by colony formation assays (FIG. 25B). HO-1-Myc clones, but not HO-1-Hygro clones, showed resistance to inhibition of colony formation by IFN- β . These results implicate IFN- β modulation of c-myc as an important factor associated with IFN- β -induced growth suppression in HO-1 cells.

[0215] These interesting findings were corroborated by cell cycle analysis. Treatment with IFN- β induced an initial G₁ arrest and eventually apoptosis in HO-1-Hygro clones (FIG. 29-Table 3). The HO-1-Myc clones showed a slight increase in the percentage of G₁ phase and apoptotic cells following IFN- β treatment, which was not statistically significant. These findings indicate that both upregulation of hPNPase^{old-35} and downregulation of c-myc are central events in mediating the ability of IFN- β to inhibit growth in human melanoma cells (FIG. 26).

[0216] 8.3 Discussion

[0217] Microarray studies have revealed a plethora of genes that are modulated by IFN treatment (115) (<http://bioinfo.cnio.es/data/oncochip/>). IFNs can directly affect gene expression by ISRE and GAS sequences in the promoters of target genes (97). In addition, IFNs can also affect gene expression by their ability to induce proteins involved in RNA metabolism, such as 2',5'-oligoadenylate synthetase/RNase L, double stranded RNA-dependent protein kinase (PKR), melanoma differentiation associated gene-5 (mda-5), retinoic acid inducible gene-I (RIG-I) and hPNPase^{old-35} (97, 99, 114, 116). Inhibition of gene expression by IFNs at a post-transcriptional level has been described for the heavy chain of immunoglobulin mu (117), the IL-4 receptor (118) and c-myc (110, 111), the focus of the present studies.

[0218] The observation that type I IFN selectively reduces c-myc mRNA has been described in multiple studies using several model cell culture systems. Jonak and Knight first hypothesized that IFN-β mediated downregulation of c-myc mRNA might mediate growth inhibition in Daudi human lymphoblastoid cells (110). As a follow-up study, Dani et al. demonstrated in Daudi cells that IFN c/5 did not affect the transcription rate of c-myc mRNA, but rather reduced the half-life of this mRNA (111). A posttranscriptional destabilization of c-myc mRNA as a mechanism of type I IFN-mediated c-myc suppression has also been described in colon carcinoma cells (119). It was shown that during terminal differentiation of hematopoietic cells, autocrine IFN-β induces c-myc suppression and induces G₀/G₁ arrest in these cells (120). Additionally, previous studies from our laboratory documented that IFN-β and mezerein-induced terminal differentiation of human melanoma cells also correlated with downregulation of c-myc mRNA (121). Moreover, studies in different cell types consistently describe the ability of type I IFN to reduce c-myc expression. However, the effect of IFN-γ on c-myc expression varies in different cell contexts. In HeLa cells, treatment with IFN-α decreased while IFN-γ increased c-myc expression (122). In a murine myeloid cell line, IFN-γ inhibited c-myc gene expression by impairing the splicing process (123). Another report described the importance of Stat-1 in IFN-γ-mediated downregulation of c-myc. Studies employing wild type and Stat-1-null mouse embryonic fibroblasts identified a gamma activated sequence element in the c-myc promoter that was necessary, but not sufficient, to suppress c-myc expression in wild type cells (124).

[0219] Although a consensus exists that type I IFNs induce post-transcriptional modulation of c-myc mRNA, the molecular mechanism underlying this process is unclear. Different components of IFN-inducible RNA degradation machinery have been implicated in this action. In colon carcinoma cells, 2',5'-oligoadenylate synthetase/RNase L system is believed to regulate IFN-β-mediated post-transcriptional processing of c-myc mRNA (119). In M1 murine myeloid leukemia cells, PKR has been shown to mediate type I IFN-induced c-myc suppression (125). A recent report indicates that in mouse monocyte/macrophage leukemia cells, IFN-β reduces steady state levels of Myc protein by increasing degradation through the 26S proteasome (126). In our previous studies, we revealed for the first time by employing recombinant hPNPase^{old-35} protein in in vitro mRNA degradation assays that a type I IFN-inducible exoribonuclease, hPNPase^{old-35}, could selectively degrade c-myc

mRNA (102, 107). In the present studies, we now confirm that hPNPase^{old-35} is the enzyme responsible for IFN-β-mediated degradation of c-myc mRNA in human melanoma cells.

[0220] Myc is an important regulator of cell proliferation (127). Expression of exogenous Myc in cultured fibroblasts promotes S-phase entry and shortens the G₁ phase of the cell cycle, while activation of a conditional Myc is sufficient to drive quiescent cells into the cell cycle (128, 129). An association between c-myc downregulation and IFN-α-mediated G₀/G₁ arrest in Daudi cells was demonstrated (130) and it was shown that IFN-α-induced G₀/G₁ arrest correlated with upregulation of cyclin-dependent kinase inhibitors (CDKI), such as p21 and p15 early in this process and p27 in the late stage of growth arrest (131). Type I IFN treatment of Daudi cells induced p21 expression and G₁ arrest and these events were preceded by a strong reduction in c-myc levels (132). Myc can directly suppress the transcription of p21 and p27 and promote the ubiquitination of phosphorylated p27 (133-135). Our previous experiments confirm that overexpression of hPNPase^{old-35} downregulates c-myc and upregulates p27 (102) and in the present study we document that inhibition of hPNPase^{old-35} as well as overexpression of c-myc protects melanoma cells from IFN-β-mediated G₁ arrest. These findings firmly establish functional and mechanistic links between hPNPase^{old-35} induction by IFN-β, c-myc mRNA degradation by hPNPase^{old-35} and IFN-β-induced cell cycle arrest and eventual apoptosis (FIG. 26).

[0221] What is the practical significance of our observations? Myc is overexpressed in multiple tumor subtypes, including melanomas (127). The expression level of Myc inversely correlates with patient survival and thus may be used as a prognostic marker in cutaneous, subungual, acral lentiginous, scalp, head and neck melanomas (136-141). Indeed, antisense inhibition of c-myc significantly inhibited the growth of melanoma cells in *in vitro* cultures (142) and improved the response to chemotherapy in human melanoma xenografts in nude mice (143). Type I IFNs have been used as adjuvant therapy for malignant melanoma with significant but limited success and high toxicity (144). Experimental overexpression of Myc in mouse fibroblasts and myeloblastic cells renders these cells resistant to cell cycle arrest by type I IFNs (145, 146). Uveal melanomas with high c-myc expression are also associated with IFN-α resistance (147). In these contexts and based on the poor survival of patients with malignant melanoma, improved therapies are mandated and cancer cell specific expression of hPNPase^{old-35}, by means of the telomerase or progression elevated gene-3 promoter (148, 149), might prove beneficial as an innovative adjuvant therapeutic approach that exploits the ability of hPNPase^{old-35} to degrade c-myc mRNA, thus inducing target cancer cell-specific growth arrest culminating in apoptosis.

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tgggaggccg tggcggttgg attgcctgag gtcaggagtt caagaccaac ctggccaaca	1380
tgatgaaacc ccatctctac taaaaatcca aaaaattccc cggggtttgt gggcagccct	1440
tttactccca actttggagg ggggctgagg gggggaaatt tgcatcccc ccggacgccc	1500
gtgttccagt gagctgagat cacgccactg cactctagcc tggcaacaa gagcaaaact	1560
ctgtctgaa aaaaacgaaa aggctggatg aaagcttaat ttaccattta ttgagtgtt	1620
acaatgtgct aggtactatt atatcatctg acactttttt tttttttttt tttttttaga	1680
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ctgcaagctc cggttcccg gttcacgcca ttctctgccc tcagcctccc gagtagctgg	1800
gactacaggo gccccctacc gcgcgggct aattttttgt attttttagta gagacgggg	1860
ttcacccctgt tagccaggat ggtctcaatc tcctgacctc atgatccacc cgcctcgcc	1920
tcccaaagtg ctaggattac aggccgtgagc caccgcgccc ggccgacact tttaaatcctt	1980
acaaaaaccc tattagattc atattactat ctgttttat ggggaaactg agactcagag	2040
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taattttttt tttttttttt tttttttttt gatggagtct cggctggag tgcagtggcg	2580
cgatctcgcc tcactgcaac ctccgcctcc tggattcaag cgattctcat gcctcagecct	2640
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cccgccctcg cctcccgccg tgctgggatt acaggcatga gccactgcgc ccggactttt	2820
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cctcgccctc ccaaagtgtct gggattacag ggctgagcca cagcgccag ccaacgtttt	3120
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taaggccgtt atcaccttac acaaaaaatt tacatcataa tatgaaaatg cccattgggtt	3240
cattcaagga aaaacataaa gaatttctaa cagaatggat taatggggtg gaagaaattt	3300
atccattctc taaaaatttt tcaaatccca aaaaactttt tttttcattt tacaaaatata	3360
ttacttc当地 cagtagctgg cattttgc当地 ttaagagttc gaccaaaagg gacagaaata	3420
taaatttgctaa attattagca atggaaatga aaatgaatca gaagtgc当地 taaagatttca	3480

