# Cytokine Induced Expression of Porcine Inhibitor of Apoptosis Protein (iap) Family Member Is Regulated by NF-κB

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The inhibitor of apoptosis (iap) proteins belong to a gene family that protect certain cell to undergo programmed cell death in response to a variety of stimuli. By differential screening we have identified a cDNA clone, designated piap, in porcine aortic endothelial cells (PAEC) that turned out by sequence comparison to be a porcine member of the iap family. The expression of piap is strongly up-regulated upon treatment of endothelial cells (EC) with inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , and LPS. In EC these stimuli lead to the activation of nuclear transcription factor kappa B (NF- $\kappa$ B) that plays a role in countering TNF- $\alpha$  induced apoptosis. We demonstrate that adenovirus mediated overexpression of  $I\kappa B\alpha$ , an inhibitor of NF- $\kappa B$  suppresses the expression of piap in response to  $TNF-\alpha$ suggesting that piap is one of the NF-kB regulated genes that operates to prevent programmed cell death of EC in inflammation. © 1998 Academic Press

The inhibitor of apoptosis proteins (iap) are a family of anti-apoptotic proteins that are conserved across several species. Iap genes were first described in baculovirus (op-iap, cp-iap and ac-iap) (1,2). Subsequently human (naip, hiap1/ciap2/MIHC, hiap2/ciap1/MIHB, xiap/ilp, survivin), chicken (ch-iap1), drosophila (diap1 and diap2/dilp/DIHA), and mouse (MIHA/miap3) homologues were cloned (3-11, reviewed in 12). Two structural features are characteristic for iap proteins. At the N-terminus there are up to three imperfect amino acid repeats approximately 65 residues in length, termed baculovirus iap repeat (BIR), a sequence motif that is unique to the iap proteins (2). Except for naip and survivin, all other known iap family members also contain a RING finger domain at the C-terminal end. RING

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finger motifs have been found in a variety of other proteins and suggested to be involved in protein-DNA as well as protein-protein interactions. For baculovirus iap proteins, both the BIR and the RING finger domains are required to block apoptosis in insect cells (13-15). However, the human iap proteins naip and survivin which lack a RING finger domain are capable to suppress apoptosis. Furthermore, it has been demonstrated that removal of the RING finger domain results in enhanced protection suggesting that the BIR domain(s) play an essential role in the inhibition of apoptosis (3).

TNF- $\alpha$  is a pro-inflammatory cytokine whose pleiotropic biological effects are signaled through two distinct cell surface receptors, TNF-receptor 1 and TNFreceptor 2 (16). TNF- $\alpha$  treatment of EC generates two cellular responses: one is activation of NF-*k*B, the central mediator of gene regulation in inflammatory responses of ECs leading to leukocyte adhesion and thrombosis (17,18), the other is induction of programmed cell death (19,20). Two of the human iap proteins, hiap1 and hiap-2 have been found recruited to the cytosolic domain of TNF receptor 2 via their association with the TRAF-N domain of TRAF 2 (TNF receptor associated factor 2) (4), a central component involved in TNF- $\alpha$  mediated activation of NF- $\kappa$ B. The function of hiap 1 and hiap 2 in the TNF-receptor family signal transduction cascade is at present unknown.

The studies presented here demonstrate that the porcine homologue of the iap gene family (piap) is strongly up-regulated in response to inflammatory cytokines TNF- $\alpha$ , IL-1 and lipopolysaccharide (LPS) in primary porcine aortic PAEC. Adenovirus mediated overexpression of I $\kappa$ B $\alpha$ , an inhibitor of NF- $\kappa$ B abolishes piap gene up-regulation, indicating that piap expression is dependent on NF- $\kappa$ B activation. We conclude that piap is a protective gene that is involved to counteract TNF- $\alpha$  induced apoptosis in EC.

