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Abstract

The PAS domain is a versatile protein fold found in many archaeal, bacterial, and plant proteins capable of sensing environmental changes in light intensity, oxygen concentration, and redox potentials. The oxygen sensor FixL from Rhizobium species contains a heme-bearing PAS domain and a histidine kinase domain that couples sensing to signaling. We identified a novel mammalian PAS protein (PASKIN) containing a domain architecture resembling FixL. PASKIN is encoded by an evolutionarily conserved single-copy gene which is ubiquitously expressed. The human PASKIN and mouse Paskin genes show a conserved intron-exon structure and share their promoter regions with another ubiquitously expressed gene that encodes a regulator of protein phosphatase-1. The 144-kDa PASKIN protein contains a PAS region homologous to the FixL PAS domain and a serine/threonine kinase domain which might be involved in signaling. Thus, PASKIN is likely to function as a mammalian PAS sensor protein.
Mammalian PASKIN, a PAS-Serine/Threonine Kinase Related to Bacterial Oxygen Sensors

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Footnotes:

The following mouse Paskin genomic nucleotide sequence has been deposited in the EMBL/GenBank/DDBJ databases (accession number AJ318757).

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ABSTRACT

The PAS domain is a versatile protein fold found in many archaeal, bacterial and plant proteins capable of sensing environmental changes in light intensity, oxygen concentration and redox potentials. The oxygen sensor FixL from *Rhizobium* species contains a heme-bearing PAS domain and a histidine kinase domain that couples sensing to signalling. We identified a novel mammalian PAS protein (PASKIN) containing a domain architecture resembling to FixL. PASKIN is encoded by an evolutionarily conserved single-copy gene which is ubiquitously expressed. The human *PASKIN* and mouse *Paskin* genes show a conserved intron-exon structure and share their promoter regions with another ubiquitously expressed gene that encodes a regulator of protein phosphatase-1. The 144 kDa PASKIN protein contains a PAS region homologous to the FixL PAS domain and a serine/threonine kinase domain which might be involved in signalling. Thus, PASKIN is likely to function as a mammalian PAS sensor protein.

KEY WORDS

Gene expression; hypoxia-inducible factor; iron; LOV-domain; nitrogen fixation; oxygen sensor; PAS-domain; protein phosphatase.
INTRODUCTION

Adaptation to environmental changes is essential to all organisms for survival and evolution. This adaptation requires specific sensors coupled to signalling cascades. While the sensing principles for some environmental parameters (such as light, sound, tension or temperature) are relatively well known, others are just being detected. Until recently, for instance, it has been unclear how mammalian cells sense oxygen. Even slight reductions in normal oxygen concentrations (hypoxia) can cause the induction of specific genes involved in mammalian oxygen homeostasis (reviewed in refs. 1, 2). Under hypoxic conditions, hypoxia-inducible factors (HIFs) serve as transcriptional regulators of these genes. Under normoxic conditions, HIF-α subunits are bound by the von Hippel-Lindau protein (pVHL) and subjected to rapid ubiquitinylation and proteasomal degradation (3). Binding of pVHL requires oxygen- and iron-dependent prolyl-hydroxylation of a conserved proline residue of HIF-α, suggesting that a prolyl-4-hydroxylase serves as the oxygen sensor that regulates the stability of HIF-α subunits (4, 5). However, because both the transactivation activity of HIF-α as well as the activity of many kinases are also regulated by oxygen concentrations (6-10), we postulate additional oxygen sensor(s) that regulate hypoxia-inducible phosphorylation of HIF-α.

In the nitrogen-fixing bacteria Bradyrhizobium japonicum and Rhizobium meliloti, the oxygen sensor is a histidine kinase termed FixL which under anaerobic conditions phosphorylates and thereby activates the transcription factor FixJ (11). The oxygen sensing domain of FixL is a so-called PAS domain, a versatile protein fold resembling to a left-handed glove that encloses a heme co-factor (12). When oxygen is bound to ferrous heme, this domain is in the flattened "off" state, resulting in a change of the protein conformation and inactivation of the kinase domain. The
PAS domain is very often found in sensor proteins of archaea, bacteria and plant species, where the corresponding sensor proteins measure light, oxygen and reduction-oxidation (redox)-potentials. These PAS sensor proteins often contain kinase domains, allowing to link sensing with signalling. While the structure of the PAS domain is highly conserved, there is only little primary sequence conservation (reviewed in refs. 13-16).