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taaaaattaca agtcgatgaa aaatgaattt actgtaaatt ggttaacaca aatcgctgt	3540
gtaacatatac tgctatgtga aatttttaaa ataaaaacca gggcggagaa ggggagaagg	3600
agatctcttt ccaaggccca ccacccaaat tctaattctt agttcacaga tccaaataat	3660
aataatgtgt tggttatgta cttagtat taaaagtaaa tataaattta ctctgcata	3720
taaacattta cattattcta ggacttctcc tttaaattac gatagtacac acacaaaaac	3780
cttatttatac taattgcctt tcgttgcac ataaagacaa agatcgtttgc acacataatc	3840
gcgttctgat acgtttgacc ctctctagtc ttctgttagt gtggccaagg ttccgttac	3900
cccgccgtcc ctcgggcacc gcggaaacga aactccatca ggctccgcc cacggtctgc	3960
ggagtgagcc aatcagggca cagcctgcgt tgaccgcgtg ccgggtgtca tggccgcctg	4020
caggtaactgc tgctcggtcc tccggctccg gc	4052

<210> SEQ ID NO 2
<211> LENGTH: 410
<212> TYPE: DNA
<213> ORGANISM: Homo Sapien

<400> SEQUENCE: 2

aggcccacca cccaaattct aattcttagt tcacagatcc aaataataat aatgtgttgt	60
tctatgactt ctagtattaa aagtaaatat aaatttactc tgcataattaa acatttacat	120
tattcttagga ctctctttt aaattacat agtacacaca caaaaacctt atttatctaa	180
ttgccttgc ttggcacata aagacaaaga tcgttgcaa cataatcgcg ttctgatacg	240
tttgcacctc tctagtcttc gtagaagggtt gccaagggtt ccgttacccc ggctccctc	300
gggcaccgcg gaaacgaaac tccatcaggc tccgccccac ggtctgcggg gtgagccat	360
cagggcacag cctgcgttga ccgcgtgcgg ggtgtcatgg cggcctgcag	410

<210> SEQ ID NO 3
<211> LENGTH: 9
<212> TYPE: DNA
<213> ORGANISM: Homo Sapien
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (5)...(0)
<223> OTHER INFORMATION: N = any nucleotide

<400> SEQUENCE: 3

gaaangaaa	9
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<210> SEQ ID NO 4
<211> LENGTH: 9
<212> TYPE: DNA
<213> ORGANISM: Homo Sapien

<400> SEQUENCE: 4

gaaacgaaa	9
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<210> SEQ ID NO 5
<211> LENGTH: 2615
<212> TYPE: DNA
<213> ORGANISM: Homo Sapien

<400> SEQUENCE: 5

gggtgccggg tgtcatggcg gcctgcaggt actgctgctc gtgcctccgg ctccggcccc	60
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tgagegatgg	tccttcctt	ctgccacggc	gggatcgccc	actcacccag	ttgcaagtgc	120
gagcactatg	gagtagcgca	gggtctcgag	ctgtggccgt	ggacttaggc	aacagggaaat	180
tagaaatatc	ttctggaaag	ctggccagat	ttgcagatgg	ctctgctgta	gtacagtca	240
gtgacactgc	agtaatggtc	acagcggtca	gtaaaacaaa	acttcccc	tcccagttt	300
tgcccttggt	ggttgactac	agacaaaaag	ctgctgcago	aggtagaatt	cccacaaaact	360
atctgagaag	agaggttgtt	acttctgata	aagaaattct	aacaagtcga	ataatagatc	420
gttcaattag	accgctctt	ccagctggct	acttctatga	tacacagggt	ctgtgtaatc	480
tgttagcagt	agatggtgta	aatgagcctg	atgtccttagc	aattaatggc	gcttccgtag	540
ccctctcatt	atcagatatt	ccttggatg	gacctgttgg	ggcagtagcga	ataggaataa	600
ttgatggaga	atatgttgtt	aacccaacaa	aaaaagaaaat	gtcttctagt	actttaaatt	660
tagtggttgc	tggagcacct	aaaagtca	ttgtcatgtt	ggaaggctct	gcagagaaca	720
ttttacagca	ggactttgc	catgctatca	aagtggaggt	gaaatatacc	caacaaataa	780
ttcagggcat	tcagcagttg	gtaaaagaaa	ctgggtttac	caagaggaca	cctcagaagt	840
tatTTACCCC	ttcggccagag	attgtgaaat	atactcataa	acttgctatg	gagagactct	900
atgcagttt	taagattac	gagcatgaca	aagttccag	agatgaagct	gttaacaaaa	960
taagattaga	tacgaggaa	caactaaaag	aaaaatttcc	agaagccat	ccatatgaaa	1020
taatagaatc	cttcaatgtt	gttcaaagg	aagtttttag	aagtattgtt	ttgaatgaat	1080
acaaaagggt	cgatggtcgg	gatttgaett	cacttaggaa	tgtaagttgt	gaggtagata	1140
tgtttaaaac	ccttcatgga	tcagcattat	ttcaaagagg	acaaacacag	gtgctttgt	1200
ccgttacatt	tgattcatta	gaatctggta	ttaagtcaga	tcaagttata	acagctataa	1260
atgggataaa	agataaaaaat	ttcatgctgc	actacgagtt	tcctccttat	gcaactaatg	1320
aaattggcaa	agtcactgg	ttaaatagaa	gagaacttgg	gcatgggtct	cttgctgaga	1380
aagctttgt	tcctgttatt	cccagagatt	ttccttcac	cataagagtt	acatctgaag	1440
tccttagagtc	aaatgggtca	tcttcatgg	catctgcatt	tggcggaaagt	tttagcattaa	1500
tggattcagg	ggttccaatt	tcatctgctg	ttgcaggcgt	agcaatagga	ttggtcacca	1560
aaaccgatcc	tgagaagggt	gaaatagaag	attatcgtt	gctgacagat	atttggaa	1620
ttgaagatta	caatgggtac	atggacttca	aaatagctgg	cactaataaa	ggaataactg	1680
cattacaggc	tgatattaaa	ttacctggaa	taccaataaa	aattgtgtatg	gaggctattc	1740
aacaagcttc	agtggcaaaa	aaggagatat	tacagatcat	gaacaaaact	atttcaaaac	1800
ctcgagcatc	tagaaaagaa	aatggacctg	ttgttagaaac	tgttcaggtt	ccattatcaa	1860
aacgagcaaa	atttggtgg	cctggggct	ataacttaaa	aaaacttcag	gctgaaacag	1920
gtgttaactat	tagtcagg	gatgaagaaa	cgtttctgt	atttgcacca	acacccagt	1980
ttatgcatt	ggcaagagac	ttcattactg	aaatctgcaa	ggatgtatc	gagcagcaat	2040
tagaatttgg	agcagtatat	accggccacaa	taactgaaat	cagagatact	ggtgtatgg	2100
taaaaattata	tccaaatatg	actgcggta	tgcttcataa	cacacaactt	gatcaacgaa	2160
agattnaaca	tcctactgcc	ctaggattag	aagttggca	agaaattcag	gtgaaatact	2220
ttggacgtga	cccagccat	ggaagaatga	ggcttctcg	aaaagtgcct	cagtcgcccag	2280
ctacaaccgt	ggtcagaact	ttgaatgaca	gaagtagtat	tgtatggg	gaacctattt	2340

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cacagtcatc atctaattct cagtgatttt tttttttaa agagaattct agaattctat	2400
tttgtctagg gtgatgtgct gtagagcaac attttagtag atcttccatt gtgttagattt	2460
ctatataata taaatacatt ttaatttattt gtactaaaat gctcattac atgtgccatt	2520
tttttaattc gagtaaccca tatttgttta attgtattt cattataaat caagaaatat	2580
tttattttaa aagtaagtca tttatacato ttaga	2615

