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A highly conserved proapoptotic gene, IKIP, located next to the APAF1 gene locus, is regulated by p53

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Abstract

We describe here the identification and initial characterization of a novel human gene termed IKIP (I kappa B kinase interacting protein) that is located on chromosome 12 in close proximity to APAF1 (apoptotic protease-activating factor-1). IKIP and APAF1 share a common 488 bp promoter from which the two genes are transcribed in opposite directions. Three IKIP transcripts are generated by differential splicing and alternative exon usage that do not show significant homology to other genes in the databases. Similar to APAF1, expression of IKIP is enhanced by X-irradiation, and both genes are dependent on p53. Moreover, IKIP promotes apoptosis when transfected into endothelial cells. We conclude that IKIP is a novel p53 target gene with proapoptotic function.

Introduction

Apoptosis plays a major role in several pathophysiological settings, including development, immunology and tumor biology. Analysis of the molecular components of the apoptotic signalling pathways is therefore of considerable interest for basic science, but has also substantial medical significance.

In the initiation phase of apoptosis, extrinsic and intrinsic pathways have been defined.[1] Whereas the former is induced by the ligand-mediated activation of tumor necrosis factor (TNF) family member receptors leading to activation of the initiator caspase, caspase-8, the latter is initiated by damage to mitochondria or as an amplification mechanism, and results in apoptosome-mediated activation of caspase-9. Thereby, several mitochondrial proteins residing in the intermembrane space are released into the cytoplasm in a bcl-2 family-dependent manner. Among these, cytochrome *c*, a component of the mitochondrial electron transfer chain, plays a prominent role, as it associates with apoptotic protease-activating factor 1 (APAF1) leading to oligomerization to form the apoptosome, and activation of caspase-9.[2,3] Supported by genetic experiments using knockout mice, APAF1 has been recognized as a central component of the intrinsic, mitochondrial pathway.[4] Another component of this pathway is p53, a transcription factor of central importance for a variety of cellular functions, which is activated by different physical, chemical and biological stress inducers and leads, via expression of its target genes Bax and p53AIP1, to cytochrome *c* release from mitochondria. p53 and also E2F have been shown to induce the expression of APAF1 at the transcriptional level.[5,6]

Besides mitochondria, the endoplasmic reticulum (ER) has recently gained much attention for its role in apoptosis.[7,8] First, the ER can generate death signals of its own as the last consequence of the unfolded protein response. This can occur through activation of caspase-12, a caspase that is remarkably specific for ER stress-mediated apoptosis;[9] however, a caspase-12 independent pathway is also activated. In addition, reminiscent of the TNFα situation where also antiapoptotic mechanisms such as NF-κB are activated, survival signals are generated as well, which involve the ER membrane proteins Ire1, PERK and ATF6. Second, the ER can release calcium during many forms of apoptosis, including radiation-induced apoptosis.[10] Calcium can lead to the activation of Bad, to upregulation of the proapoptotic transcription factor NAK1/Nur77, and may sensitize mitochondria to the effects of bcl-2 family members.[11,12] Third, bcl-2 family members, including bcl-2 itself, Bax and Bak, as well as the BH3-only protein BIK, localize not only to mitochondria but also to the ER.[13–15] Therefore, multiple pathways exist by which the ER can contribute to pro- or antiapoptotic stimuli.

In the context of a study aiming at the identification of genes involved in NF-κB activation, we identified a gene, which we termed IKIP (I kappa B kinase interacting protein), that is located within 0.5 kb upstream of APAF1. Given the multiple links between the NF-κB and apoptotic signalling pathways, we have analyzed here the structure, regulation and function of IKIP.

Results

Identification of IKIP and localization to APAF1

Initially, we performed yeast two-hybrid screens to identify novel genes involved in the NF-κB signalling cascade. Using
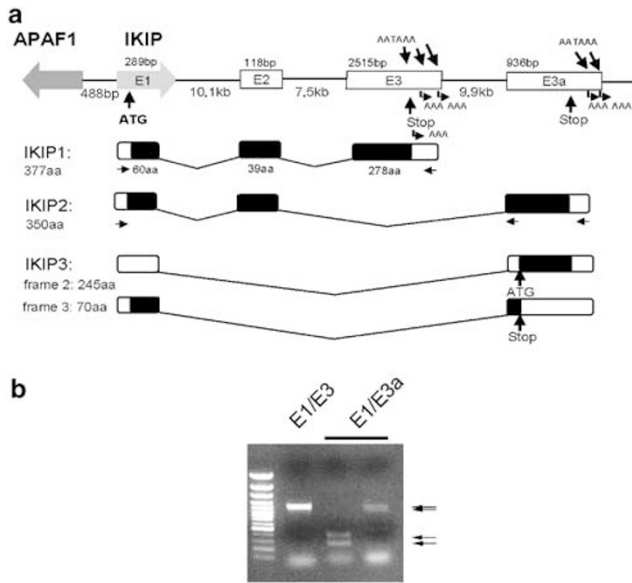
a

b

Figure 1 Schematic presentation of the human IKIP genomic organization and mRNA transcripts IKIP1–3. (**a**) Exons are numbered and represented as boxes. Filled boxes within the transcripts correspond to coding regions and open boxes to untranslated regions; start and stop codons are indicated. The positions of the polyadenylation signals are indicated by arrows. Arrows below the transcript boxes designate the positions of the primers used for RT-PCR. The first exon of the APAF1 gene is also indicated. Exons and introns are not drawn to scale. (**b**) RT-PCR analysis of IKIP transcripts using mRNA from endothelial cells with the primers indicated in (**a**)

IKK2, a central component of this pathway as a bait, we isolated a gene that was termed IKIP (IKK-interacting protein). A search across nucleotide databases revealed that IKIP is located on chromosome 12, just 488 bp upstream of the APAF1 transcriptional start site, such that the two genes are transcribed in opposite directions (Figure 1a). This close association suggested that APAF1 and IKIP might also share common regulation through the use of common promoter elements, and possibly their involvement in common biological functions. Whereas the function of IKIP in NF-κB signalling will be the subject of a separate manuscript (Schmid *et al.*, in preparation), we present here the initial characterization of this novel gene and its relation to apoptosis.