In mammalian proteins, the PAS domains identified so far seem to serve primarily as heterodimerization interfaces of transcription factors involved in the xenobiotic response, circadian rhythm and hypoxic adaptation. In fact, both HIF-α and HIF-β subunits contain N-terminal PAS domains that are essential for heterodimerization. Because the PAS domain turned up again in signalling pathways related to sensor mechanisms, we wondered whether it might be evolutionarily conserved also in the sensing mechanism itself. We identified a single mammalian gene containing a PAS domain and a serine/threonine kinase domain.
MATERIALS AND METHODS

Cloning and sequencing of PASKIN. The cDNA clone DKFZp434O1522 encoding full-length human PASKIN was obtained from the Resource Center/Primary Database (Berlin, Germany). The cDNA clone HA0/203 encoding KIAA0135 was a kind gift from T. Nagase (Chiba, Japan). The I.M.A.G.E. cDNA clone IMAGp998H022323 encoding part of mouse PASKIN was obtained from Research Genetics (Invitrogen) and the insert was completely sequenced. Part of the mouse PASKIN cDNA was amplified by RT-PCR using the primers Paskin5.1 (5'-CCACCTTCCCTCAGTTTG-3') and Paskin3.1 (5'-CAGCTCCAACTGAGCTTCCT-3'). Total RNA derived from mouse erythroleukaemia (MEL) cells served as template for the RT-PCR. The PCR product was subcloned into the Smal site of pUC19 resulting in the plasmid pmPaskin-PCR1.

The mouse Paskin gene was cloned from a genomic λ phage library (kind gift of U. Müller, Zürich, Switzerland) prepared from DNA which has been isolated from the mouse strain 129Sv(ev)-derived embryonic stem cell line AB-1, partially digested with Sau3AI and ligated into the vector LambdaGEM-11 (Promega). This library was screened twice by plaque hybridization to gel-isolated probes derived from the mouse (resulting in the λ phage clones λ.P1 to λ.P21) or the human (λ.P22 to λ.P36) cDNA. The mouse probe was the 1386 bp SalI-NotI fragment from mouse IMAGp998H022323 and the human probe was the 1060 bp XhoI-BamHI fragment from human HA0/203, labeled by random-primed labeling with (α-32P)dCTP (Hartmann). Positive λ clones were plaque purified, and the XhoI fragments were subcloned into pBluescript vectors (Stratagene). The Paskin gene was sequenced on both strands using automated sequencing procedures according to the instructions provided by the manufacturer (Applied Biosystems).
**DNA blot analysis.** Genomic DNA was isolated and analyzed using standard techniques (17). Bacterial DNA was a kind gift of H.-M. Fischer (Zürich, Switzerland). If not otherwise indicated, genomic DNA (10 µg) was digested overnight with EcoRI, separated by electrophoresis through 0.7% agarose gels, blotted to uncharged Biodyne A membranes (Pall) and cross-linked by UV irradiation (Stratalinker, Stratagene). The blots were hybridized to the human PASKIN probe in 6 x SSC, 10 x Denhardt's, 0.1% SDS, 1.1 mM Na₄P₂O₇, 17 mM Na₂HPO₄/NaH₂PO₄ (pH 7.7) and 200 µg/ml sonicated salmon sperm DNA (Sigma) for 15 h at 63°C. The blots were washed for 1 h in 0.1 x SSC, 0.2% SDS at 45°C, exposed, and re-washed for 1 h at 65°C. Radioactive signals were recorded by phosphoimaging (Molecular Dynamics).