<210> SEQ ID NO 6
<211> LENGTH: 783
<212> TYPE: PRT
<213> ORGANISM: Homo Sapien

<400> SEQUENCE: 6

Met Ala Ala Cys Arg Tyr Cys Cys Ser Cys Leu Arg Leu Arg Pro Leu	
1 5 10 15	
Ser Asp Gly Pro Phe Leu Leu Pro Arg Arg Asp Arg Ala Leu Thr Gln	
20 25 30	
Leu Gln Val Arg Ala Leu Trp Ser Ser Ala Gly Ser Arg Ala Val Ala	
35 40 45	
Val Asp Leu Gly Asn Arg Lys Leu Glu Ile Ser Ser Gly Lys Leu Ala	
50 55 60	
Arg Phe Ala Asp Gly Ser Ala Val Val Gln Ser Gly Asp Thr Ala Val	
65 70 75 80	
Met Val Thr Ala Val Ser Lys Thr Lys Pro Ser Pro Ser Gln Phe Met	
85 90 95	
Pro Leu Val Val Asp Tyr Arg Gln Lys Ala Ala Ala Gly Arg Ile	
100 105 110	
Pro Thr Asn Tyr Leu Arg Arg Glu Val Gly Thr Ser Asp Lys Glu Ile	
115 120 125	
Leu Thr Ser Arg Ile Ile Asp Arg Ser Ile Arg Pro Leu Phe Pro Ala	
130 135 140	
Gly Tyr Phe Tyr Asp Thr Gln Val Leu Cys Asn Leu Leu Ala Val Asp	
145 150 155 160	
Gly Val Asn Glu Pro Asp Val Leu Ala Ile Asn Gly Ala Ser Val Ala	
165 170 175	
Leu Ser Leu Ser Asp Ile Pro Trp Asn Gly Pro Val Gly Ala Val Arg	
180 185 190	
Ile Gly Ile Ile Asp Gly Glu Tyr Val Val Asn Pro Thr Arg Lys Glu	
195 200 205	
Met Ser Ser Ser Thr Leu Asn Leu Val Val Ala Gly Ala Pro Lys Ser	
210 215 220	
Gln Ile Val Met Leu Glu Ala Ser Ala Glu Asn Ile Leu Gln Gln Asp	
225 230 235 240	
Phe Cys His Ala Ile Lys Val Gly Val Lys Tyr Thr Gln Gln Ile Ile	
245 250 255	
Gln Gly Ile Gln Gln Leu Val Lys Glu Thr Gly Val Thr Lys Arg Thr	
260 265 270	
Pro Gln Lys Leu Phe Thr Pro Ser Pro Glu Ile Val Lys Tyr Thr His	
275 280 285	
Lys Leu Ala Met Glu Arg Leu Tyr Ala Val Phe Thr Asp Tyr Glu His	
290 295 300	

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Asp Lys Val Ser Arg Asp Glu Ala Val Asn Lys Ile Arg Leu Asp Thr
 305 310 315 320
 Glu Glu Gln Leu Lys Glu Lys Phe Pro Glu Ala Asp Pro Tyr Glu Ile
 325 330 335
 Ile Glu Ser Phe Asn Val Val Ala Lys Glu Val Phe Arg Ser Ile Val
 340 345 350
 Leu Asn Glu Tyr Lys Arg Cys Asp Gly Arg Asp Leu Thr Ser Leu Arg
 355 360 365
 Asn Val Ser Cys Glu Val Asp Met Phe Lys Thr Leu His Gly Ser Ala
 370 375 380
 Leu Phe Gln Arg Gly Gln Thr Gln Val Leu Cys Thr Val Thr Phe Asp
 385 390 395 400
 Ser Leu Glu Ser Gly Ile Lys Ser Asp Gln Val Ile Thr Ala Ile Asn
 405 410 415
 Gly Ile Lys Asp Lys Asn Phe Met Leu His Tyr Glu Phe Pro Pro Tyr
 420 425 430
 Ala Thr Asn Glu Ile Gly Lys Val Thr Gly Leu Asn Arg Arg Glu Leu
 435 440 445
 Gly His Gly Ala Leu Ala Glu Lys Ala Leu Tyr Pro Val Ile Pro Arg
 450 455 460
 Asp Phe Pro Phe Thr Ile Arg Val Thr Ser Glu Val Leu Glu Ser Asn
 465 470 475 480
 Gly Ser Ser Ser Met Ala Ser Ala Cys Gly Gly Ser Leu Ala Leu Met
 485 490 495
 Asp Ser Gly Val Pro Ile Ser Ser Ala Val Ala Gly Val Ala Ile Gly
 500 505 510
 Leu Val Thr Lys Thr Asp Pro Glu Lys Gly Glu Ile Glu Asp Tyr Arg
 515 520 525
 Leu Leu Thr Asp Ile Leu Gly Ile Glu Asp Tyr Asn Gly Asp Met Asp
 530 535 540
 Phe Lys Ile Ala Gly Thr Asn Lys Gly Ile Thr Ala Leu Gln Ala Asp
 545 550 555 560
 Ile Lys Leu Pro Gly Ile Pro Ile Lys Ile Val Met Glu Ala Ile Gln
 565 570 575
 Gln Ala Ser Val Ala Lys Lys Glu Ile Leu Gln Ile Met Asn Lys Thr
 580 585 590
 Ile Ser Lys Pro Arg Ala Ser Arg Lys Glu Asn Gly Pro Val Val Glu
 595 600 605
 Thr Val Gln Val Pro Leu Ser Lys Arg Ala Lys Phe Val Gly Pro Gly
 610 615 620
 Gly Tyr Asn Leu Lys Lys Leu Gln Ala Glu Thr Gly Val Thr Ile Ser
 625 630 635 640
 Gln Val Asp Glu Glu Thr Phe Ser Val Phe Ala Pro Thr Pro Ser Val
 645 650 655
 Met His Glu Ala Arg Asp Phe Ile Thr Glu Ile Cys Lys Asp Asp Gln
 660 665 670
 Glu Gln Gln Leu Glu Phe Gly Ala Val Tyr Thr Ala Thr Ile Thr Glu
 675 680 685
 Ile Arg Asp Thr Gly Val Met Val Lys Leu Tyr Pro Asn Met Thr Ala
 690 695 700
 Val Leu Leu His Asn Thr Gln Leu Asp Gln Arg Lys Ile Lys His Pro

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705 710 715 720
Thr Ala Leu Gly Leu Glu Val Gly Gln Glu Ile Gln Val Lys Tyr Phe
725 730 735

Gly Arg Asp Pro Ala Asp Gly Arg Met Arg Leu Ser Arg Lys Val Leu
740 745 750

Gln Ser Pro Ala Thr Thr Val Val Arg Thr Leu Asn Asp Arg Ser Ser
755 760 765

Ile Val Met Gly Glu Pro Ile Ser Gln Ser Ser Ser Asn Ser Gln
770 775 780

<210> SEQ ID NO 7
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo Sapien

<400> SEQUENCE: 7

agttgagggg actttcccg gc 22

<210> SEQ ID NO 8
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo Sapien

<400> SEQUENCE: 8

agttgaggcg actttcccg gc 22

<210> SEQ ID NO 9
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Homo Sapien

<400> SEQUENCE: 9

ccacacagac agccactcac c 21

<210> SEQ ID NO 10
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Homo Sapien

<400> SEQUENCE: 10

tggcatttgt gggtgggtca g 21

<210> SEQ ID NO 11
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Homo Sapien

<400> SEQUENCE: 11

ggtgcatggg gttgtggaga a 21

<210> SEQ ID NO 12
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Homo Sapien