MNTEKDRLLTF11 TCCAGATGTGGCCATTGACCTTTCTGTCGCCAGCAGATCTGGCAAAAGCAGGCTTTTACTACATAGGACCTGGAGACA O M W P L T F L S P A D L A K A G F Y Y I G P G D R 37 157 GAGTGGCTTGCCTTGCCTGTGGTGGAAAATTGAGCAATTGGGAACCAAAGGATGATGCTATGACAGAACACTTACGAC A C F A C G G K L S N W E P K D D A M T E H L R H 63 ATTTCCCCAACTGCCCATTTTTGGGAAATCAGCTTCAAGACTCTTCAAGATACACTGTTTCTAACCTGAGCATGCAGA 235 F P N C P F L G N O L O D S S R Y T V S N L S M O T 89 313 CATATGCAGCCCGCTTTAAAACATTCTGTAACTGGCCTTCTAGTATTCCAGTTCATCCTGAACAGCTTGCAAGTGCAG YAARFKTFCNWPSSIPVHPEOLASAG 115 391 GTTTTTATTATATGGGTCACAGTGATGATGTGAAGTGCTTCTGCTGTGATGGTGGGCTGAGGTGTTGGGAATCTGGAG YYMGHSDDVKCFCCDGGLRCWESGD141 ATGATCCATGGGTGGAACATGCCAAGTGGTTTCCAAGGTGTGAGTACTTGATACGAATTAAAGGACAGGAGTTCATCA 469 WVEHAKWFPRCEYLIRIKGQEFIS 167 D P GTCGCGTTCAAGCCAGTTACCCTCATCTACTTGAACAGCTATTGTCTACTTCAGACAATCCAGAAGATGAAAATGCAG 447 RVOASYPHLLEOLLSTSDNPEDENAE 193 625 AGCCACCAAATGACCTATCATTGATCCGGAAGAACAGAATGGCACTTTTTCAACACTTGACTTGTGTGCTTCCAATCC PNDLSLIRKNRMALFQHLTCVLPIL 219 Þ TGGATAGTCTACTAATTGCCAGAGTGATTAGTGAACAAGAACATGATGTTATTAAACAGAAAACACAGACATCTTTAC 703 SLLIARVISEOEHDVIKOKTOTSLO245 781 AAGCAAGAGAACTGATTGATATTATTTTAGTAAAAGGAAATTATGCAGCCACCATATTCAAAAAACTCTCTACAAGAAA A R E L I D I I L V K G N Y A A T I F K N S L O E I 271 859 TCGATCCCATGTTATACAAGCATTTATTTGTGCAACAAGACATAAAGTATATTCCCACAGAAAATGTTTCAGATTTAT DPMLYKHLFVOODIKYIPTENVSDLS 297 CAATGGAAGAACAATTAAGGAGACTACAGGAAGAAGAACATGCAAAGTGTGTATGGACAAAGAAGTGTCCATAGTGT 937 M E E O L R R L O E E R T C K V C M D K E V S I V F 323 1015 TTATTCCGTGTGGTCATCTGGTAGTCTGCAAAGATTGTGCCCCCTTCTCTAAGAAAATGTCCTATTTGTAGAGGTACAA PCGHLVVCKDCAPSLRKCPICRGTI349 TCAAGGGTACAGTTCGTACATTTCTTTCATGAAGATCTAAAACTTTGCCTAAACTTTAGAACTAATGGATTAAATGTA 1093 KGTVRTFLS • 358 1171 TTGTAATCTTAACTTTGATACTGCTTTGGTTTCTTTTAAATTTTTT<u>ATTTAA</u>CAACTCAGAAAATTTTGTTTTATA TAAATGT<u>ATTTA</u>TATAAATATATATATCTAAAACATGAACATATATATTTTATATCGTAAGGGAATGATAGGATTTTGTT 1249 1327 CTTGTGAAMWAAGAATAGGGAAAGCACTACAAGCACAATACTAAATGAAAATTATAGTACTATTGATATTGTAACTGA 1405 CTGTCTTATACATAGGAAGGTCTTGATGTTTGTTGAATGACTTTTTCAGGACATGATGTTTTTGTAAAAA 1483

**FIG. 1.** Nucleotide and deduced amino acid sequence of piap. Nucleotides are numbered on the left, amino acid residues on the right. The AUUUA motifs are underlined.

