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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-α, IL-1β, and LPS. In EC these stimuli lead to the activation of nuclear transcription factor kappa B (NF-κB) that plays a role in countering TNF-α induced apoptosis. We demonstrate that adenovirus mediated overexpression of IκBα, an inhibitor of NF-κB suppresses the expression of piap in response to TNF-α suggesting that piap is one of the NF-κB regulated genes that operates to prevent programmed cell death of EC in inflammation.

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 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-α is a pro-inflammatory cytokine whose pleiotropic biological effects are signaled through two distinct cell surface receptors, TNF-receptor 1 and TNF-receptor 2 (16). TNF-α treatment of EC generates two cellular responses: one is activation of NF-κB, 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-α mediated activation of NF-κB. 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-α, IL-1 and lipopolysaccharide (LPS) in primary porcine aortic PAEC. Adenovirus mediated overexpression of IκBα, an inhibitor of NF-κB abolishes piap gene up-regulation, indicating that piap expression is dependent on NF-κB activation. We conclude that piap is a protective gene that is involved to counteract TNF-α induced apoptosis in EC.

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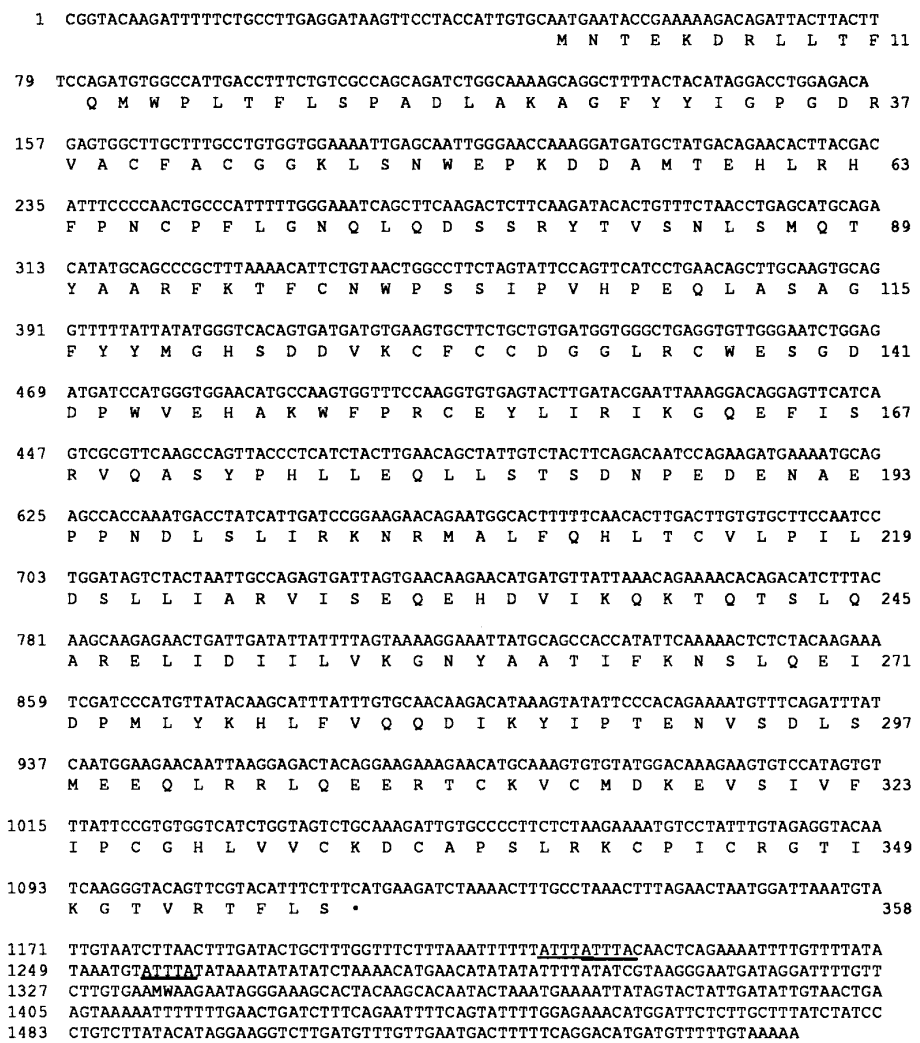

FIG. 1. Nucleotide and deduced amino acid sequence of piap. Nucleotides are numbered on the left, amino acid residues on the right. The AUUUA motifs are underlined.