<400> SEQUENCE: 12

gcagactagg gttgccagat t 21

<210> SEQ ID NO 13

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<211> LENGTH: 24

<212> TYPE: DNA

<213> ORGANISM: Homo Sapien

<400> SEQUENCE: 13

atgggaaagg tgaaggctcg agtc

24

<210> SEQ ID NO 14

<211> LENGTH: 24

<212> TYPE: DNA

<213> ORGANISM: Homo Sapien

<400> SEQUENCE: 14

gctgatgtac ttgaggctgt tgtc

24

<210> SEQ ID NO 15

<211> LENGTH: 132

<212> TYPE: PRT

<213> ORGANISM: Homo Sapien

<400> SEQUENCE: 15

Gly Asn Arg Lys Leu Glu Ile Ser Ser Gly Lys Leu Ala Arg Phe Ala
1 5 10 15

Asp Gly Ser Ala Val Val Gln Ser Gly Asp Thr Ala Val Met Val Thr
20 25 30

Ala Val Ser Lys Thr Lys Pro Ser Pro Ser Gln Phe Met Pro Leu Val
35 40 45

Val Asp Tyr Arg Gln Lys Ala Ala Ala Ala Gly Arg Ile Pro Thr Asn
50 55 60

Tyr Leu Arg Arg Glu Val Gly Thr Ser Asp Lys Glu Ile Leu Thr Ser
65 70 75 80

Arg Ile Ile Asp Arg Ser Ile Arg Pro Leu Phe Pro Ala Gly Tyr Phe
85 90 95

Tyr Asp Thr Gln Val Leu Cys Asn Leu Leu Ala Val Asp Gly Val Asn
100 105 110

Glu Pro Asp Val Leu Ala Ile Asn Gly Ala Ser Val Ala Leu Ser Leu
115 120 125

Ser Asp Ile Pro
130

<210> SEQ ID NO 16

<211> LENGTH: 136

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 16

Ser Leu Arg Asn Val Ser Cys Glu Val Asp Met Phe Lys Thr Leu His
1 5 10 15

Gly Ser Ala Leu Phe Gln Arg Gly Gln Thr Gln Val Leu Cys Thr Val
20 25 30

Thr Phe Asp Ser Leu Glu Ser Gly Ile Lys Ser Asp Gln Val Ile Thr
35 40 45

Ala Ile Asn Gly Ile Lys Asp Lys Asn Phe Met Leu His Tyr Glu Phe
50 55 60

Pro Pro Tyr Ala Thr Asn Glu Ile Gly Lys Val Thr Gly Leu Asn Arg
65 70 75 80

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Arg Glu Leu Gly His Gly Ala Leu Ala Glu Lys Ala Leu Tyr Pro Val
85 90 95

Ile Pro Arg Asp Phe Pro Phe Thr Ile Arg Val Thr Ser Glu Val Leu
100 105 110

Glu Ser Asn Gly Ser Ser Ser Met Ala Ser Ala Cys Gly Gly Ser Leu
115 120 125

Ala Leu Met Asp Ser Gly Val Pro
130 135

<210> SEQ ID NO 17

<211> LENGTH: 601

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 17

Pro Trp Asn Gly Pro Val Gly Ala Val Arg Ile Gly Ile Ile Asp Gly
1 5 10 15

Glu Tyr Val Val Asn Pro Thr Arg Lys Glu Met Ser Ser Ser Thr Leu
20 25 30

Asn Leu Val Val Ala Gly Ala Pro Lys Ser Gln Ile Val Met Leu Glu
35 40 45

Ala Ser Ala Glu Asn Ile Leu Gln Gln Asp Phe Cys His Ala Ile Lys
50 55 60

Val Gly Val Lys Tyr Thr Gln Gln Ile Ile Gln Gly Ile Gln Gln Leu
65 70 75 80

Val Lys Glu Thr Gly Val Thr Lys Arg Thr Pro Gln Lys Leu Phe Thr
85 90 95

Pro Ser Pro Glu Ile Val Lys Tyr Thr His Lys Leu Ala Met Glu Arg
100 105 110

Leu Tyr Ala Val Phe Thr Asp Tyr Glu His Asp Lys Val Ser Arg Asp
115 120 125

Glu Ala Val Asn Lys Ile Arg Leu Asp Thr Glu Glu Gln Leu Lys Glu
130 135 140

Lys Phe Pro Glu Ala Asp Pro Tyr Glu Ile Ile Glu Ser Phe Asn Val
145 150 155 160

Val Ala Lys Glu Val Phe Arg Ser Ile Val Leu Asn Glu Tyr Lys Arg
165 170 175

Cys Asp Gly Arg Asp Leu Thr Ser Leu Arg Asn Val Ser Cys Glu Val
180 185 190

Asp Met Phe Lys Thr Leu His Gly Ser Ala Leu Phe Gln Arg Gly Gln
195 200 205

Thr Gln Val Leu Cys Thr Val Thr Phe Asp Ser Leu Glu Ser Gly Ile
210 215 220

Lys Ser Asp Gln Val Ile Thr Ala Ile Asn Gly Ile Lys Asp Lys Asn
225 230 235 240

Phe Met Leu His Tyr Glu Phe Pro Pro Tyr Ala Thr Asn Glu Ile Gly
245 250 255

Lys Val Thr Gly Leu Asn Arg Arg Glu Leu Gly His Gly Ala Leu Ala
260 265 270

Glu Lys Ala Leu Tyr Pro Val Ile Pro Arg Asp Phe Pro Phe Thr Ile
275 280 285

Arg Val Thr Ser Glu Val Leu Glu Ser Asn Gly Ser Ser Ser Met Ala
290 295 300

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Ser Ala Cys Gly Gly Ser Leu Ala Leu Met Asp Ser Gly Val Pro Ile
305 310 315 320

Ser Ser Ala Val Ala Gly Val Ala Ile Gly Leu Val Thr Lys Thr Asp
325 330 335

Pro Glu Lys Gly Glu Ile Glu Asp Tyr Arg Leu Leu Thr Asp Ile Leu
340 345 350

Gly Ile Glu Asp Tyr Asn Gly Asp Met Asp Phe Lys Ile Ala Gly Thr
355 360 365

Asn Lys Gly Ile Thr Ala Leu Gln Ala Asp Ile Lys Leu Pro Gly Ile
370 375 380

Pro Ile Lys Ile Val Met Glu Ala Ile Gln Gln Ala Ser Val Ala Lys
385 390 395 400

Lys Glu Ile Leu Gln Ile Met Asn Lys Thr Ile Ser Lys Pro Arg Ala
405 410 415

Ser Arg Lys Glu Asn Gly Pro Val Val Glu Thr Val Gln Val Pro Leu
420 425 430

Ser Lys Arg Ala Lys Phe Val Gly Pro Gly Gly Tyr Asn Leu Lys Lys
435 440 445

Leu Gln Ala Glu Thr Gly Val Thr Ile Ser Gln Val Asp Glu Glu Thr
450 455 460

Phe Ser Val Phe Ala Pro Thr Pro Ser Val Met His Glu Ala Arg Asp
465 470 475 480

Phe Ile Thr Glu Ile Cys Lys Asp Asp Gln Glu Gln Gln Leu Glu Phe
485 490 495

Gly Ala Val Tyr Thr Ala Thr Ile Thr Glu Ile Arg Asp Thr Gly Val
500 505 510

Met Val Lys Leu Tyr Pro Asn Met Thr Ala Val Leu Leu His Asn Thr
515 520 525

Gln Leu Asp Gln Arg Lys Ile Lys His Pro Thr Ala Leu Gly Leu Glu
530 535 540

Val Gly Gln Glu Ile Gln Val Lys Tyr Phe Gly Arg Asp Pro Ala Asp
545 550 555 560

Gly Arg Met Arg Leu Ser Arg Lys Val Leu Gln Ser Pro Ala Thr Thr
565 570 575

Val Val Arg Thr Leu Asn Asp Arg Ser Ser Ile Val Met Gly Glu Pro
580 585 590

Ile Ser Gln Ser Ser Ser Asn Ser Gln
595 600

<210> SEQ ID NO 18
<211> LENGTH: 490
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 18