#### MATERIALS AND METHODS

*Cell culture.* PAECs were grown at 37°C and 5% CO<sub>2</sub> in gelatin (0,2%) coated culture flasks in DMEM supplemented with 10% fetal calf serum, L-glutamine, Penicillin, and Streptomycin. Confluent cells were split in a 1:3 ratio and used to the 15<sup>th</sup> passage. Treatment of cells with different stimuli was for four hours: lipopolysaccharide (LPS) 600ng/ml; tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) 500u/ml; IL-1 $\beta$  300u/ml; IL-6 200u/ml; IL-8 200ng/ml; IL-10 2ng/ml; IL-11 10ng/ml; leukaemia inhibitory factor (LIF) 20ng/ml; oncostatin M (OM) 20ng/ml; epidermal growth factor (EGF) 10ng/ml; transforming growth factor  $\beta$  (TGF- $\beta$ ) 2ng/ml, Thrombin 4u/ml.

*cDNA cloning.* A cDNA library was constructed using pooled mRNAs from PAEC induced with TNF- $\alpha$ , LPS, IL-1 $\beta$  for 4 and 9 hours. cDNA synthesis was performed in the presence of oligo(dT) and random primers and cDNAs were cloned into the *Eco*RI site of the lambda ZAPII vector (Stratagene). Propagation of recombinant phages was carried out in the E.coli strain PLK-F, plaque lifting and hybridization were done according to the protocol provided by Stratagene.  $\alpha$ [<sup>32</sup>P]dATP labeled cDNA probes (prime-it II, Stratagene) or polyAtailed oligonucleotides (terminal transferase, Boehringer Mannheim)

specific to piap were used for hybridization at 65°C in Quickhyb-solution (Stratagene). Membranes were washed at high stringency (65°C/1% SDS/0,2×SSC) and exposed on a X-OmatAR Film (Kodak). Positive phages were re-screened and sequenced.

DNA sequence analysis. Plasmid DNA was prepared using Quiagen plasmid kit. Nucleic acid sequencing was performed utilizing the Prism Ready Reaction Taq Cycle Sequencing Kit (Perkin Elmer) and an automatic sequencer (model 373, Applied Biosystems). Nucleotide and amino acid sequences were analysed and compared using Mac-Vector (Oxford Molecular LTD), GCG programmes (Genetics Computer Group, Inc. University of Wisconsin), and GenEMBL data base. Primers were synthesized on a 392 RNA/DNA synthesizer (Applied Biosystems).

Genomic DNA. For preparation of genomic porcine DNA PAEC were incubated for 3 hours at 55 °C in 100 mM NaCl/10 mM Tris-HCl pH 8,0/25 mM EDTA/0,5% SDS/0,47 mg/ml Proteinase K and then incubated with 100 mg/ml RNAseA for 1 hour at 37° C. After phenol-chloroform extraction and isopropanol precipitation genomic DNA was dissolved in 50  $\mu$ l TE.

*Southern and Northern blots.* Southern and Northern blot analysis was performed essentially as described (21). Signals were analysed on a PhosphorImager SF (Molecular Dynamics).