**RNA blot analysis.** Total RNA isolation and Northern blotting procedures were performed as described previously (18). Mouse tissue RNA was subsequently hybridized to the mouse PASKIN cDNA probe and to a ribosomal protein L28 cDNA probe (18). The membranes were washed to a final stringency of 50°C in 0.1 x SSC, 0.2% SDS. A multiple human tissue dot blot containing 115 to 659 ng polyA⁺ RNA per dot was hybridized to the human PASKIN probe according to the manufacturer's instruction (Clontech). Final washing stringency was 1 h at 65°C in 0.1 x SSC, 0.2% SDS. The amount of polyA⁺ RNA was normalized to the mRNA expression levels of eight different housekeeping genes.

**In vitro translation, stable transfection and immunoblotting.** The oligonucleotide 5'-CATGGTGAGAGGATCGCATCACCATCACCATCACGC-3', encoding the peptide sequence MVRGS(H)₆A, was annealed to its antisense strand
and inserted into the *Ncol* site of the human PASKIN cDNA clone DKFZp434O1522. The resulting plasmid (pSportHis2PASKIN) was linearized with *NotI* and used as a template for coupled *in vitro* transcription/translation (IVTT) in wheat germ extracts according to the manufacturer's instructions (Promega).

The expression vector pcDNA3His2PASKIN was constructed by cloning the blunted *MluI* fragment from pSportHis2PASKIN into the *EcoRV* site of pcDNA3 (Invitrogen). pcDNA3His2PASKIN was linearized with *PvuI* and stably transfected into Chinese hamster ovary (CHO) cells as described previously (19). Total cellular protein was extracted with 10 mM Tris/HCl (pH 8.0), 1 mM EDTA (pH 8.0), 400 mM NaCl, 0.1% NP-40, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na$_3$VO$_4$ and 1 µg/ml each aprotinin/leupeptin/pepstatin A.

IVTT and CHO protein extracts were fractionated by 6% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Amersham-Pharmacia). The membrane was treated with blocking solution (4% defatted milk powder in PBS) and incubated for 1 h with a monoclonal anti-RGS-His antibody (Qiagen), diluted 1:1000 in blocking solution, followed by a rabbit anti-mouse horseradish peroxidase-coupled secondary antibody (SantaCruz), diluted 1:1000, which was detected by chemiluminescence with 100 mM Tris-HCl pH 8.5, 2.65 mM H$_2$O$_2$, 0.45 mM luminol, and 0.625 mM coumaric acid for 1 min, and exposure to X-ray films (Fuji).
RESULTS

Cloning of human PASKIN

With the aim of identifying mammalian proteins related to FixL, a BLAST search was performed using the *Bradyrhizobium japonicum* FixL sequence (EMBL/GenBank/DDBJ accession number P23222) as query. A single mammalian homologue was identified which we named PASKIN. The sequence of the human PASKIN cDNA was assembled from overlapping cDNA sequences deposited in the databases as a result of several large scale sequencing projects. The cDNA clone HA0/203 encoding KIAA0135 (accession number D50925) contained most of the PASKIN sequence and was completed using the DKFZp434O1522 cDNA (accession number AL043004) which contained additional 309 bp (88 amino acids) at the 5' end, including a consensus TCCCATGG translation initiation codon (20). This assembled cDNA sequence, designated human PASKIN, was 4306 bp long and encoded a predicted protein of 1323 amino acids (Fig. 1A). In addition, the HSU79240 cDNA clone (accession number U79240) was extended at the non-translated 3' terminus by 193 bp, most probably because of the use of alternative polyadenylation sites.

The human PASKIN gene was located to a previously reported 175 kb fragment of the human genome (accession number AC005237). Sequence comparison confirmed the assembled cDNA sequence. Recently, a novel PASKIN cDNA has been reported (accession number AF387103) which was extended by 29 bp at its 5' end. This fragment represents the untranslated first exon which was located 6.4 kb upstream of the second exon containing the translation initiation codon. Thus, PASKIN consists of 18 exons spread over 43.3 kb (Fig. 1B). Interestingly, the first exon of the PPP1R7 gene (accession number AF067130), encoding a putative mitotic regulator of protein phosphatase-1 (21), was located only
1.0 kb upstream of the first exon of PASKIN (Fig. 1B).