Met Ala Ala Cys Arg Tyr Cys Cys Ser Cys Leu Arg Leu Arg Pro Leu
1 5 10 15

Ser Asp Gly Pro Phe Leu Leu Pro Arg Arg Asp Arg Ala Leu Thr Gln
20 25 30

Leu Gln Val Arg Ala Leu Trp Ser Ser Ala Gly Ser Arg Ala Val Ala
35 40 45

Val Asp Leu Gly Asn Arg Lys Leu Glu Ile Ser Ser Gly Lys Leu Ala

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50	55	60
Arg Phe Ala Asp Gly Ser Ala Val Val Gln Ser Gly Asp Thr Ala Val		
65	70	75
Met Val Thr Ala Val Ser Lys Thr Lys Pro Ser Pro Ser Gln Phe Met		
85	90	95
Pro Leu Val Val Asp Tyr Arg Gln Lys Ala Ala Ala Ala Gly Arg Ile		
100	105	110
Pro Thr Asn Tyr Leu Arg Arg Glu Val Gly Thr Ser Asp Lys Glu Ile		
115	120	125
Leu Thr Ser Arg Ile Ile Asp Arg Ser Ile Arg Pro Leu Phe Pro Ala		
130	135	140
Gly Tyr Phe Tyr Asp Thr Gln Val Leu Cys Asn Leu Leu Ala Val Asp		
145	150	155
Gly Val Asn Glu Pro Asp Val Leu Ala Ile Asn Gly Ala Ser Val Ala		
165	170	175
Leu Ser Leu Ser Asp Ile Pro Trp Asn Gly Pro Val Gly Ala Val Arg		
180	185	190
Ile Gly Ile Ile Asp Gly Glu Tyr Val Val Met Asp Ser Gly Val Pro		
195	200	205
Ile Ser Ser Ala Val Ala Gly Val Ala Ile Gly Leu Val Thr Lys Thr		
210	215	220
Asp Pro Glu Lys Gly Glu Ile Glu Asp Tyr Arg Leu Leu Thr Asp Ile		
225	230	235
Leu Gly Ile Glu Asp Tyr Asn Gly Asp Met Asp Phe Lys Ile Ala Gly		
245	250	255
Thr Asn Lys Gly Ile Thr Ala Leu Gln Ala Asp Ile Lys Leu Pro Gly		
260	265	270
Ile Pro Ile Lys Ile Val Met Glu Ala Ile Gln Gln Ala Ser Val Ala		
275	280	285
Lys Lys Glu Ile Leu Gln Ile Met Asn Lys Thr Ile Ser Lys Pro Arg		
290	295	300
Ala Ser Arg Lys Glu Asn Gly Pro Val Val Glu Thr Val Gln Val Pro		
305	310	315
Leu Ser Lys Arg Ala Lys Phe Val Gly Pro Gly Gly Tyr Asn Leu Lys		
325	330	335
Lys Leu Gln Ala Glu Thr Gly Val Thr Ile Ser Gln Val Asp Glu Glu		
340	345	350
Thr Phe Ser Val Phe Ala Pro Thr Pro Ser Val Met His Glu Ala Arg		
355	360	365
Asp Phe Ile Thr Glu Ile Cys Lys Asp Asp Gln Glu Gln Gln Leu Glu		
370	375	380
Phe Gly Ala Val Tyr Thr Ala Thr Ile Thr Glu Ile Arg Asp Thr Gly		
385	390	395
Val Met Val Lys Leu Tyr Pro Asn Met Thr Ala Val Leu Leu His Asn		
405	410	415
Thr Gln Leu Asp Gln Arg Lys Ile Lys His Pro Thr Ala Leu Gly Leu		
420	425	430
Glu Val Gly Gln Glu Ile Gln Val Lys Tyr Phe Gly Arg Asp Pro Ala		
435	440	445
Asp Gly Arg Met Arg Leu Ser Arg Lys Val Leu Gln Ser Pro Ala Thr		
450	455	460

-continued

Thr Val Val Arg Thr Leu Asn Asp Arg Ser Ser Ile Val Met Gly Glu
465 470 475 480

Pro Ile Ser Gln Ser Ser Ser Asn Ser Gln
485 490

<210> SEQ ID NO 19
<211> LENGTH: 507
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 19

Met Ala Ala Cys Arg Tyr Cys Cys Ser Cys Leu Arg Leu Arg Pro Leu
1 5 10 15

Ser Asp Gly Pro Phe Leu Leu Pro Arg Arg Asp Arg Ala Leu Thr Gln
20 25 30

Leu Gln Val Arg Ala Leu Trp Ser Ser Ala Gly Ser Arg Ala Val Ala
35 40 45

Val Asp Leu Gly Asn Arg Lys Leu Glu Ile Ser Ser Gly Lys Leu Ala
50 55 60

Arg Phe Ala Asp Gly Ser Ala Val Val Gln Ser Gly Asp Thr Ala Val
65 70 75 80

Met Val Thr Ala Val Ser Lys Thr Lys Pro Ser Pro Ser Gln Phe Met
85 90 95

Pro Leu Val Val Asp Tyr Arg Gln Lys Ala Ala Ala Ala Gly Arg Ile
100 105 110

Pro Thr Asn Tyr Leu Arg Arg Glu Val Gly Thr Ser Asp Lys Glu Ile
115 120 125

Leu Thr Ser Arg Ile Ile Asp Arg Ser Ile Arg Pro Leu Phe Pro Ala
130 135 140

Gly Tyr Phe Tyr Asp Thr Gln Val Leu Cys Asn Leu Leu Ala Val Asp
145 150 155 160

Gly Val Asn Glu Pro Asp Val Leu Ala Ile Asn Gly Ala Ser Val Ala
165 170 175

Leu Ser Leu Ser Asp Ile Pro Trp Asn Gly Pro Val Gly Ala Val Arg
180 185 190

Ile Gly Ile Ile Asp Gly Glu Tyr Val Val Asn Pro Thr Arg Lys Glu
195 200 205

Met Ser Ser Ser Thr Leu Asn Leu Val Val Ala Gly Ala Pro Lys Ser
210 215 220

Gln Ile Val Met Leu Glu Ala Ser Ala Glu Asn Ile Leu Gln Gln Asp
225 230 235 240

Phe Cys His Ala Ile Lys Val Gly Val Lys Tyr Thr Gln Gln Ile Ile
245 250 255

Gln Gly Ile Gln Gln Leu Val Lys Glu Thr Gly Val Thr Lys Arg Thr
260 265 270

Pro Gln Lys Leu Phe Thr Pro Ser Pro Glu Ile Val Lys Tyr Thr His
275 280 285

Lys Leu Ala Met Glu Arg Leu Tyr Ala Val Phe Thr Asp Tyr Glu His
290 295 300

Asp Lys Val Ser Arg Asp Glu Ala Val Asn Lys Ile Arg Leu Asp Thr
305 310 315 320

Glu Glu Gln Leu Lys Glu Phe Pro Glu Ala Asp Pro Tyr Glu Ile

-continued

325	330	335	
Ile Glu Ser Phe Asn Val Val Ala Lys Glu Val Phe Arg Ser Ile Val			
340	345	350	
Leu Asn Glu Tyr Lys Arg Cys Asp Gly Arg Asp Leu Thr Ser Leu Arg			
355	360	365	
Asn Val Ser Cys Glu Val Asp Met Phe Lys Thr Leu His Gly Ser Ala			
370	375	380	
Leu Phe Gln Arg Gly Gln Thr Gln Val Leu Cys Thr Val Thr Phe Asp			
385	390	395	400
Ser Leu Glu Ser Gly Ile Lys Ser Asp Gln Val Ile Thr Ala Ile Asn			
405	410	415	
Gly Ile Lys Asp Lys Asn Phe Met Leu His Tyr Glu Phe Pro Pro Tyr			
420	425	430	
Ala Thr Asn Glu Ile Gly Lys Val Thr Gly Leu Asn Arg Arg Glu Leu			
435	440	445	
Gly His Gly Ala Leu Ala Glu Lys Ala Leu Tyr Pro Val Ile Pro Arg			
450	455	460	
Asp Phe Pro Phe Thr Ile Arg Val Thr Ser Glu Val Leu Glu Ser Asn			
465	470	475	480
Gly Ser Ser Ser Met Ala Ser Ala Cys Gly Gly Ser Leu Ala Leu Met			
485	490	495	
Asp Ser Gly Val Pro Ile Ser Ser Ala Val Ala			
500	505		

<210> SEQ ID NO 20

<211> LENGTH: 45

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 20

Met Ala Ala Cys Arg Tyr Cys Cys Ser Cys Leu Arg Leu Arg Pro Leu			
1	5	10	15
Ser Asp Gly Pro Phe Leu Leu Pro Arg Arg Asp Arg Ala Leu Thr Gln			
20	25	30	
Leu Gln Val Arg Ala Leu Trp Ser Ser Ala Gly Ser Arg			
35	40	45	

<210> SEQ ID NO 21

<211> LENGTH: 75

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 21

Lys Leu Ala Met Glu Arg Leu Tyr Ala Val Phe Thr Asp Tyr Glu His			
1	5	10	15
Asp Lys Val Ser Arg Asp Glu Ala Val Asn Lys Ile Arg Leu Asp Thr			
20	25	30	
Glu Glu Gln Leu Lys Glu Lys Phe Pro Glu Ala Asp Pro Tyr Glu Ile			
35	40	45	
Ile Glu Ser Phe Asn Val Val Ala Lys Glu Val Phe Arg Ser Ile Val			
50	55	60	
Leu Asn Glu Tyr Lys Arg Cys Asp Gly Arg Asp			
65	70	75	