The IKIP gene is composed of four exons, E1, E2, E3 and E3a, spanning 31 kb with intron sizes ranging from 7.5 to 10.1 kb (Figure 1a). A survey of ESTs revealed that three transcripts are generated, termed IKIP1–3. IKIP1 is transcribed from exons E1, E2 and E3, whereas IKIP2 is generated by the alternative use of exon E3a instead of E3. A splice variant of IKIP2, termed IKIP3, was found that resulted from the omission of E2; however, no corresponding splice variant omitting E2 was found for IKIP1 (Figure 1a). The presence of all three putative IKIP transcripts in endothelial cells was confirmed by real-time PCR (RT-PCR) using primers specific for the corresponding mRNAs and sequencing (Figure 1b). The exact transcription start site has not been determined experimentally; however, a group of EST sequences starts closely to the 5'-end of mRNA BC029415, which subsequently was used as a reference for the

transcription start. Further EST data analysis showed that at least three different sites (positions 1244, 1605, 2922) within the IKIP1 3'-UTR are used for polyadenylation, each preceded by a perfect polyadenylation signal (AATAAA). Transcripts containing exon E3a (IKIP2 and 3) are terminated at two different polyadenylation sites (positions 1322 and 1343 in IKIP2), each preceded by a perfect polyadenylation signal. The sequences of human IKIP1, 2 and 3 have been deposited in the EMBL database under the accession numbers AJ539425, AJ539426, and AJ539427, respectively.

IKIP protein domains

The predicted open reading frame of IKIP2 is slightly shorter (350 amino acids (aa)) when compared to IKIP1 (377 aa). The omission of exon 2 in IKIP3 leads to a frameshift, producing two potential open reading frames of 70 and 245 aa (Figure 1a); however, it is unclear whether these are actually translated *in vivo*. No striking homologies to other proteins were found in the databases; however, a weak homology between IKIP1 and myosin heavy chain (smooth muscle isoform) was detected (25% identity over 291 aa). Further, IKIP contains a hydrophobic region (aa 45–63) that may represent a signal anchor sequence, and two coiled-coil domains (116–259, 287–323). IKIP2 also contains the hydrophobic region but only one short coiled-coil domain (184–212). The region preceding the hydrophobic region carries a net positive charge, suggesting type I membrane orientation. In contrast to IKIP1, IKIP2 contains a potential leucine zipper (280–308) domain in the region encoded by exon E3a. A search for conserved protein domains in the NCBI-Conserved Domain Database revealed a significant hit, both in IKIP1 and IKIP2, within the regions encoded by exons E3 and E3a: Smc (NCBI COG database number COG1196) is a domain found in ATPases involved in chromosome segregation, recombination and DNA repair, and is predicted for IKIP1 at position 59–336 and for IKIP2 at position 84–350.

Evolutionary conservation of IKIP

The IKIP gene family was also found in other species including mouse, rat, pig, cattle and *Xenopus laevis*. No IKIP3-specific transcripts were found in these species. Full-length coding sequences were available or could be generated from EST and genomic data only for mouse and rat. Mouse and rat IKIP1 and 2 show predicted open reading frames of 373 and 345 aa, respectively. In the mouse, the IKIP gene is located on chromosome 10 and the exon–intron structure is conserved. Mouse IKIP1 and 2 are represented in the NCBI database under Gene ID 67454 and 69435, respectively. For rat IKIP, only partial sequences were available from EST data; nevertheless the complete coding region could be restored by alignment of the mouse transcripts to the genomic sequence AC095650 from rat chromosome 7. The sequences of rat IKIP1 and 2 have been deposited in the EMBL database under the accession numbers BN000112 and BN000113, respectively. A multiple sequence alignment of IKIP1 and 2 from man, mouse and rat is shown in Figure 2.