Cloning of mouse PASKIN

The IMAGE clone 934321 (accession number AA544838) was identified by BLAST analysis and was obtained from an IMAGE clone depository. Sequencing of the insert revealed that it encodes 1.5 kb of the 3' end of mouse PASKIN beginning at exon 14. The insert was used as a probe to screen a genomic mouse λ phage library and 21 phage clones (λP1 to λP21) were isolated. This library was then re-screened using a human PASKIN 5' probe derived from the DKFZp434O1522 cDNA clone, resulting in 15 additional phage clones (λP22 to λP36). The location of the phage clones was mapped (Fig. 1C) and the mouse Paskin gene was sequenced on both strands by a combination of restriction fragment subcloning and primer walking.

The intron-exon structure is well conserved between the human and mouse genes (Fig. 1B). The intron 5' and 3' splice sites of both genes conformed all with the GT/AG rule (22). As deduced by sequencing of the plasmid pmPaskin-PCR1, containing part of the mouse cDNA, the only difference in the splice site locations is the presence of additional 5 bp at the end of mouse exon 9 and additional 208 bp at the beginning of exon 10 (numbering according to the human sequence). This results in additional 71 amino acids in the predicted mouse PASKIN sequence, and the assembled mouse cDNA encodes for a predicted protein of 1390 amino acids.

An alternative splice acceptor 21 bp downstream of the start of intron 14 was identified in both the human and mouse genes, leading to a 7 amino acid insertion in the serine/threonine kinase domain of KIAA0135 which is not present in other database entries of human PASKIN cDNAs. By sequence comparison with the tentative mouse genome sequence (Celera database), we found that the mouse
Ppp1r7 gene (accession number AF067129) is located at a similar position relative to
Paskin gene as it is found in the corresponding human locus. Therefore, we predict
the existence of an untranslated, short mouse first exon at a position similar to the
human first exon (Fig. 1B).

PASKIN is encoded by an evolutionarily conserved single-copy gene
As shown by Southern blot analysis, the human PASKIN PAS domain probe
hybridized to a single band of the expected size with human genomic DNA, and
cross-hybridized also with genomic DNA derived from several rodent species (Fig.
2A), making it suitable for screening the mouse λ phage library (see above). A
detailed restriction mapping of mouse genomic DNA derived from 129 or C57Bl/6
strains and hybridization with a mouse probe resulted in bands of the expected size
without polymorphisms between the two strains, suggesting that mouse Paskin is a
single copy gene (Fig. 2B). These data could be confirmed by recent database
analyses of the human and mouse genome entries, which did not result in additional
matches. Of note, the human PAS domain probe also cross-hybridized with genomic
DNA derived from distinct bacterial species, including E. coli and B. japonicum (Fig.
2C), under the same hybridization stringency used for the human and rodent blots.
These results indicate evolutionary conservation of the gene encoding PASKIN.

Mammalian PASKIN is related to FixL
Using ProfileScan analysis, two N-terminal consensus PAS repeats, a PAS
associated sequence in-between them, and a C-terminal serine/threonine kinase
domain could be identified (Fig. 3). Human and mouse PASKIN are highly similar
within these domains, but share less similarity outside of them. An alignment of
human and mouse PASKIN to the *B. japonicum* FixL sequence using the Clustalw program revealed sequence conservation of the PAS repeats (Fig. 3). The FixL histidine kinase region weakly aligns to the long exon 10 of both human and mouse PASKIN, rather than to the serine/threonine kinase regions (Fig. 3).