									BIR 1			
aciap cpiap						<b>Г</b>						
opiap diap1												
diap2 chiap1				MNIMDSSPLL	ASVMKONAHC	.MTELGMELE GELKYDLSCE	SVRLATFGEW LYRMSTFSTF	PLNAPVSAED PVNVPVSERR	LVANGFFATG LARAGFYYTG	NWLEAECHFC VQDKVKCFSC	HVRIDRWEYG GLVLDNWQPG	
miha piap			MTF	NSFEGTRTFV	LADTNKDE	EFVEE	FNRLKTFANF	PSSSPVSAST	LARAGFLYTG	EGDTVQCFSC	HAAIDRWQYG	
survivin hiapl				MNIVENSIFL	SNLMKSANT	FELKYDLSCE	LYRMSTYSTF	PAGVEVSERS	LARAGFYYTG	VNDKVKCFCC	GLMLDNWKRG	
hiap2 xiap		MHKTASQR	LFPGPSYQN1	KSIMEDSTIL NSFEGSKTCV	.SDWINS.NK	QKMKYDFSCE EFVEE	LYRMSTYSTF FNRLKTFANF	PAGVPVSERS PSGSPVSAST	LARAGFYYTG LARAGFLYTG	VNDKVKCFCC EGDTVRCFSC	GLMLDNWKLG HAAVDRWQYG	
naip	MATQQKASDE	RISQFDHNLL	PELSALLGLD	AVQLAKELEE	EEQKERAKMQ	KGYNSQMRS	AKRLKTFVTY SF	EPYSSWIPOE	MAAAGFYFTG	VKSGIQCFCC	SLILFGAGLT	
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aciap		<del></del>					MNE	DTPPFYFISV	CENFRENTAE	HVFEMLIERH	SSFENYP.IE	
opiap			· · · · · · · · · · · · · · ·					MS	SRAIGAPQEG	ADMKNKAARL	GTYTNWPV	
diap1 diap2	DQVAERHRRS	SPICSMV AP	NHCGNVPRSQ	ES		MASVVADLE	S YGPIAFDQV	D NNINATOL	VVDSPESCSC	PDLLLEANRL	VTFKDWPN	
miha	DSAVGRHRRI	SPNCRFINGF	YFENGAAQST	NPGIQNGQYK	SENCVGNRNP	FAPDRPPETH	AD	YLLRTGQVVD	ISDT.IYPRN	PAMOSEEARL	KSFQNWP.DY	
piap survivin									PSALKISSIN		LIFORWE	
hiap1 hiap2	DSPTEKHKKL DSPIQKHKQL	YPSCRFVDSL YPSCSF1QNL	VS.ASLGSTS	KNTSP	.MRNSF.AHS	LEPGTENSGY	FSGSYSNSPS	NPUNSRANGE	ISSSRINPYS	YAMSPEEARF	LTYHMWPL	
xiap naip	RLPIEDHKRF	HPDCGFL N.	TLENSATUST	NSGIQNGQIK	VENT LGSRDH	FALLRPSEIN	KDVG	NIAKYDIRVK	NLKSRLRGGK	MRYCEEEARL	ASFRNWPFYV	
	рн	c		•••••	•••••			•••••	•••••	R.	•TY •••••	
RID 2												
aciap	NTAFINSLIV	NGFKYNQVDD	HVVCEYCEAE	IKNWSEDECI	EYAHVTLSPY	САЧА						
cpiap opiap	SFLSPETMAK QFLEPSRMAA	NGFYYLGRSD SGFYYLGRGD	EVRCAFCKVE EVRCAFCKVE	IMRWKEGEDP ITNWVRGDDP	AADHKKWAPQ ETDHKRWAPQ	CPFVK CPFVR				· · · · · · · · · · · · · · · · · · ·		
diap1 diap2	DWLDKRQLAQ PNITPQALAK	TGMYFTHAGD AGFYYLNRLD	KVKCFFCGVE HVKCVWCNGV	IGCWEQEDQP IAKWEKNENA	VPEHQRWSPN FEEHKRFFPQ	CPLLRRRTTN CPR/Q	NVPINAEALD	RILPPISYDI	CGANDSTLEM	REHAYAEGVI	PMSQLIQSIG .MGPLIEFAT	
chiap1 miha	MFLSPAELAK AHLTPRELAS	AGLYYLGTAD AGLYYTGADD	KVACFTCGGQ QVQCFCCGGK	LSNWEPKDNA LENWEPCDRA	MSEHRRHFPN WSEHRRHFPN	CPF/ CFF/						
<b>piap</b> survivin	TFLSPADLAK	AGFYYIGPGD	RVACFACGGK	LSNWEFKDDA	MTEHLRHFPN	CPFL	•••••			•••••	• • • • • • • • • • • • • • • • • • •	