Although low, there is still a detectable degree of similarity between exons E3 and E3a (11% identity between the human
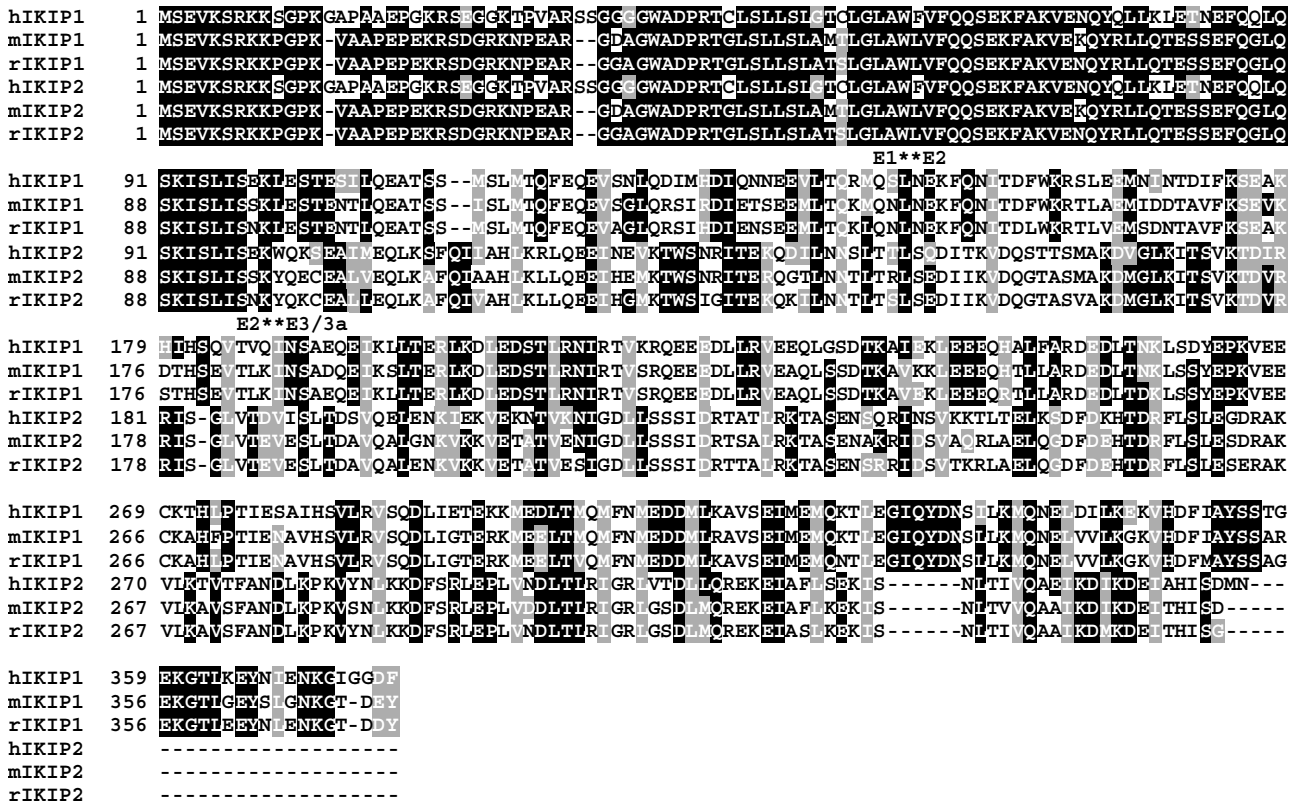
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hIKIP1    1 MSEVKSRKKSGPKGAPAAEFGKRSEGGKTFVARSSGGGWADPRTCLSLLSLGTCLGLAWFVFQQSEKFAKVENQYQLLKLETNEFCQLQ
mIKIP1    1 MSEVKSRKKPGPK-VAAPEPEKRSDGRKNPEAR--GDAGWADPRTGLSLLSLAMILGLAWLVFQQSEKFAKVEKQYRLLQTESSEFQGLQ
rIKIP1    1 MSEVKSRKKPGPK-VAAPEPEKRSDGRKNPEAR--GGAGWADPRTGLSLLSLATSLGLAWLVFQQSEKFAKVENQYRLLQTESSEFQGLQ
hIKIP2    1 MSEVKSRKKSGPKGAPAAEFGKRSEGGKTFVARSSGGGWADPRTCLSLLSLGTCLGLAWFVFQQSEKFAKVENQYQLLKLETNEFCQLQ
mIKIP2    1 MSEVKSRKKPGPK-VAAPEPEKRSDGRKNPEAR--GDAGWADPRTGLSLLSLAMILGLAWLVFQQSEKFAKVEKQYRLLQTESSEFQGLQ
rIKIP2    1 MSEVKSRKKPGPK-VAAPEPEKRSDGRKNPEAR--GGAGWADPRTGLSLLSLATSLGLAWLVFQQSEKFAKVENQYRLLQTESSEFQGLQ
                                                                                       E1**E2
hIKIP1   91 SKISLISEKLESTESILQEATSS--MSLVTQFEQEVSNLQDIMHDIQNNEEVLTRLQSLNEKFQNITDFWKRSLEIMNINTDIFKSEAK
mIKIP1   88 SKISLISSKLESTENTLQEATSS--ISLVTQFEQEVSGIQRSIIDIETSEEMLTKIQNLNEKFQNITDFWKRTLAIMIDDTAVFKSEVK
rIKIP1   88 SKISLISNKLESTENTLQEATSS--MSLVTQFEQEVAGIQRSIHDIENSEEMLTKIQNLNEKFQNITDLWKRTLVIMSDNTAVFKSEAK
hIKIP2   91 SKISLISEKWQKSEAIMEQLKSFQILAHIKRLQEEINEVKTWSNRITEIQDILNISITTLSQDITKIDQSTTSMAKDVGLKITSVKTDIR
mIKIP2   88 SKISLISSKYQECEALVLEQLKAFQIAAHIKLLQEEIHEIKTWSNRITEIQGTLNITTRLSEDIIKIDQGTASMAKDMGLKITSVKTDVR
rIKIP2   88 SKISLISNKYQKCEALIEQLKAFQIVAHIKLLQEEIHGIKTWSIGITEIQKILNITTSLSEDIIKIDQGTASVAKDMGLKITSVKTDVR
             E2**E3/3a
hIKIP1  179 HIHSQVTVQINSAEQEIKILTERLKDIEDSTLRNIRTVKRQEEIDLLRVEEQLGSDTKAIEKIEEEQIALFARDIDLTNKLSDYEPKVEE
mIKIP1  176 DTHSEVTLKINSADQEIKSLTERLKDIEDSTLRNIRTVSRQEEIDLLRVEAQLSSDTKAVKKIEEEQITLIARDIDLTNKLSSYEPKVEE
rIKIP1  176 STHSEVTLKINSAEQEIKILTERLKDIEDSTLRNIRTVSRQEEIDLLRVEAQLSSDTKAVEKIEEEQITLIARDIDITDKLSSYEPKVEE
hIKIP2  181 RIS-GLVTDVISLTDSVQEIENKIEKVEKNTVKNIGDILSSSIDRTATIRKTASENIQRINSVKKTLTELKSDFIKHTDRFLSIEGDRAK
mIKIP2  178 RIS-GLVEESLTDAVQAIGNKVKKVETATVENIGDILSSSIDRTSAIRKTASENAKRIDSVAQRLAELQGDFIDEHTDRFLSIESDRAK
rIKIP2  178 RIS-GLVEESLTDAVQAIENKVKKVETATVESIGDILSSSIDRTTAIRKTASENSRRIDSVTKRLAELQGDFIDEHTDRFLSIESERAK

hIKIP1  269 CKTHIPTIESAIHSVLRYSQDLIETEKKIEDLTMQIFNIEDDMLKAVSEIMEMQKTIEGIQYDNSILKMQNEIDILKEKVHDFIAYSSTG
mIKIP1  266 CKAHFPTIEIAVHSVLRYSQDLIGTERKIEELTYQIFNIEDDMLKAVSEIMEMQKTIEGIQYDNSILKMQNELVVLKGKVHDFIAYSSAR
rIKIP1  266 CKAHIPTIEIAVHSVLRYSQDLIGTERKIEELTVQIFNIEDDMLKAVSEIMEMQNTIEGIQYDNSILKMQNELVVLKGKVHDFMAYSSAG
hIKIP2  270 VLKTVTFANDLKPKVYNIKKDFSRLEPLVNDLTIRIGRIVTDLLOREKEIAFLSEKIS------NITIVQAEIKDIKDEIAHISDMN---
mIKIP2  267 VLKAVSFANDLKPKVSNIKKDFSRLEPLVNDLTIRIGRIGSDLMQREKEIAFLKEKIS------NITVVQAAIKDIKDEITHISD-----
rIKIP2  267 VLKAVSFANDLKPKVYNIKKDFSRLEPLVNDLTIRIGRIGSDLMQREKEIASLKEKIS------NITIVQAAIKDIKDEITHISG-----