**PASKIN is ubiquitously expressed**

Northern blot analyses were performed to determine the mRNA size and the expression pattern of PASKIN in both adult and embryonic mouse tissues. As shown in Fig. 4, the mouse PASKIN hybridization probe revealed a relatively weak band of approx. 4.5 kb in all tissues examined. To obtain a more complete overview on the tissue expression pattern, a human multiple tissue dot blot was hybridized with the human PAS domain probe under high stringency conditions that allowed hybridization with human but not mouse PASKIN DNA (data not shown). The dot blot contains normalized levels of polyA⁺ mRNA from a total of 61 different human adult tissues, 7 fetal tissues and 8 cell lines. As depicted in Fig. 5, the PASKIN probe hybridized with all of these samples, yielding relatively uniform signal intensities. As determined by phosphoimaging analysis, 2 to 4-fold higher expression levels compared to the mean value were found in caudate nucleus and putamen of the brain, in prostate and in testis; and a 5-fold reduced expression level was found in placenta. The PASKIN probe did not cross-hybridize with yeast RNA, human poly r(A) or human C₀t-1 DNA, confirming the specificity of the hybridization. In contrast, as shown by genomic Southern blotting (Fig. 2), the PASKIN probe cross-hybridized with *E. coli* DNA.
PASKIN is a soluble 144 kDa protein without extensive post-translational modifications

The open reading frame of the human PASKIN cDNA encodes for a protein of a predicted molecular weight of 144 kDa. To examine whether PASKIN is post-translationally modified, a RGSH$_6$ tag was added to the N-terminus of the human PASKIN cDNA allowing the detection of the fusion protein with a monoclonal anti-RGSH$_6$ tag antibody. RGSH$_6$-tagged PASKIN protein was synthesized in vivo and in vitro, by stable transfection of CHO cells (resulting in the cell line PKCHO) and by coupled in vitro transcription/translation (IVTT), respectively. Recombinant PASKIN protein was analyzed by immunoblotting as shown in Fig. 6. The detection of a band with a relative molecular weight of about 146 kDa (including the RGSH$_6$ tag) following protein synthesis both in vitro and in vivo, but not in maternal CHO cells, demonstrated that PASKIN is not extensively post-translationally modified. As expected from the primary sequence data, PASKIN remained in the supernatant following centrifugation at 20000 x g for 30 min, suggesting that PASKIN is soluble (data not shown).
DISCUSSION

The PASKIN protein reported in this work is an attractive candidate for a mammalian cellular sensor protein because first, it represents the first mammalian PAS protein containing a kinase domain that might link sensing to signalling; second, its PAS domain is more similar to the PAS domain of the oxygen sensor FixL than to any other known mammalian PAS protein; and third, it is ubiquitously expressed. Apart from this circumstantial evidence, we also tried to obtain experimental evidence for this hypothetical sensor function. The PAS domain of FixL contains heme as the oxygen-binding co-factor. Thus, the identification of a prosthetic group within the PAS domain would have given a further hint as to what sensing function of PASKIN might be. However, while bacterially expressed FixL showed the expected absorption spectrum, we did not observe a chromophore in bacterially expressed fragments of PASKIN containing the PAS domain (data not shown). Whether this is an artifact of the bacterial expression system or whether PASKIN does indeed not contain a chromophore (or even any co-factor at all) is currently a matter of investigation.

Although there are many different co-factors known for PAS domains of archaeal and bacterial species (13-16), the only ligand known so far for a mammalian PAS protein is dioxin, or dioxin-related xenobiotics, which bind the aryl hydrocarbon receptor, another heterodimerization partner of the HIF-1β subunit.

As shown by a multiple alignment, the PAS sensor domains have been structurally conserved between the *Rhizobium* FixL and mammalian PASKIN proteins, but the histidine kinase domain (weakly) matched to the intervening domain rather than to the serine/threonine kinase domain of PASKIN. Interestingly, mouse *Paskin* exon 10 to 12, which compose the intervening domain, are part of one long open reading frame (810 amino acids) that is interrupted by single stop regions within
small introns. Thus, it is tempting to speculate that this region is the remnant of an ancestor protein where the histidine kinase-like domain became inactivated during evolution and now spaces the PAS domain from the newly gained serine/threonine kinase domains in the mammalian PASKIN proteins, suggesting that PASKIN and FixL share a common ancestor. The idea of this "evolutionary exon erosion" is further supported by the fact, that the human gene contains a shortened exon 10 when compared to the mouse gene, whereas the positions of all other splice sites has been conserved.