-continued

<210> SEQ ID NO 22
<211> LENGTH: 63
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 22

```
Pro Val Val Glu Thr Val Gln Val Pro Leu Ser Lys Arg Ala Lys Phe
 1           5          10          15

Val Gly Pro Gly Gly Tyr Asn Leu Lys Lys Leu Gln Ala Glu Thr Gly
 20          25          30

Val Thr Ile Ser Gln Val Asp Glu Glu Thr Phe Ser Val Phe Ala Pro
 35          40          45

Thr Pro Ser Val Met His Glu Ala Arg Asp Phe Ile Thr Glu Ile
 50          55          60
```

<210> SEQ ID NO 23
<211> LENGTH: 75
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 23

```
Leu Glu Phe Gly Ala Val Tyr Thr Ala Thr Glu Ile Arg Asp
 1           5          10          15

Thr Gly Val Met Val Lys Leu Tyr Pro Asn Met Thr Ala Val Leu Leu
 20          25          30

His Asn Thr Gln Leu Asp Gln Arg Lys Ile Lys His Pro Thr Ala Leu
 35          40          45

Gly Leu Glu Val Gly Gln Glu Ile Gln Val Lys Tyr Phe Gly Arg Asp
 50          55          60

Pro Ala Asp Gly Arg Met Arg Leu Ser Arg Lys
 65          70          75
```

<210> SEQ ID NO 24
<211> LENGTH: 396
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 24

```
ggcaacagga aattagaaat atcttctgga aagctggcca gatttcaga tggctctgtc      60
gtatgtacgt caggtgacac tgcagtaatg gtcacagccg tcagtaaac aaaaccttcc      120
ccttcccagt ttatgccttt ggtgggttac tacagacaaa aagctgtgc agcaggtaga      180
atccccacaa actatctgag aagagaggaa ggtacttctg ataaagaaat tctaacaagt      240
cgaataatag atcggtcaat tagaccgctc tttccagctg gctacttcta tgatacacag      300
gttctgtgtt atctgttagc agtagatggt gttaatgagc ctgtatgtcc agcaattaat      360
ggcgcttcccg tagccctctc attatcagat attctc                                396
```

<210> SEQ ID NO 25
<211> LENGTH: 408
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 25

```
tcacttagga atgtaagttg tgaggttagat atgtttaaaa cccttcatgg atcagcattt      60
tttcaaagag gacaaacaca ggtgctttgt accgttacat ttgattcatt agaatctggt      120
```

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attaagtca	atcaagttat aacagctata aatggataa aagataaaaa ttcatgctg	180
cactacgagt	tccctccta tgcactaat gaaattggca aagtca	240
agagaacttg	ggcatggtgc tcttgctgaa agagcttgc atcctgttat tcccagat	300
tttccttca	ccataagagt tacatctgaa gtcctagat caaatgggtc atcttctatg	360
gcatctgcat	gtggcggaag ttttagcatta atggattca gggttcca	408
 <210> SEQ ID NO 26		
<211> LENGTH: 1803		
<212> TYPE: DNA		
<213> ORGANISM: Homo sapiens		
 <400> SEQUENCE: 26		
ccttggatg	gacctgttgg ggcagtaega ataggataa ttgatggaga atatgttgg	60
aacccaacaa	gaaaagaaat gtcttctagt actttaatt tagtgggtgc tggagcacct	120
aaaagtca	gatgtatgtt ggaaggctct gcagagaaca ttttacagca ggactttgc	180
catgctatca	aagtgggat gaaatatacc caacaataaa ttcagggcat tcagcagttg	240
gtaaaagaaa	ctgggttac caagaggaca ctcagaatg tatttacccc ttgcagag	300
atttgtaaat	atactcataa acttgctatg gagagactct atgcagttt tacagattac	360
gagcatgaca	aagtttccag agatgaagct gttacaaaa taagattaga tacggaggaa	420
caactaaaag	aaaaatttcc agaagccat ccatacgaaa taatagaatc cttcaatgtt	480
gttgcacagg	aagttttag aagtattgtt ttgaatgaat acaaaagggtg cgatggtcgg	540
gatttgactt	cacttagaa tgtaagttgt gaggttagata tgtttaaac cttcatgga	600
tcagcattat	ttcaaagagg acaaaccacag gtgcattgtt ccgttacatt tgattcatta	660
gaatctggta	ttaagtcaga tcaagttata acagctataa atggataaa agataaaaaat	720
ttcatgctgc	actacgagtt tcctccttat gcaactaatg aaattggcaa agtca	780
ttaaatagaa	gagaacttgg gcatgggtct cttgtgaga aagcttgc ttctgttatt	840
cccagagatt	ttcccttcac cataagagtt acatctgaa tccttagatc aaatgggtca	900
tcttcatgg	catctgcattt gggcgaaatg ttagcattaa tggatcagg ggttcaatt	960
tcatctgctg	ttgcaggcgt agcaatagga ttggtcacca aaaccgatcc tgagaagggt	1020
gaaatagaag	attatcgat gctgacagat attttggaa ttgaagatca aatgggtac	1080
atggacttca	aaatagctgg cactaataaa ggaataactg cattacaggc tgatattaaa	1140
ttacctggaa	taccaataaa aattgtgtatc gaggctattc aacaagttc agtggcaaaa	1200
aaggagat	atcagatcat gaacaaaact atttcaaaac ctgcagatc tagaaaagaa	1260
aatggacctg	ttgttagaaac tggtcaggat ccattatcaa aacgagcaaa atttggat	1320
cctggggct	ataacttaaa aaaacttcag gctgaaacag gtgtactat tagtcagg	1380
gatgaagaaa	cgtttctgt atttgcacca acaccagtg ttatgcattt ggcaagagac	1440
ttcattactg	aatatctgcaa ggtatgtatc gagcagcaat tagaattgg agcgtat	1500
accggcacaa	taactgaaat cagagatact ggtgtatgg taaaattata tccaaatatg	1560
actgcggta	tggtcataa cacacaactt gatcaacgaa agatcaaaca tcctactgc	1620
ctaggattag	aagtggca agaaaatcag gtgaaatact ttggacgtga cccagccat	1680
ggaagaatga	gggtttctcg aaaagtgcctt cagtcggcag ctacaaccgt ggta	1740

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ttgaatgaca gaagtagtat tgtaatggga gaacattt cacagtcatc atctaattct	1800
cag	1803

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<210> SEQ ID NO 27
<211> LENGTH: 1470
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

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<400> SEQUENCE: 27

atggcgccct gcaggtactg ctgctgtgc ctccggctcc ggccccctgag cgatggtcct	60
ttccttctgc cacggcgaaa tcgggactc acccagttgc aagtgcgagc actatggagt	120
agcgcagggt ctcgagctgt ggccgtggac ttaggcaaca gggaaattaga aatatcttct	180
ggaaagctgg ccagatttgc agatggctct gctgttagtac agtcaggtga cactgcagta	240
atggtcacag cggtcagtaa aacaaaacct tcccctccc agtttatgcc tttggtggtt	300
gactacagac aaaaagctgc tgcagcaggt agaattccca caaactatct gagaagagag	360
gttggtaactt ctgataaaga aattctaaca agtcaataa tagatcggtc aatttagaccg	420
ctcttccag ctggctactt ctatgataca caggttctgt gtaatctgtt agcagtagat	480
ggtgttaatg agcctgtatgt cctagcaatt aatggcgctt ccgtageccct ctcattatca	540
gatattccctt ggaatggacc tggggggca gtacgaatag gaataattga tggagaatat	600
gttggtaatgg attcagggggt tccaaatttca tctgtgttg caggcgtagc aataggattt	660
gtcacaaaaa ccgatcctga gaagggtgaa atagaagatt atcggttgct gacagatatt	720
ttggaaattt aagattacaa tggtgacatg gacttcaaaa tagctggcac taataaaggaa	780
ataactgcat tacaggctga tattaaatattt cctggataac caataaaaaat tggatggag	840
gctattcaac aagcttcagt ggcaaaaaag gagatattac agatcatgaa caaaactatt	900
tcaaaaacctc gaggatcttag aaaagaaaaat ggacctgttg tagaaactgt tcagggttcca	960
ttatcaaaaac gagcaaaatt tggggaccc ggtggctata actaaaaaaa acttcaggct	1020
gaaacagggtg taactattag tcagggtggat gaagaaacgt tttctgtatt tgccaccaaca	1080
cccagtgtta tgcgtggc aagagacttc attactgaaa tctgcaaggaa tgatcaggag	1140
cagcaatttag aattttggagc agtatatacc gccacaataa ctgaaatcag agatactgg	1200
gtaatggtaa aatttatatcc aaatatgact ggggtactgc ttccataaac acaaacttgat	1260
caacgaaaga tttaaacatcc tactggctta ggattagaag ttggcaaga aattcagggt	1320
aaatactttg gacgtgaccc agccgatgga agaatgaggc tttctcgaaa agtgcgttc	1380
tcggccagcta caaccgtggc cagaactttg aatgacagaa gtagtattgt aatgggagaa	1440
cctatttcac agtcatcatc taattctcag	1470