hIKIP1  359 EKGTLKEYNIENKGIGGDF
mIKIP1  356 EKGTLGEYSIGNKGT-DEY
rIKIP1  356 EKGTLEEYNIENKGT-DDY
hIKIP2      -------------------
mIKIP2      -------------------
rIKIP2      -------------------
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Figure 2 Multiple sequence alignment of IKIP1 and IKIP2. Protein sequences from human (h), mouse (m) and rat (r) were aligned using CLUSTALW. Identical residues are highlighted as white text on a black background, and similar residues as white text on a gray background. The boundaries between individual exons are marked by asterisks below the sequences

protein sequences), which is significantly above the value obtained when comparing E3 with randomized sequences of E3a (between 5 and 9%). The randomization was performed using the EMBOSS-tool SHUFFLESEQ,[16] which randomizes a sequence maintaining its composition. Concerning the full-length protein sequences, there is 77% (77%) identity between human and mouse IKIP1 (IKIP2), 79% (77%) between human and rat, and 91% (91%) between mouse and rat. The notion that exons E3 and E3a share a low but significant homology is further supported by the detection of the Smc domain in both corresponding protein sequences.

Expression analysis

The expression of IKIP1 was investigated by Northern blotting using an IKIP1-specific probe and revealed expression predominantly in the human lung, kidney, spleen, thymus and skeletal muscle (Figure 3), as well as human umbilical vein endothelial cells (HUVECs) (not shown). IKIP1 was not regulated by the inflammatory stimuli IL-1, TNFα or bacterial lipopolysaccharide (LPS) in endothelial cells over a period of 4 h as revealed by RT-PCR (data not shown). The sizes of the three predicted human IKIP1 transcripts (1.3, 1.6, 2.9 kb) correspond largely to the three main bands seen on the Northern blot. The longest transcript appears to be the predominant one.
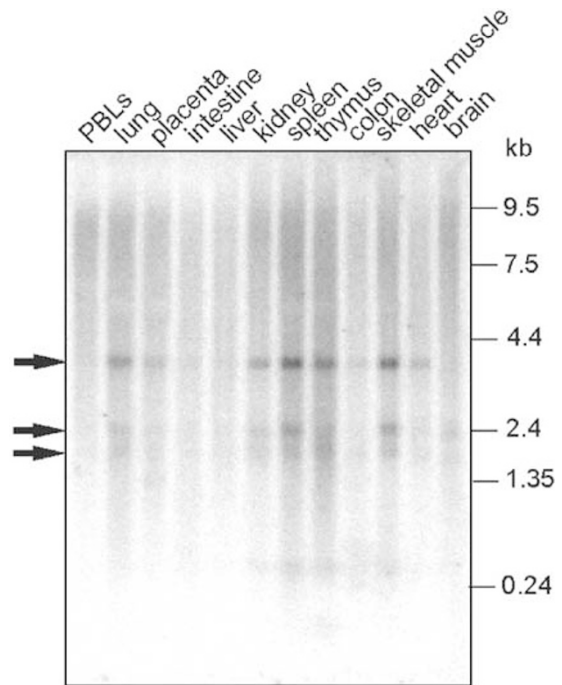
Figure 3 Northern blot analysis of IKIP1. An IKIP1-specific cDNA probe was used to analyze the expression of IKIP1 in different tissues as indicated using an MTN blot (Clontech). Arrows on the left indicate the main IKIP transcripts

IKIP localizes to the endoplasmic reticulum

To investigate the subcellular distribution of IKIP, we transfected a fusion gene of IKIP1 with the yellow variant of green fluorescent protein (GFP) (EYFP-IKIP1) into HUVECs, followed by detection by fluorescence microscopy. Following transfection into HUVECs, EYFP-IKIP1 localized to perinuclear structures in the cytoplasm. By cotransfection of subcellular localization markers that are fusion genes with the cyan variant of GFP (ECFP) and that can be distinguished by fluorescence microscopy from EYFP due to their different excitation and emission spectra, we found that IKIP1 localizes to the ER (Figure 4a). A similar localization was found for IKIP2 (Figure 4b). To investigate the intracellular localization of endogenous IKIP, we generated an antibody against an N-terminal peptide of IKIP1 (see Materials and Methods). Immunofluorescence showed a similar intracellular localization as the transfected construct (Figure 4c).

Next, we generated a set of myc-tagged N- and C-terminal deletion mutants that lack parts of the protein corresponding to the coiled-coil domains or the hydrophobic stretch (Figure 5a). Deletions affecting the hydrophobic region
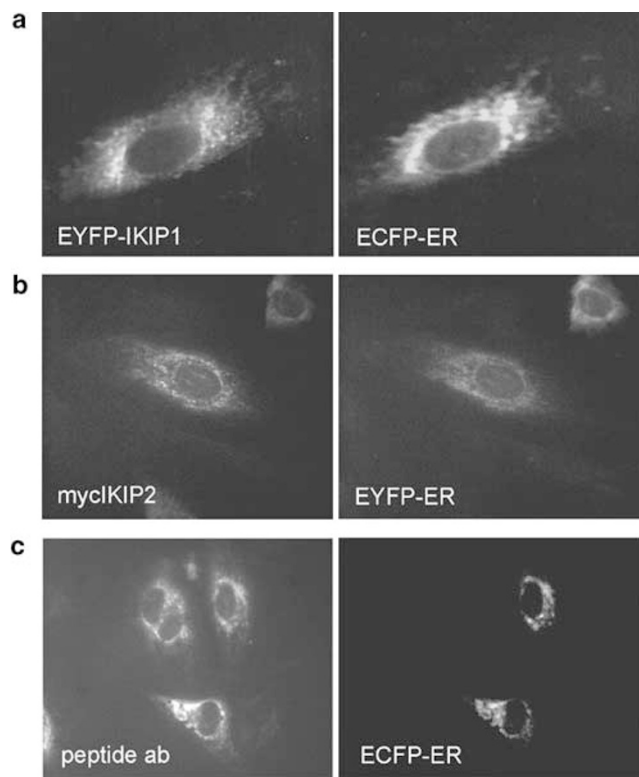
Figure 4 Subcellular localization of IKIP. (**a**) An EYFP-IKIP1 fusion gene was cotransfected together with an ECFP-tagged marker for the ER into HUVECs, and subcellular localization was revealed by fluorescence microscopy using band-pass filters for EYFP (left) and ECFP (right). (**b**) Myc-IKIP2 and EYFP-ER expression vectors were cotransfected. Left: IKIP was detected with the anti-myc antibody and APC-conjugated anti-mouse secondary antibody; right: EYFP-ER. (**c**) Cells were transfected with the ECFP-ER vector alone, and (left) endogenous IKIP was detected by an affinity-purified antibody raised against a common N-terminal peptide of IKIP1 and 2, followed by Alexa568-cojugated anti-rabbit immunostaining; right: ECFP. Note that only transfected cells express the ECFP-ER marker, whereas all cells express IKIP

between aa 45 and 63 resulted in uniform distribution throughout the cell, suggesting that this region is responsible for ER localization (Figure 5b).