MATERIALS AND METHODS

Cell culture. PAECs were grown at 37°C and 5% CO_2 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 15th passage. Treatment of cells with different stimuli was for four hours: lipopolysaccharide (LPS) 600ng/ml; tumor necrosis factor α (TNF-α) 500u/ml; IL-1β 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 ß (TGF-ß) 2ng/ml, Thrombin 4u/ml.

cDNA cloning. A cDNA library was constructed using pooled mRNAs from PAEC induced with TNF-α, LPS, IL-1β 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. $\alpha[^{32}P]$dATP labeled cDNA probes (prime-it II, Stratagene) or polyA-tailed 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 MacVector (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 μl 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).

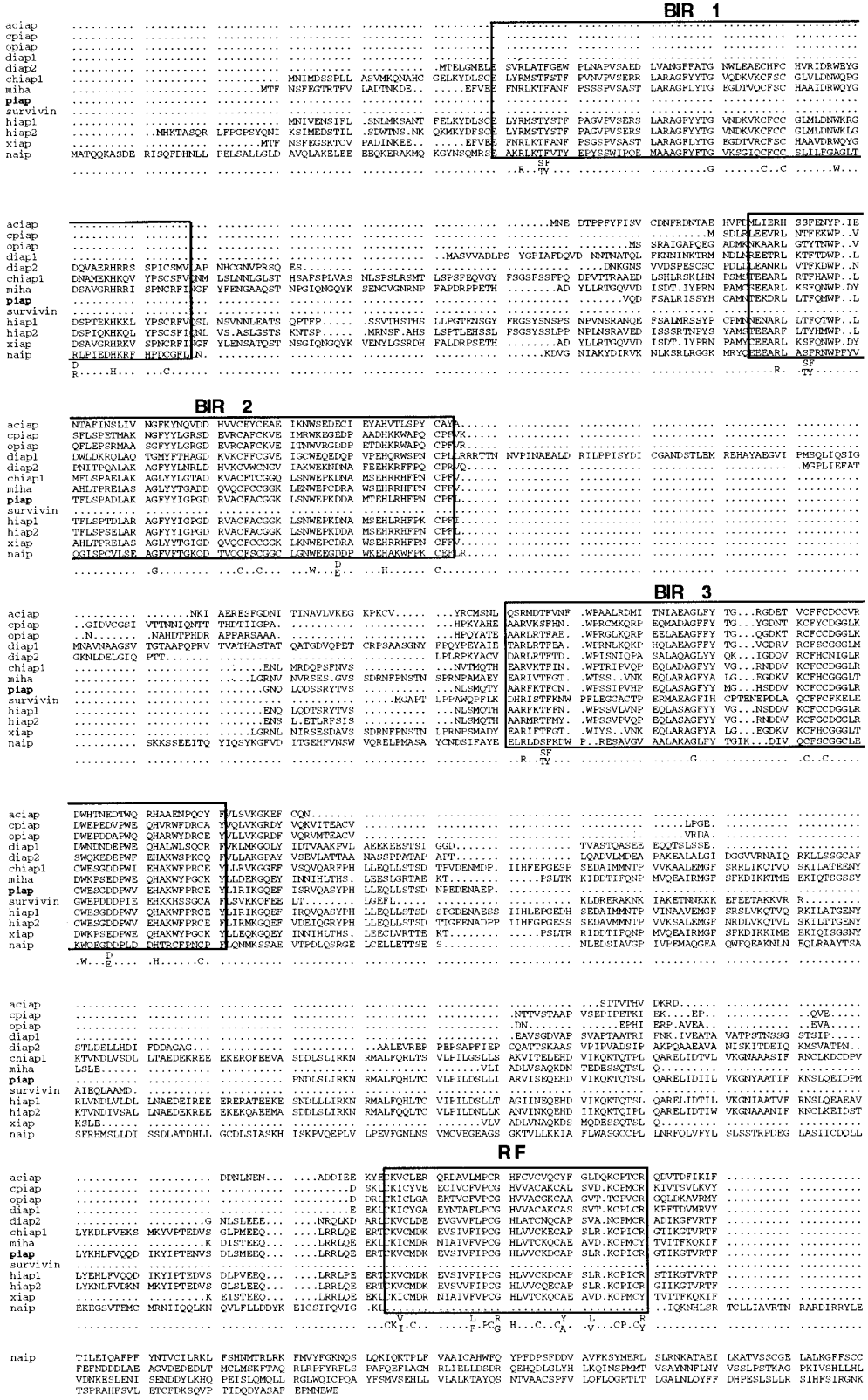

FIG. 2. Comparison of amino acid sequences of thirteen iap gene family members. The deduced consensus sequence is given in the bottom line. N-terminal BIR domains and the C-terminal RING finger domain are boxed. The GeneBank accession numbers are M96361 (aciap), L05494 (cpiap), L224564 (opiap), L49440 (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.