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<210> SEQ ID NO 28
<211> LENGTH: 1521
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

```

<400> SEQUENCE: 28

atggcgccct gcaggtactg ctgctgtgc ctccggctcc ggccccctgag cgatggtcct	60
ttccttctgc cacggcgaaa tcgggactc acccagttgc aagtgcgagc actatggagt	120
agcgcagggt ctcgagctgt ggccgtggac ttaggcaaca gggaaattaga aatatcttct	180

-continued

ggaaagctgg ccagattgc agatggctct gctgttagtac agtcagggtga cactgcgat	240
atggtcacag cggtcagtaa aacaaaacct tccccttccc agtttatgcc tttggtggtt	300
gactacagac aaaaagctgc tgcccgaggt agaattccca caaaactatct gagaagagag	360
gttggtactt ctgataaaga aattctaaca agtcaataa tagatcggtc aatttagaccg	420
ctcttccag ctggctactt ctatgataca caggttctgt gtaatctgtt agcagtagat	480
ggtgtaaatg agcctgtatgt cctagcaatt aatggcgctt ccgtagccct ctcattatca	540
gatattcctt ggaatggacc tggggggca gtacgaatag gaataattga tggagaatat	600
gttggtaacc caacaagaaa agaaaatgtct tctagttactt taaaattgtt gggtgctgga	660
gcacctaataa gtcagattgt catgttgaa gcctctgcag agaacatccc acaggcaggac	720
ttttgcattt ctatcaaagt gggagtggaaa tatacccaac aaataattca gggcatttcg	780
cagttggtaa aagaaactgg tggatccaag aggacacccctc agaagtttattt tacccttcg	840
ccagagattt tgaaatatac tcataaactt gctatggaga gactctatgc agtttttaca	900
gattacgagc atgacaaaatgttccagat gaagctgtt acaaaataag attagatacg	960
gaggaacaac taaaagaaaa atttccagaa gccgatccat atgaaataat agaatccctc	1020
aatgttggttt caaaggaaatgtttagaagt attgttttta atgaatacaa aagggtgcgt	1080
ggtcgggatt tgacttcact taggaatgtt agttgtgagg tagatatgtt taaaaccctt	1140
catggatcag cattatttca aagaggacaa acacaggtgc ttgttaccgt tacatttgc	1200
tcattagaat ctggattttaa gtcagatcaa gttataacag ctataatgg gataaaagat	1260
aaaaatttca tgctgcacta cgagtttccct ccttatgcaa ctaatgaaat tggcaaagtc	1320
actgggtttaa atagaagaga acttgggcattt ggtgttcttgc ctgagaaagc ttgttatcc	1380
gttattccca gagattttcc ttccaccata agagttacat ctgaagtccct agagtcaaat	1440
gggtcatctt ctatggcattt tgcatgtggc ggaagtttag cattaatggat ttcagggtt	1500
ccaatttcat ctgctgttgc a	1521

<210> SEQ ID NO 29

<211> LENGTH: 135

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 29

atggggcct gcaggtactg ctgtctgtc ctccggctcc ggccccctgag cgatggctt	60
ttccttctgc cacggcggga tcgggcactc acccagttgc aagtgcgagc actatggagt	120
agcgcagggt ctgcga	135

<210> SEQ ID NO 30

<211> LENGTH: 225

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 30

aaacttgcta tggagagact ctatgcgtt ttacagatt acgagcatga caaagtttc	60
agagatgaag ctgttaacaa aataagatta gatacgagg aacaactaaa agaaaaattt	120
ccagaagccg atccatatga aataatagaa tccttcaatgtt tttttttttt ggaagttttt	180
agaagtattt ttttgcattttt atacaaaagg tgcgtatggc gggat	225

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<210> SEQ ID NO 31
<211> LENGTH: 189
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 31
cctgtttag aaactgttca gttccatta tcaaaacgag caaaatttg tggacctggt      60
ggctataact taaaaaaaaact tcaggctgaa acagggtgtaa ctattagtca ggtggatgaa    120
gaaacgtttt ctgtatttgc accaacaccc agtgttatgc atgaggcaag agacttcatt     180
actgaaatc                                         189

<210> SEQ ID NO 32
<211> LENGTH: 225
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 32
ttagaatttg gaggcgtata taccgccaca ataactgaaa tcagagatac tgggtataatg      60
gtaaaaattat atccaaatat gactgcggta ctgcatttata acacacaact tgatcaacga    120
aagattaaac atcctactgc cctaggatta gaagttggcc aagaaattca ggtgaaatac     180
tttggacgtg acccagecgta tggagaatg aggcttctc gaaaa                         225

<210> SEQ ID NO 33
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Sense Primer

<400> SEQUENCE: 33
gctagcatgg cggcctgcag gtac                                         24

<210> SEQ ID NO 34
<211> LENGTH: 54
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic AntiSense Primer

<400> SEQUENCE: 34
ggatcctcaa gcgtaatctg gaacatcgta tgggtactga gaatttagatg atga        54

<210> SEQ ID NO 35
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Sense Primer

<400> SEQUENCE: 35
gctagcatgc ttggaaatgg acctgttggg                                         30

<210> SEQ ID NO 36
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Sense Primer
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<400> SEQUENCE: 36

gttaacatgg attcaggggt tcccaatt

27

<210> SEQ ID NO 37

<211> LENGTH: 27

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Synthetic Sense Primer

<400> SEQUENCE: 37

gctagcatgg attcaggggt tcccaatt

27

<210> SEQ ID NO 38

<211> LENGTH: 58

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Synthetic Primer

<400> SEQUENCE: 38

ggatcctcaa gcgtaatctg gaacatcgta tgggtactgc aacagcagat gaaattgg

58

<210> SEQ ID NO 39

<211> LENGTH: 9

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Synthetic C-terminal Hemagglutinin (HA)-epitope

<400> SEQUENCE: 39

Tyr Pro Tyr Asp Val Pro Asp Tyr Ala

1 5

<210> SEQ ID NO 40

<211> LENGTH: 21

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Synthetic Control Sequence

<400> SEQUENCE: 40

aacaaaacct tccccttccc a

21

<210> SEQ ID NO 41

<211> LENGTH: 23

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Synthetic Control Sequence

<400> SEQUENCE: 41

aagggtcgta tatagggatc gat

23

1-44. (canceled)

45. A method for identifying an agent that inhibits inflammation, comprising administering a test agent that is a putative anti-inflammatory agent to a system comprising an old-35 promoter element operatively linked to a reporter gene and determining whether the exposure to the test agent increases transcription of the reporter gene, wherein a decrease in transcription of the reporter gene indicates that the test agent inhibits inflammation.

46. The method of claim 45, wherein the old-35 promoter comprises a sequence as set forth in SEQ ID NO: 2.

47. The method of claim 45, wherein the old-35 promoter comprises a sequence as set forth in SEQ ID NO: 4.

48. The method of claim 45, wherein the reporter gene is selected from the group consisting of green fluorescent protein and luciferase.

49. A method for identifying an agent that inhibits inflammation, comprising administering a test agent to a cell comprising an old-35 gene operatively linked to a promoter element, wherein the old-35 gene is transcribed and expressed as OLD-35 protein, and determining whether the exposure to the test agent decreases the amount of reactive oxygen species in the cell.

50. The method of claim 49, wherein the OLD-35 protein has a sequence as set forth in SEQ ID NO: 6.

51. The method of claim 49, wherein the cell is a test cell into which an old-35 gene operatively linked to a promoter element has been introduced, and the amount of reactive oxygen species in the test cell exposed to the test agent is decreased relative to the amount of reactive oxygen species in a first control cell into which the old-35 gene operatively linked to the promoter has been introduced, but where the first control cell is not exposed to the test agent; wherein the amount of reactive oxygen species in the first control cell is greater than that in a second control cell which does not contain the old-35 gene operatively linked to the promoter element and which is not senescent or terminally differentiated.

52. The method of claim 51, wherein the OLD-35 protein has a sequence as set forth in SEQ ID NO: 6.

53. A kit for detecting inflammation in a subject, comprising a probe that binds to an old-35 gene product selected from the group consisting of old-35 mRNA and OLD-35 protein and a probe that binds to a cytokine selected from the group consisting of interleukin-6, interleukin-8, TNFR1, RANTES and MMP-3.

54. A method of inhibiting inflammation in a subject in need of such treatment, comprising administering, to the subject, an effective amount of an agent selected from the group consisting of an antibody that binds to OLD-35 protein, an old-35 RNA-i, an antisense old-35 nucleic acid, and a nucleic acid comprising an old-35 promoter element operatively linked to a gene that inhibits inflammation.

55. An anti-inflammatory composition, comprising an agent that antagonizes old-35 activity selected from the group consisting of an old-35 RNA-i, an old-35 antisense RNA, and an antibody directed toward OLD-35, and another anti-inflammatory agent.