Glycosylation of IKIP

We noted that in Western blots of high-resolution gels, IKIP1 migrated as a double band. Bioinformatic analysis predicted one potential N-linked glycosylation site in IKIP1 at aa position 154, and two sites at aa positions 144 and 328 in IKIP2. We therefore transfected myc-IKIP1 and 2 into HUVECs, and 2 days later treated the cells with tunicamycin (TN) for 12 h and analyzed IKIP by Western blotting. Whereas in untreated cells IKIP1 migrated as a double band, the intensity of the upper band was reduced in TN-treated cells. Furthermore, incubation of cell extracts with endoglycosidase H led to complete disappearance of the upper band, demonstrating that the higher molecular weight band is due to glycosylation (Figure 6a). Likewise, IKIP2 with two predicted N-linked glycosylation sites migrated as three distinct bands on high-resolution gels, and the upper bands were removed by either incubation of the cells with TN or by treatment of the extracts with endoglycosidase H. After mutation of the Asn residue in position 154 to Ala, this mutant (termed IKIP1mut154) migrated as a single band that corresponded to the deglycosylated form of the protein, confirming that this residue is the site for glycosylation (Figure 6, right panel). Mutation of this glycosylation site did not change the subcellular localization, as IKIP1mut154 also localized to the ER (Figure 6b).

Analysis of the common APAF1/IKIP promoter reveals regulation by p53 and X-irradiation

From published and EST data, the distance between the APAF1 and IKIP transcription start sites was found to be only 488 bp. This close linkage was also conserved in the mouse. Using APAF1 and IKIP-specific primers, this region was amplified by PCR from human DNA, sequenced and found to be identical to the published genomic sequence. This close physical linkage suggests that APAF1 and IKIP may share a common promoter, common regulatory elements, and even indicate a functional relationship of the two genes. The presence of potential transcription factor binding sites was determined using MatInspector professional in combination with the TRANSFAC database.[17] With classical TATA and CCAAT boxes being absent, the promoter 'backbone' is mainly built by a series of SP-1 sites. From the variety of potential transcription factor binding sites (see Figure 7a), p53 and E2F-1 have been found to be functional and lead to increased APAF1 expression.[18,19] We therefore tested the p53 dependence of IKIP by transfecting a p53 expression vector into HEK 293 cells and analyzed IKIP expression by Western blotting using an antiserum generated against an N-terminal peptide of IKIP1. Transfection of p53, but not of NF-AT that was used as a negative control, increased IKIP1 protein levels in a dose-dependent manner (Figure 7b). Similar results were obtained in HUVECs (not shown).

To further support these data, we compared IKIP levels in p53-expressing and -deficient cells. As a model system we
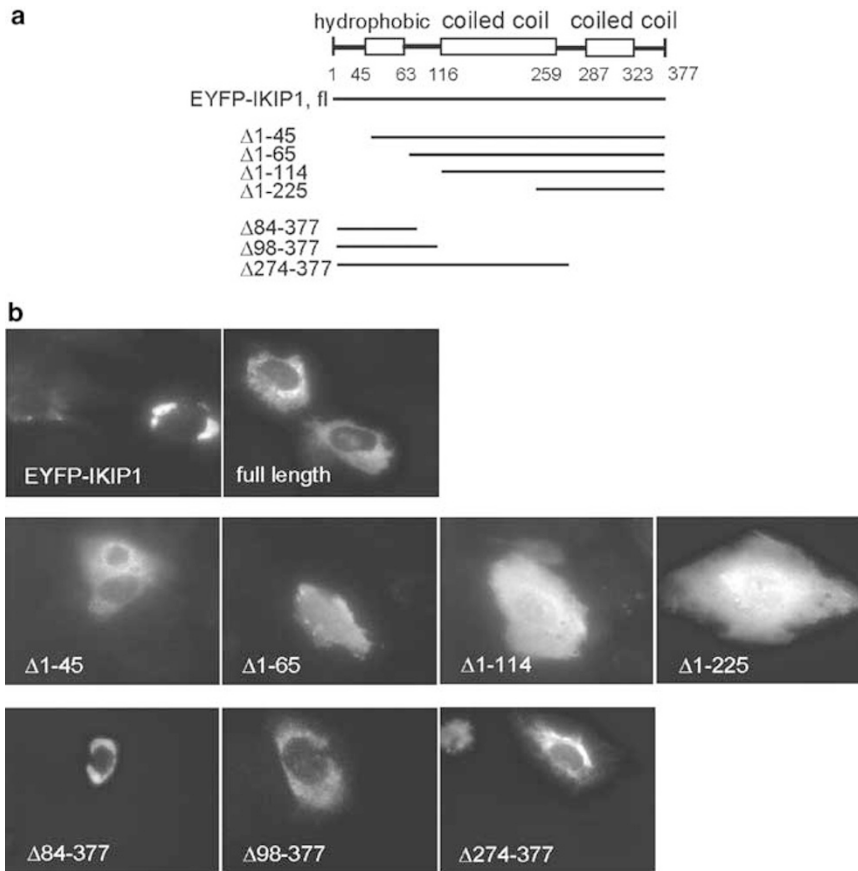
Figure 5 Subcellular localization of IKIP mutants. (**a**) Schematic representation of the different IKIP1 N- and C-terminal deletion mutants. (**b**) Myc-tagged IKIP1 mutants as well as the EYFP-IKIP1 fusion construct were transfected into endothelial cells followed by immunofluorescence detection