hiap1 hiap2	TFLSPTDLAR TFLSPSELAR	AGFYYIGPGD AGFYYIGPGD	RVACFACGGK RVACFACGGK	LSNWEPKINA LSNWEPKDDA	MSEHLRHFPK MSEHRRHFPN	CPFL				•••••		
xiap naip	AHLTPRELAS OGISPCVLSE	AGLYYTGIGD AGFVFTGKOD	QVQCFCCGGK TVQCFSCGGC	LKNWEPCDRA LGNWEEGDDP	WSEHRRHFPN WKEHAKWFPK	CFFV CEFLR	•••••	•••••			• • • • • • • • • • • • •	
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									BIR 3			
aciap cpiap	GIDVCGST	NKI	AERESFGDNI THDTIIGPA.	TINAVLVKEG	KPKCV	YRCMSNL	QSRMDTFVNF AARVKSFHN.	.WPAALRDMI .WPRCMKORP	TNIAEAGLFY EOMADAGFFY	TGRGDET TGYGDNT	VCFFCDCCVR KCFYCDGGLK	
opiap diapl	N	.NAHDTPHDR TGTAAPOPRV	APPARSAAA.	OATGDVOPET	CRESAASGNY	HPQYATE FPOYPEYAIE	AARLRTFAE . TARLRTFEA .	.WPRGLKORP .WPRNLKOKP	EELAEAGFFY HOLAEAGFFY	TGQGDKT TGVGDRV	RCFCCDGGLK RCFSCGGGLM	
diap2	GKNLDELGIQ	PTT		MRDOPSENVS		LPLRPKYACV	DARLETFTD. EARVKTFIN.	.WPISNIQPA .WPTRIPVOP	SALAQAGLYY EOLADAGFYY	QKIGDQV VGRNDDV	RCFHCNIGLR KCFCCDGGLR	
miha piap			LGRNV	NVRSES.GVS LODSSRYTVS	SDRNFPNSTN	SPRNPAMAEY	EARIVTEGT. AAREKTECN.	.WTSSVNK .WPSSIPVHP	EQLARAGFYA EQLASAGFYY	LGEGDKV MGHSDDV	KCFHCGGGLT KCFCCDGGLR	
survivin hiapl				LODTSRYTVS	MGAPT	LPPAWQPFLK NLSMQTH	DHRISTFKNW AARFKTFFN.	PFLEGCACTP .WPSSVLVNP	ERMAEAGFIH EQLASAGFYY	CPTENEPDLA VGNSDDV	QCFFCFKELE KCFCCDGGLR	
hiap2 xiap				L.ETLRFSIS NIRSESDAVS	SDRNFPNSTN	NLSMOTH LPRNFSMADY	AARMRTFMY . EARIFTFGT .	.WPSSVPVQP .WIYSVNK	EQLASAGFYY EQLARAGFYA	VGRNDDV LGEGDKV	KCFGCDGGLR KCFHCGGGL/T	
naip		SKKSSEEITQ	YIQSYKGFVD	ITGEHFVNSW	VQRELPMASA	YCNDSIFAYE	ELRLDSFKDW SF	PRESAVGV	AALAKAGLFY	TGIKDIV	QCFSCGGCLE	
	•••••						· · · · · · ŢŶ · · ·		G			
aciap	DWHTNEDTWO	RHAAENPQCY	FVLSVKGKEF	CQN				••••	LPGE			
opiap	DWEPDDAPWQ	QHARWYDRCE OHALWI SOCR	YVLLVKGRDF	VORVMTEACV	AFEKFEGTGT	GGD		TVASTOASEE	VRDA.			
diap2	SWQKEDEPWF	EHAKWSPKCQ	FULLAKGPAY	VSEVLATTAA	NASSPPATAP	APT	THEFFORESP	LQADVLMDEA SEDA IMMNTE	PAKEALALGI	DGGVVRNAIQ	RKLLSSGCAF	
miha	DWKPSEDPWE	QHAKWYPGCK	YLLDEKGQEY	INNIHLTHS.	LEESLGRTAE	KT	PSLTK	KIDDTIFQNP	MVQEAIRMGF	SFKDIKKTME	EKIQISGSSY	
survivin	GWEPDDDPIE	EHKKHSSGCA	FLSVKKQFEE VLTRIKCOFF	LT.	LGEFL	SPODENAESS	THEREBREDH	KLDRERAKNK SEDA IMMNTP	IAKETNNKKK	EFEETAKKVR SRSLVKOTVO	R RKILATGENY	
hiap2	CWESGDDFWV	EHAKWFPRCE	FLIRMKGQEF	VDEIQGRYPH INNIHUTHS.	LLEQLLSTSD LEECLVRTTE	TIGEENADPP KT.	IIHFGPGESS PSLTR	SEDAVMMNTP RIDDTIFONP	VVKSALEMGF MVOEAIRMGF	NRDLVKQTVL SFKDIKKIME	SKILTTGENY EKIOISGSNY	