56. An OLD-35 variant protein comprising one, but not two, RPH domain and having an activity selected from the group consisting of anti-proliferative activity, PNPase activity, RNA degradation activity, cell-cycle slowing activity, senescence-inducing activity, immunity-inducing activity, and a combination thereof.

57. The OLD-35 variant of claim 56 comprising amino acid residues 52-183 (SEQUENCE ID NO: 15) of native OLD-35 protein, or a sequence that is at least 90 percent, preferably at least 95 percent homologous to residues 52-183.

58. The OLD-35 variant of claim 56 comprising amino acid residues 366-501 (SEQUENCE ID NO: 16) of native OLD-35 protein, or a sequence that is at least 90 percent, preferably at least 95 percent homologous to residues 366-501.

59. The OLD-35 variant of claim 57, further comprising residues 289-363 (SEQUENCE ID NO: 21) of native OLD-35 protein, or a sequence that is at least 90 percent, preferably at least 95 percent homologous to residues 289-363.

60. The OLD-35 variant of claim 58, further comprising residues 289-363 (SEQUENCE ID NO: 21) of native OLD-35 protein, or a sequence that is at least 90 percent, preferably at least 95 percent homologous to residues 289-363.

61. A method for identifying an agent that inhibits inflammation, comprising administering a test agent to a cell comprising an old-35 gene operatively linked to a promoter element, wherein the old-35 gene is transcribed and expressed as OLDS 5 protein, and determining whether the exposure to the test agent decreases the amount of binding between NF-[kappa]B and its target sequence in the cell.

62. The method of claim 61, wherein the OLD-35 protein has a sequence as set forth in SEQ ID NO: 6.

63. The method of claim 61, wherein the cell is a test cell into which an old-35 gene operatively linked to a promoter element has been introduced, and the amount of binding between NF-KB and its target sequence in the test cell exposed to the test agent is decreased relative to the amount of binding between NF-[kappa]B and its target sequence in a first control cell into which the old-35 gene operatively linked to the promoter has been introduced, but where the first control cell is not exposed to the test agent; wherein the amount of binding between NF-[kappa]B and its target sequence in the first control cell is greater than that in a second control cell which does not contain the old-35 gene operatively linked to the promoter element and which is not senescent or terminally differentiated.

64. The method of claim 63, wherein the OLD-35 protein has a sequence as set forth in SEQ ID NO: 6.

65. A method for identifying an agent that inhibits inflammation, comprising administering a test agent to a cell comprising an old-35 gene operatively linked to a promoter element, wherein the old-35 gene is transcribed and expressed as OLD-35 protein, and determining whether the exposure to the test agent decreases the amount of translocation of a NF-[kappa]B protein from the cytoplasm into the nucleus of the cell.

66. The method of claim 65, wherein the OLD-35 protein has a sequence as set forth in SEQ ID NO: 6.

67. The method of claim 65 wherein the cell is a test cell into which an old-35 gene operatively linked to a promoter element has been introduced, and the amount of translocation of a NF-[kappa]B protein from the cytoplasm into the nucleus in the test cell exposed to the test agent is decreased relative to the amount of translocation of a NF-[kappa]B protein from the cytoplasm into the nucleus in a first control cell into which the old-35 gene operatively linked to the promoter has been introduced, but where the first control cell is not exposed to the test agent; wherein the amount of translocation of a NF-[kappa]B protein from the cytoplasm

into the nucleus in the first control cell is greater than that in a second control cell which does not contain the old-35 gene operatively linked to the promoter element and which is not senescent or terminally differentiated.

68. The method of claim 67, wherein the OLD-35 protein has a sequence as set forth in SEQ ID NO: 6.

69. A method for identifying an agent that inhibits inflammation, comprising administering a test agent to a cell comprising an old-35 gene operatively linked to a promoter element, wherein the old-35 gene is transcribed and expressed as OLD-35 protein, and determining whether the exposure to the test agent decreases the amount of a cytokine selected from the group consisting of interleukin-6, interleukin-8, TNFR1, RANTES and MMP-3 in the cell.

70. The method of claim 69, wherein the OLD-35 protein has a sequence as set forth in SEQ ID NO: 6.

71. The method of claim 69 wherein the cell is a test cell into which an old-35 gene operatively linked to a promoter element has been introduced, and the amount of a cytokine selected from the group consisting of interleukin-6, interleukin-8, TNFR1, RANTES and MMP-3 in the test cell exposed to the test agent is decreased relative to the amount of a cytokine selected from the group consisting of interleukin-6, interleukin-8, TNFR1, RANTES and MMP-3 in a first control cell into which the old-35 gene operatively linked to the promoter has been introduced, but where the first control cell is not exposed to the test agent; wherein the amount of a cytokine selected from the group consisting of interleukin-6, interleukin-8, TNFR1, RANTES and MMP-3 in the first control cell is greater than that in a second control cell which does not contain the old-35 gene operatively linked to the promoter element and which is not senescent or terminally differentiated.

72. The method of claim 71, wherein the OLD-35 protein has a sequence as set forth in SEQ ID NO: 6.

73. A model system of arthritis, comprising a non-human animal carrying a transgene comprising an old-35 gene operatively linked to a promoter element that is selectively active in cells comprised in a joint of the animal.

74. A model system for atherosclerosis, comprising a non-human animal carrying a transgene comprising an old-35 gene operatively linked to a promoter element that is selectively active in cells of the vascular system.

75. A model system for Alzheimer's disease, comprising a non-human animal carrying a transgene comprising an old-35 gene operatively linked to a promoter element that is selectively active in cells of the central nervous system.

76. A method for evaluating inflammation in a transgenic non-human animal carrying a transgene comprising an old-35 gene operatively linked to a promoter element, comprising determining, in a cell, tissue, or fluid of the animal, whether the amount of reactive oxygen species is increased.

77. A method for evaluating inflammation in a transgenic non-human animal carrying a transgene comprising an old-35 gene operatively linked to a promoter element, comprising determining, in a cell of the animal, whether the amount of binding of a NF-[kappa]B protein to its target sequence is increased.

78. A method for evaluating inflammation in a transgenic non-human animal carrying a transgene comprising an old-35 gene operatively linked to a promoter element, comprising determining, in a cell of the animal, whether the amount of a NF-[kappa]B protein translocated into the nucleus is increased.

79. A method for evaluating inflammation in a transgenic non-human animal carrying a transgene comprising an old-35 gene operatively linked to a promoter element, comprising determining, in a cell, tissue, or fluid of the animal, whether the amount of a cytokine selected from the group consisting of interleukin-6, interleukin-8, TNFR1, RANTES and MMP-3 is increased.

80. A method of detecting inflammation in a subject, comprising determining whether there is an increase in the expression of an old-35 gene in a cell of the subject relative to a control cell.

81. A kit for detecting inflammation in a subject, comprising a probe that binds to an old-35 gene product selected from the group consisting of old-55 mRNA and OLD-35 protein and a probe that binds to a cytokine mRNA selected from the group consisting of interleukin-6 mRNA, interleukin-8 mRNA, TNFR1 mRNA, RANTES mRNA and MMP-3 mRNA.

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