used the human osteogenic sarcoma cell lines U-2 OS and Saos-2, which are p53-wt and -deficient, respectively.[20] As shown by Western blotting using the anti-IKIP1 antiserum, the p53-deficient Saos-2 cells expressed significantly lower amounts of (endogenous) IKIP1 (Figure 7c). No changes were detected in response to TNFα. In order to test whether inducers of p53 (and of APAF1) would also enhance IKIP expression, we irradiated HUVECs with X-radiation. A 5 Gy radiation caused increased expression of (endogenous) APAF1 and IKIP1 4 h after irradiation, as determined by Western blotting (Figure 7d). Consistent with published data, p53 levels started to increase already at 2 h after irradiation,[21] thus preceding APAF1 and IKIP expression, and consistent with the notion that p53 is a transcription factor involved in the regulation of both of these genes. To test for a functional relationship between p53 and IKIP, we inhibited p53 expression in U-2 OS cells using siRNA, followed by X-irradiation. Western analysis demonstrated that in p53 knockdown cells IKIP expression was significantly reduced, demonstrating that IKIP is a p53-dependent gene.

Overexpression of IKIP1 promotes apoptosis

Upon transfection of IKIP into cells, we noted increased cell death. To investigate whether this is due to apoptosis, HUVECs were transfected with a myc-IKIP1 construct followed by TUNEL staining. As shown in Figure 8a, IKIP1-positive cells also showed positive staining in the TUNEL stain, indicating that overexpression of IKIP1 leads to enhanced apoptosis. When transfecting IKIP1 mutants, we found that the N-terminal deletion mutant (Δ1–65) still promoted apoptosis, whereas the C-terminal mutant (Δ274–377) did not, indicating that the ability of IKIP1 to promote apoptosis resides specifically in its C-terminal part. In contrast, glycosylation of IKIP1 appeared not to be necessary, since the gylcosylation mutant IKIP1mut154 showed a similar ability to promote cell death as the wild-type (wt) protein in two different cell types, HUVECs and U-2 OS cells (Figure 8b).

Discussion

The intriguingly close linkage of IKIP to APAF1, a key component of the intrinsic pathway of apoptosis, has prompted our analysis of the regulation, subcellular localization and function of IKIP. Since the two genes share a common 488 bp intergenic region from which they are transcribed in opposite directions, it could be expected that regulatory mechanisms that are operative in this region affect the expression of both genes. Thereby both common but also mutually exclusive regulation can be envisaged, the latter especially since the described promoter region of APAF1 extends into the transcribed region of IKIP.
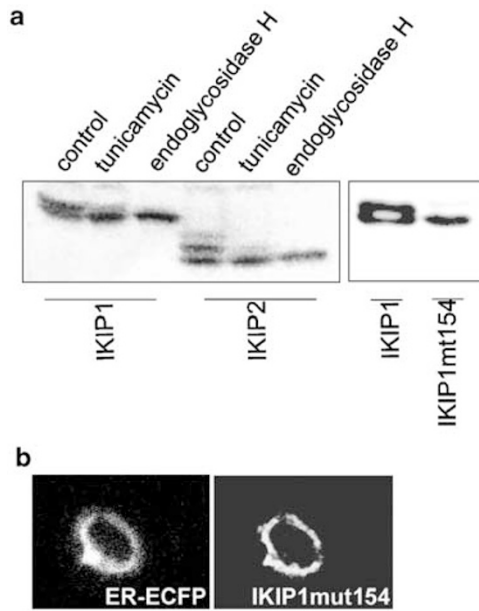
Figure 6 (**a**) Western blot analysis of IKIP1 and 2 glycosylation. Myc-IKIP1 and 2 were transfected into HUVECs, and after 2 days the cells were either left untreated or treated with TN for 12 h as indicated. Extracts were analyzed by Western blotting using an anti-myc antibody. Endoglycosidase H treatment of extracts from untreated, IKIP-transfected cells was carried out as indicated. The right panel shows an IKIP1 mutant (IKIP1mut154) where the potential glycosylation site was changed to Ala, in comparison to IKIP1 wt. (**b**) Myc-tagged IKIP1mut154 was transfected together with the ER marker ER-ECFP into HUVECs and subcellular localization was revealed by immunostaining with APC-conjugated anti-myc antibody

A recent survey of transcribed sequences in the human genome has revealed that bidirectional promoters occur rather frequently;[22] however, the mechanisms of regulation of all but a few bidirectional genes are unknown. The same study shows that the transcripts of many bidirectional pairs are coexpressed, whereas some are antiregulated, and further that many of the promoter segments between two bidirectional genes contain shared elements that regulate both genes.

Expression of APAF1 has been shown to be regulated by p53 and E2F-1.[6,18,19] In our experiments using transient transfections, p53-deficient cells, as well as p53 siRNA, we could demonstrate that IKIP expression is dependent on p53 (Figure 7b, c and e). However, no regulation by E2F-1 could be observed (not shown). X-irradiation, a commonly used inducer of p53, also upregulated IKIP, and IKIP expression was diminished in p53 knockdown cells. Therefore, in these settings, common regulatory mechanisms appear to control the expression of APAF1 and IKIP.