naip	KWOEGDDPLD	DHTRCFPNCP	FLONMKSSAE	VTPDLQSRGE	LCELLETTSE	s		NLEDSIAVGP	IVFEMAQGEA	QWFQEAKNLN	EQLRAAYTSA	
	.w <u>ē</u>	.нС.	• • • • • • • • • • • • •					• • • • • • • • • • • •	•••••			
anim								STURMERS	DKRD			
cpiap					· · · · · · · · · · · · · · · · · · ·		NTTVSTAAP	VSEPIPETKI	EKEP		QVE	
diap1	GULDELLHOT	FDDAGAG			AALEVREP	PEPSAPFIEP	.EAVSGDVAP	SVAPTAATRI VPIPVADSIP	FNK.IVEATA	VATPSTNSSG	STSIP	
chiapl miha	KTVNDLVSDL	LTAEDEKREE	EKERQFEEVA	SDDLSLIRKN	RMALFORLTS	VLFILGSLLS	AKVITELEHD	VIKQKTQTPL TEDESSOTSL	QARELIDTVL	VKGNAAASIF	RNCLKDCDPV	
piap	ATEOLAAMD			PNDLSLIRKN	RMALFQHLTC	VLPILDSLLI	ARVISEQEND	VIKQKTQTSL	QARELIDIIL	VKGNYAATIF	KNSLQEIDPM	
hiap1 hiap2	RLVNDLVLDL KTVNDIVSAL	LNAEDEIREE	ERERATEEKE	SNDLLLIRKN	RMALFQHLTC RMALFOOLTC	VIPILDSLLT	AGIINEQEHD ANVINKOEHD	VIKOKTOIPL	QARELIDTIL OARELIDTIW	VKGNIAATVF VKGNAAANIF	RNSLQEAEAV KNCLKEIDST	
xiap	KSLE	SCOLATOHLL	CODI GLORING	TSKEVOFELV	LPEVECNENS	VMCVEGEAGS	ADLVNAQKDS	MODESSOTSL FLWASGCOPL	Q	SUSSTREDEG	LASTICDOLL	
	5176270EED1		Son and the MI				RF					
acian			DONLNEN.	ADDIFF	KYECKVCLER	ORDAVLMPCR	- HFCVCVOCYF	GLEQKCPTCR	QDVTDFIKIF			
cpiap				D	SKLCKICYVE	ECIVOFVPCG EKTVOFVPCG	HVVACAKCAL HVVACGKCAA	SVD.KCPMCR GVT.TCPVCR	KIVTSVLKVY GQLDKAVRMY			
diap1 diap2		G	NLSLEEE.	E	EKLCKICYGA ARLCKVCLDE	EYNTAFLPCG	HVVACAKCAS HLATCNOCAP	SVT.KCPLCR SVA.NCPMCR	KPFTDVMRVY ADIKGFVRTF			
chiap1 miha	LYKDLFVEKS	MKYVPTEDVS	GLPMEEQ DISTEEO.	LRRLQE	ERTCKVCMDK	EVSIVFIPCG	HLVVCKECAP HLVTCKOCAF	SLR.KCPICR AVD.KCPMCY	GTIKGTVRTF TVITFKOKIF			
piap survivin	LYKHLFVQQD	IKYIPTENVS	DLSMEEQ	LRRLQE	ERTCKVCMDK	EVSIVFIPCG	HLVVCKDCAP	SLR.KCPICR	GTIKGTVRTF			
hiap1 hiap2	LYEHLFVQQD LYKNLFVDKN	IKYIPTEDVS MKYIPTEDVS	DLPVEEQ	LRRLPE	ERTCKVCMDK ERTCKVCMDK	EVSIVFIFCG	HLVVCKDCAP HLVVCOECAP	SLR.KCPICR SLR.KCPICR	STIKGTVRTF			
xiap naip	EKEGSVTEMC	MRNI IQOLKN	EISTEEQ QVLFLLDDYK	LRRLOE EICSIPOVIG	EKLCKICMDR .KL	NIAIVFVPCG	HLVTCKQCAE	AVD. KCPMCY	TVITFKQKIF IQKNHLSR	TCLLIAVRTN	RARDIRRYLE	
					ску.с		нсс.	.VCP.CY				
	m** m= =		nauna marco		1.001.0000		VEDEPERSON	1100000-	01 010 000 000	1 1/ 100 1000	I M KORROSS	
naip	TILEIQAFPF FEFNDDDLAE	AGVDEDEDLT	F SHNMTRLRK MCLMSKFTAQ	RLRPFYRFLS	PAFQEFLAGM	VAA1CAHWFQ RLIELLDSDR	QEHQDLGLYH	LKQINSPMMT	VSAYNNFLNY	VSSLPSTKAG	PKIVSHLLHL	
	VUNKESLENI TSPRAHFSVL	SENDDYLKHQ ETCFDKSQVP	TIDQDYASAF	RGLWQ1CFQA EPMNEWE	IF SMVSEHLL	VLALKTAYQS	MIVAACSPFV	ogr loggRTLT	DOMENLQYFF	DULESPER	SINFSIRGNK	