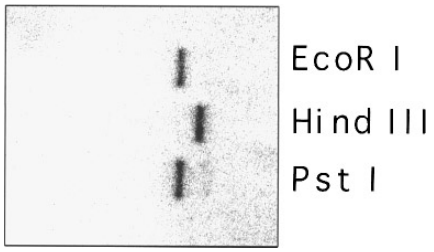

FIG. 3. Southern blot analysis of piap using porcine genomic DNA. 10 μg of genomic DNA was digested with EcoR I, Hind III, or Pst I respectively, separated on a 0,8% agarose gel, and probed with a piap-specific cDNA fragment covering both BIR domains.

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 down-regulated 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-α, and IL-1β caused pronounced up-regulation 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-α suggesting that TNF-α inducible piap expression is specific for certain cell types (Fig, 4C).

The nuclear transcription factor NF-κB is a central mediator of gene regulation in inflammation. NF-κB is

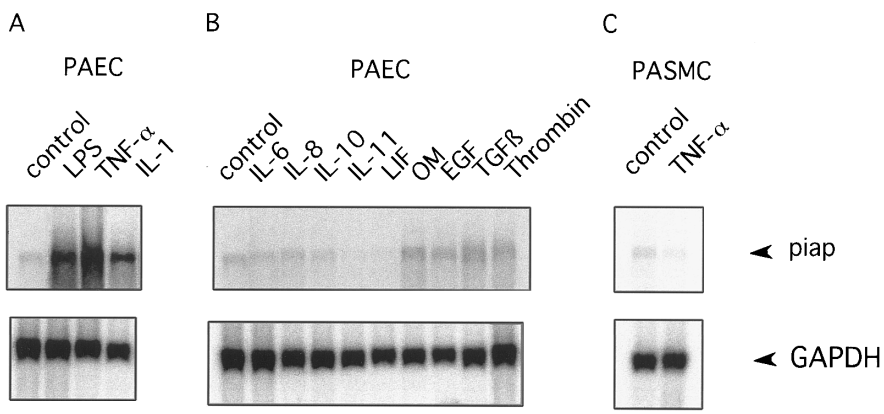

FIG. 4. Piap gene expression in PAEC (A,B) and PASMC (C) in response to different stimuli after four hours. 10 μg of total RNA were loaded per lane. Northern blots were probed at high stringency with a cDNA fragment specific to piap, stripped and reprobed with GAPDH-cDNA fragment to confirm equal loading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

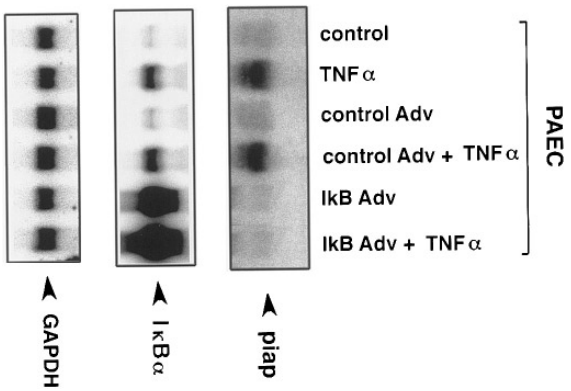

FIG. 5. NF-κB-dependent expression of piap. Piap gene expression in non-infected cells (control) and adenovirus-infected PAECs was assayed by Northern blot analysis. PAECs were infected with a control adenovirus (control Adv) or a recombinant adenovirus-IκBα (IκB Adv) construct. Non-infected cells and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

activated by TNF-α, IL-1 and LPS in EC. Therefore we tested whether NF-κB was involved in up-regulation of piap. Having shown previously that expression of IκBα, the inhibitor of NF-κB, from a recombinant adenovirus vector abolishes NF-κB 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-IκBα construct to investigate whether NF-κB inhibition also impairs piap gene expression. PAEC were infected with either a control adenovirus or the recombinant adenovirus-IκBα. After two days cells were stimulated with TNF-α 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-κB. Several groups have demonstrated that direct inhibition of NF-κB or of upstream parts of its signaling pathway during TNF-α activation results in apoptosis in a variety of cells (25-27). The existence of NF-κB 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-κB in a Jurkat T cell line and suppresses TNF-α 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-α induced apoptosis remains open. Importantly, LPS and IL-1β also induce piap gene expression. Pretreatment of a human fibrosarcoma line (HT1080V) with IL-1β (a cytokine that activates NF-κB but is not pro-apoptotic) protects these cells from apoptosis induced by the later addition of TNF-α, even in the presence of a protein synthesis inhibitor (25). In cells expressing a super-repressor form of the NF-κB inhibitor IκBα, IL-1β does not have a protective effect, suggesting that the effect of IL-1β also relies on the expression of NF-κB-regulated anti-apoptotic genes. A mechanism to overrule apoptotic signals during TNF-α 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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