The well-described proapoptotic function of APAF1 has prompted us to also test IKIP for influencing apoptosis. Indeed, when transfected into cells, IKIP1 lead to increased apoptotic cell death. Using N- and C-terminal deletion mutants we found that the ability to confer proapoptotic function to IKIP resides in its C-terminal and core domain, while its N-terminal domain is dispensable for triggering apoptosis. However, it is presently not clear how the proapoptotic function of IKIP, which resides in its C-terminus, is exerted, especially since it was not affected when changing the subcellular localization (e.g., of the ΔN mutant). A possible explanation could be a
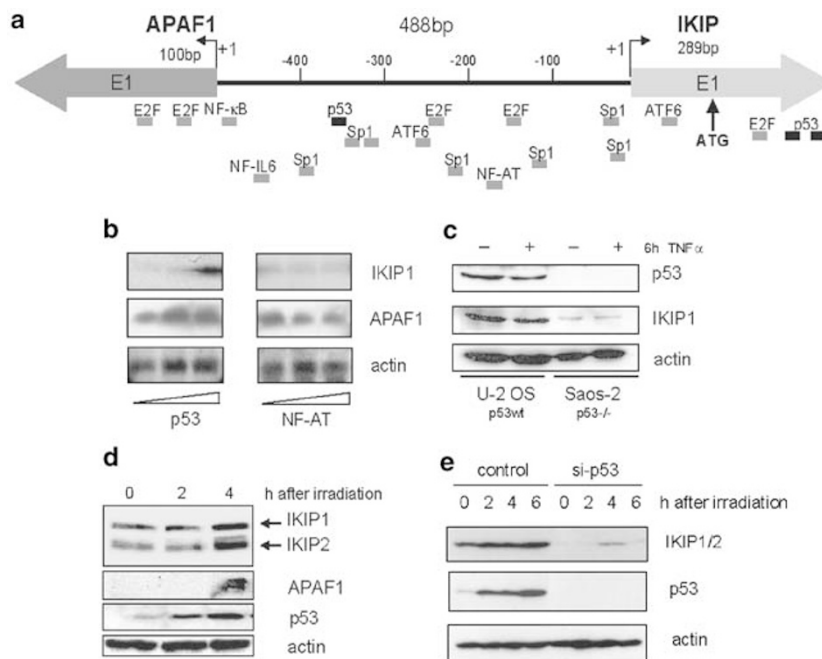

Figure 7 IKIP and APAF1 share a common promoter and are regulated by p53 and X-irradiation. (**a**) The genomic region between the human IKIP and APAF1 genes is shown. The names and positions of selected transcription factor binding sites, either predicted by MatInspector or as published,[6,18,19] are indicated. Numbers are relative to the IKIP transcription start site (+ 1). (**b**) Human 293 cells were transfected with increasing amounts of p53 and NF-AT expression vectors as indicated and cells were analyzed for APAF1 and IKIP1 expression by Western blotting. (**c**) Human p53 expressing U-2 OS and p53-deficient Saos-2 osteosarcoma cells were treated with TNFα for 6 h or left untreated as indicated, and analyzed for IKIP1, p53 and actin by Western blotting. (**d**) HUVECs were irradiated with 5 Gy for the indicated times and analyzed by Western blotting for IKIP, APAF1 and p53 expression. (**e**) U-2 OS cells were transfected with an expression vector for p53 siRNA (pSUPER-p53) or control vector, irradiated with 5 Gy for the indicated times and analyzed by Western blotting for IKIP and p53 expression
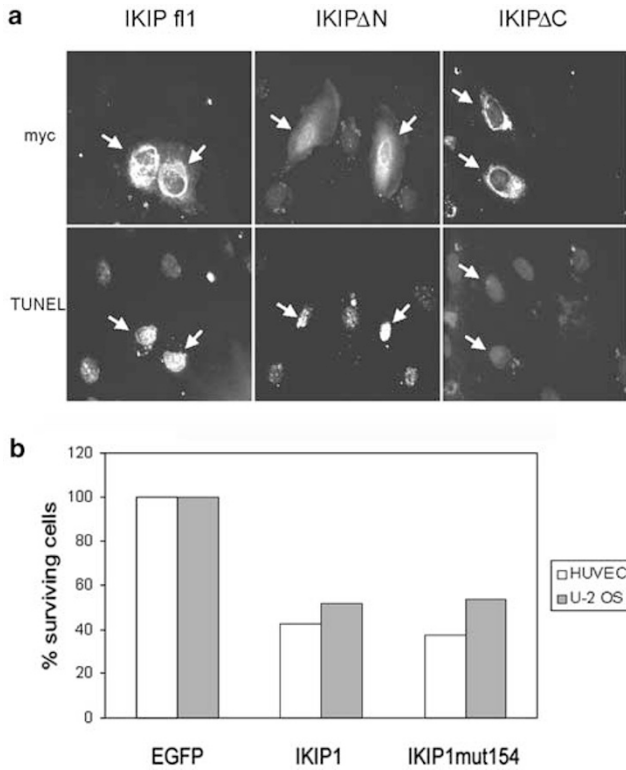
Figure 8 IKIP1 promotes apoptosis. (**a**) HUVECs were transfected with myc-tagged full-length IKIP1 and with the N- and C-terminal deletion mutants IKIPΔN (Δ1–65) and IKIPΔC (Δ84–377), and apoptotic cells were detected by TUNEL assay (lower row). Expression of IKIP1 was detected by immunofluorescence using an anti-myc antibody and APC-conjugated secondary antibody (upper row). The arrows indicate IKIP1 and TUNEL double positive cells. (**b**) Comparison of IKIP1 and its glycosylation mutant IKIP1mut154. Expression vectors for both genes together with EGFP were transfected into HUVECs (open bars) or U-2 OS cells (shaded bars), and the number of EGFP-positive cells was determined 1 and 2 days later. Values are given as % surviving cells in relation to EGFP alone on day 2 as compared to day 1 after transfection

cleavage event that results in a C-terminal fragment with altered localization. Cleavage of proteins at the ER membrane has been described;[23] however, for IKIP this has to await experimental verification.

By the use of an antibody generated against IKIP, as well as transient transfection of wt and mutant constructs, we found that IKIP localizes to the ER, and that its hydrophobic domain (aa 45–63) is necessary for this localization. This hydrophobic stretch is reminiscent of a signal anchor sequence[24] that would predict insertion of IKIP into the ER membrane. In addition, the region amino terminal to the hydrophobic stretch carries a net positive charge, predicting that the amino terminus of IKIP would face the cytoplasm and its carboxy-terminal part protrude into the lumen of the ER. This is consistent with our data demonstrating that IKIP1 (and IKIP2) are glycosylated, with the sites of glycosylation predicted (and in the case of IKIP1 experimentally verified) to be in the region that is inserted into the lumen of the ER.

It is presently not clear how IKIP is retained in the ER. The ER retention/retrieval process can be mediated through discrete short protein motifs; however, the classical ER retention signal (KDEL) is only relevant for luminal proteins.

Retention signals for ER transmembrane proteins are more complex, and cognate motifs include RXR, KKXX and XXRR,[25] none of which are present (in the correct position) in IKIP. Thus, the mechanism for IKIP retention in the ER remains to be elucidated.