**FIG. 2.** Comparison of amino acid sequences of thirteen iap gene family members. The deduced consensus sequence is given in the bottom line. N-terminal BIR domains and the C-terminal RING finger domain are boxed. The GeneBank accession numbers are M96361 (aciap), L05494 (cpiap), L224564 (opiap), L49440 (diap1), L49441 (diap2), AF008592 (ch-iap1), U36842 (MIHA), U79142 (piap), U75285 (survivin), U45878 (hiap1), U45879 (hiap2), U45880 (xiap), U19251 (naip). The PILEUP (GCG) program was used for alignment.



**FIG. 3.** Southern blot analysis of piap using porcine genomic DNA. 10  $\mu$ g of genomic DNA was digested with EcoR I, Hind III, or Pst I respectively, separated on a 0.8% agarose gel, and probed with a piap-specific cDNA fragment covering both BIR domains.

Adenovirus infection. Adenovirus infection was carried out essentially as described (22). Briefly, confluent PAEC were washed once with complete phosphate buffered saline (PBS) and incubated in PBS at a multiplicity of infection of 1000 with the respective adenovirus constructs. After 30 min at 37°C, unbound adenovirus was washed off and fresh medium was added. Cells were maintained for two additional days before assayed.

## **RESULTS AND DISCUSSION**

We have employed a modified differential screening procedure to selectively identify and clone up- or downregulated genes in PAEC. A 1550 bp cDNA that is strongly up-regulated upon LPS stimulation of PAEC turned out by sequence comparison to encode a porcine homologue of the iap gene family, therefore refered as piap. The cDNA clone contains an open reading frame of 1077 bp encoding a protein of 41 kDa (Fig. 1). The 5' end was verified by primer extension analysis (data not shown). The best overall homology was found to the human iap genes with 73,9% identidy to hiap 1 on amino acid level. Using the PILEUP program for amino acid alignment a consensus sequence for BIR domains was derived from thirteen family members. Obviously the second and the third BIR domains of the respective family members followed the consensus sequence more strictly than the first domain (Fig. 2). Both iap gene specific sequence motifs are present in piap. The RING finger domain is located close to the C-terminal end. In contrast to most other mammalian family members piap contains only two N-terminal BIR domains which is usually found in drosophila and viral family members. However, it appears that the number of BIR domains is not a crucial issue for its protective function since a single BIR element present in survivin is sufficient to protect cells from apoptosis (9). Just as for other iap family members three AUUUA motifs are present within the 3' untranslated region of the mRNA. These motifs have been suggested to destabilize mRNA species by binding a transcriptional repressor resulting in a shorter half-life of the mRNA (23,24).

Hybridization of a piap cDNA fragment covering the two BIR domains to porcine genomic DNA digested with different restriction enzymes indicated that piap is a single copy gene (Fig. 3). Moreover, the porcine probe hybridized to only one restriction fragment whereas in the human genome at least five family members exist, three of them could be detected using a human BIR cDNA fragment covering the equivalent region in hiap 1 (data not shown).

Originally we have identified piap as a LPS inducible gene. We then tested whether other cytokines can also induce piap expression. A panoply of cytokines was assayed by Northern blot analysis for their effect on piap mRNA accumulation in PAEC. Just the inflammatory stimuli LPS, TNF- $\alpha$ , and IL-1 $\beta$  caused pronounced upregulation of piap mRNA after 4 hours of treatment (Fig. 4A,B). In primary porcine aortic smooth muscle cells (PASMC) there is no induction with TNF- $\alpha$  suggesting that TNF- $\alpha$  inducible piap expression is specific for certain cell types (Fig, 4C).