The localization of a novel proapoptotic protein to the ER underscores the emerging role of this organelle in apoptosis. During the last years, the regulation of apoptosis by ER pathways has been the subject of intense investigation (for review, see Breckenridge et al.[7]). In particular, the ER has been shown to participate both in Fas- and p53/DNA damage-mediated apoptosis, and the ER membrane has been recognized as a locus where an initiator caspase is activated. Our identification and initial characterization of IKIP may therefore serve as a starting point for an additional aspect of ER-mediated apoptosis.

Materials and Methods

Cell culture

HUVECs were isolated and cultured in M199 medium supplemented with 20% FCS, antibiotics, endothelial cell growth supplement (ECGS) and heparin as described.[26] Human HEK 293 cells were obtained from ATCC and propagated in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, antibiotics and glutamine. Human U-2 OS and Saos-2 cell lines were propagated in DMEM with 10% FCS and antibiotics.

Plasmids and primers

Myc-IKIP1 was obtained by PCR using cDNA from HUVECs as template with the primers IKIP1fo 5′-aaaagtcgacctctgaggtgaagagccg-3′ and IKIP1rev 5′-aaaagcggccgcttaaaaatcaccaccaattc-3′ followed by digestion with SalI and NotI and ligation into pCMV-myc (Clontech, Palo Alto, USA). Accordingly, IKIP2 was obtained using IKIP1fo with IKIP2rev 5′-aaaagcggccgctaattcatatctgaaatgtg-3′. The primers used to confirm the expression of the different splice variants in HUVECs as shown in Figure 2 were as follows: #1: IKIP1fo; #2: IKIP1rev; #3: 5′-ctgtagacgcttttagatg-3′; #4: IKIP2rev. Primers for generating the IKIP1 mutants were as follows. For N-terminal deletions the primers were: Δ1–45: 5′-aaaagtcgaccat gacgtgcctgagcctgc-3′; Δ1–65: 5′-aaaagtcgaccatgcagtcagaaaaatttgcaaa-3′; Δ1–114: 5′-aaaagtcgaccatgtctttgatgacccagt-3′, together with the reverse primer IKIP1rev. The Δ1–255 deletion mutant was the IKIP1 fragment originally isolated from the yeast two-hybrid screen comprising aa 256–377. For the C-terminal deletions the primers were: Δ84–377: aaaagcggccgcttatggcaaatgtgtcttgca-3′; Δ98–377: 5′-aaaagcggccgctta catcaaagacatggatgat-3′; Δ274–377: aaaagcggccgcagaaaatcaccaccaat tcctt-3′, together with the forward primer IKIP1fo. The genomic region between the APAF1 and IKIP genes was amplified from human genomic DNA using the primers APIKfo 5′-cacctctggttctatcccttttg-3′ and APIKrev 5′-gcacagcggacaggaagtaac-3′. For the generation of the glycosylation mutant of IKIP1, IKIP1mut154, the primers 154left 5′-gcggatttctggaaga gaagcct-3′ and 154right 5′-aatattctgaaatttctcattaaggc-3′ were used in PCR to change the codon for Asn at aa position 154 to Ala. Expression vectors for flag-IKK2, HA-IKK1, MEKK1, NIK, TAK1, p38 and the 5xNF-kB-Luc reporter construct have been described previously.[27] The expression construct for p53 siRNA (pSUPER-p53) was obtained from OligoEngine (Seattle).

The IKIP1-EYFP fusion construct was generated by cloning the full-length IKIP1 into the EYFP-C1 vector (Clontech) resulting in a fusion gene

with the yellow variant of GFP fused to the N-terminus of IKIP1. The intracellular localization markers consisting of expression vectors for the cyan or yellow variants of GFP (ECFP and EYFP, respectively) fused to different signal sequences that target proteins to intracellular compartments were obtained from Clontech.

Transient transfections and immunofluorescence

For intracellular localization studies, the different myc-tagged IKIP constructs were transfected into HUVECs using LipofectaminePlus (InVitrogen) as described by the manufacturer, and at a ratio of DNA/Lipofectamine/Plus reagent of 1 μg/3 μl/6 μl for 2.15 h. After 24 h, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 and incubated with a monoclonal anti-myc antibody (Oncogene, #OP10L; dilution 1 : 100) followed by Alexa488-conjugated rabbit anti-mouse antibody (Molecular Probes; A-11059; dilution 1 : 5000). For viewing ECFP and EYFP, a Nikon Diaphot TMD microscope equipped with XF104 and XF114 filter sets (Omega Opticals Inc., Brattleboro, VT, USA) specific for EYFP and ECFP, respectively, was used as described previously.[28] Images were taken by means of a cooled CCD camera (Kappa DX30, Kappa GmbH, Gleichen, Germany) using the manufacturer's software (Kappa Image Base). Transfection of HEK 293 cells was carried out by the calcium phosphate method as described.[29]

Preparation of anti-IKIP antiserum

Antiserum was prepared by immunization of rabbits with a KLH-conjugated synthetic peptide corresponding to the common N-terminal 15 aa of IKIP1 and IKIP2. The serum was affinity-purified with the immunizing peptide (Eurogentec, Belgium).

Western analysis

Myc-IKIP-transfected cells were analyzed using an anti-myc antibody (Oncogene, #OP10L; dilution 1 : 1000) and peroxidase-conjugated rabbit anti-mouse second antibody (Amersham Pharmacia; NA931V) at a dilution of 1 : 5000 in PBS/1% BSA, followed by detection with ECLplus (Amersham Pharmacia). Other antibodies were obtained from Chemicon (anti-APAF1) and Santa Cruz (anti-p53, anti-actin).

Glycosylation experiments

For inhibition of glycosylation, HUVECs were incubated with TN (Sigma) at a concentration of 10 μg/ml for 12 h; for deglycosylation experiments, cell extracts were prepared and incubated with endoglycosidase H (Roche Diagnostics) at a concentration of 250 mU/ml for 12 h before Western analysis.

Apoptosis and survival assays

Apoptotic cells were detected by TUNEL staining using the *In Situ* Cell Death Detection Kit (Roche) according to the manufacturer's protocol. For determination of cell death of the IKIP1mut154 construct, HUVECs and U-2 OS cells were transfected with either the IKIP wt or mutant together with EGFP, or EGFP alone as control, and the number of EGFP-positive and -negative cells was counted 1 and 2 days later. Values were expressed as % EGFP-positive cells on day 2 as compared to day 1, with the EGFP control-transfected samples set to 100%.

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