The nuclear transcription factor NF- $\kappa$ B is a central mediator of gene regulation in inflammation. NF- $\kappa$ B is



**FIG. 4.** Piap gene expression in PAEC (A,B) and PASMC (C) in response to different stimuli after four hours. 10  $\mu$ g of total RNA were loaded per lane. Northern blots were probed at high stringency with a cDNA fragment specific to piap, stripped and reprobed with GAPDH-cDNA fragment to confirm equal loading of RNA. Signals were analysed using a PhosphorImager SF (Molecular Dynamics).



**FIG. 5.** NF- $\kappa$ B-dependent expression of piap. Piap gene expression in non-infected cells (control) and adenovirus-infected PAECs was assayed by Northern blot analysis. PAECs were infected with a control adenovirus (control Adv) or a recombinant adenovirus-I $\kappa$ B $\alpha$  (I $\kappa$ B Adv) construct. Non-infected cells and infected cells were left untreated or treated with TNF- $\alpha$  (500U/ml) for 4 hours. Membranes were probed with a cDNA fragment specific to piap. Expression of I $\kappa$ B $\alpha$  was controlled by reprobing the membranes with cDNA fragment specific to I $\kappa$ B $\alpha$ . Equal loading was confirmed by hybridization with GAPDH cDNA fragment.

activated by TNF- $\alpha$ , IL-1 and LPS in EC. Therefore we tested whether NF- $\kappa$ B was involved in up-regulation of piap. Having shown previously that expression of I $\kappa$ B $\alpha$ , the inhibitor of NF- $\kappa$ B, from a recombinant adenovirus vector abolishes NF-*k*B dependent up-regulation of inflammatory genes such as IL-1, IL-6, IL-8, and VCAM-1 in LPS-stimulated EC (22), we used this adenovirus-I $\kappa$ B $\alpha$  construct to investigate whether NF- $\kappa B$  inhibition also impairs piap gene expression. PAEC were infected with either a control adenovirus or the recombinant adenovirus-I $\kappa$ B $\alpha$ . After two days cells were stimulated with TNF- $\alpha$  for four hours and probed for piap expression. As shown in Fig. 5, the expression of piap is suppressed, indicating that the up-regulation of the piap gene is controlled by NF- $\kappa$ B. Several groups have demonstrated that direct inhibition of NF- $\kappa$ B or of upstream parts of its signaling pathway during TNF- $\alpha$  activation results in apoptosis in a variety of cells (25-27). The existence of NF-*k*B regulated anti-apoptotic genes has therefore been postulated. Here we present evidence that piap appears to be such a gene. This assumption is supported by the recent finding that expression of a human iap gene (hiap1) is controlled by activation of NF- $\kappa$ B in a Jurkat T cell line and suppresses TNF- $\alpha$  mediated apoptosis (28).

Whether under physiological circumstances the expression of iap gene(s) is sufficient or whether simultaneous expression of other anti-apoptotic genes such as A20 (29), manganese superoxide dismutase (30), plasminogen activator inhibitor type 2 (31), A1 (32) or other yet not defined genes is required to protect Ecand presumably other cell types from TNF- $\alpha$  induced apoptosis remains open. Importantly, LPS and IL-1 $\beta$  also induce

piap gene expression. Pretreatment of a human fibrosarcoma line (HT1080V) with IL-1 $\beta$  (a cytokine that activates NF- $\kappa$ B but is not pro-apoptotic) protects these cells from apoptosis induced by the later addition of TNF- $\alpha$ , even in the presence of a protein synthesis inhibitor (25). In cells expressing a super-repressor form of the NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$ , IL-1 $\beta$  does not have a protective effect, suggesting that the effect of IL-1 $\beta$ also relies on the expression of NF- $\kappa$ B-regulated antiapoptotic genes. A mechanism to overrule apoptotic signals during TNF- $\alpha$  mediated inflammation would enable EC to respond properly by up-regulation of inflammatory mediators such as tissue factor and cell adhesion molecules and at the same time survive inflammation in order to retain homeostasis.

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