Intriguingly, the analysis of the genomic structure of the human PASKIN gene revealed the presence of a short (1 kb) promoter region that is shared by the PPP1R7 gene (21). Analogously, we also found the mouse Ppp1r7 gene in the 5' region of the Paskin gene. So far, the location of the putative, very short first mouse exon could not be deduced by comparison with the human sequence. However, regarding the overall conservation of the structure of these four genes, the mouse Paskin exon 1 is likely to exist at a similar position as in the human PASKIN gene, although its definitive allocation awaits the identification of the 5' end of the cDNA. A comparison of the human PPP1R7 and mouse Ppp1r7 promoter regions has been reported by Ceulemans and colleagues (21). This promoter drives ubiquitous expression of the PPP1R7 gene (21). Assuming a bicistronic promoter activity, this is in agreement with our finding that PASKIN is ubiquitously expressed. The PPP1R7 gene had been mapped to human chromosome 2q37.3 and the Ppp1r7 gene to mouse chromosome 1 (21), hence representing also the chromosomal localizations of the human PASKIN and mouse Paskin genes.

The PPP1R7 gene encodes Sds22, a regulator of type 1 serine/threonine protein phosphatases (PP1). It will be interesting to elucidate whether co-expression
of the PASKIN and PPP1R7 genes from a single conserved genomic locus means that they are also functionally related. Phosphorylation as part of the signalling mechanism of an environmental sensor requires de-phosphorylation to turn off the signal. Intriguingly, the FixL protein is known to be regulated by autophosphorylation. Because Sds22 mediates target protein recognition of the PP1 protein phosphatase (23), it might be involved in dephosphorylation of autophosphorylated PASKIN, thereby resetting the sensor to its inactive state.

While this manuscript was in preparation, S. L. McKnight and co-workers reported the cloning of a human cDNA that is identical to PASKIN (24). This protein has been named PASK for PAS kinase. However, in order to distinguish PASKIN from an unrelated kinase which has been named PASK previously (25-27), we prefer the designation "PASKIN". McKnight and colleagues reported that the PASKIN kinase activity is activated by autophosphorylation and inhibited by the presence of the N-terminal PAS domain, whether in cis or in trans. Therefore, by assaying the kinase activity of PASKIN in an in vitro assay, it should be possible to define the nature of the environmental parameters for which PASKIN might serve as a sensor protein. Nevertheless, to unequivocally demonstrate the physiological relevance of PASKIN as a sensor protein, the generation of knock-out mice will be required. Based on the data reported here, we are currently targeting the Paskin gene in mouse embryonic stem cells.
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REFERENCES


FIGURE LEGENDS

**FIG. 1.** Primary structure of PASKIN. (A) Human PASKIN cDNA. The ATG translation initiation and TAA termination codons, respectively, as well as the PAS repeats and the serine/threonine kinase domain are indicated. (B) Gene structure of human *PASKIN* and mouse *Paskin*. Exons are indicated by black boxes numbered according to the human sequence. The mouse first exon (in parentheses) is tentative and could not be deduced by homology to the human first exon. (C) Map of the cloned λ phages encoding the mouse *Paskin* gene. Note the different scale bars.

**FIG. 2.** PASKIN is encoded by an evolutionarily conserved single-copy gene. Southern blot analyses of the genomic DNA isolated from human and rodent species (A), mouse (B) and several bacterial species (C). The blots were hybridized to a human PASKIN PAS domain probe (A, C) or to a mouse PASKIN probe (B).

**FIG. 3.** Alignment of human and mouse PASKIN. PAS domain (underlined) and PAS-associated domains (dashed line) of PASKIN aligned along with *Bradyrhizobium japonicum* FixL. The histidine kinase domain of FixL and the serine/threonine kinase domains of human and mouse PASKIN are indicated by dotted lines. Identical amino acids are shown in bold (indicated by asteriks). Colon, conserved amino acid; period, semi-conserved amino acid. Pairs of amino acids shown in italics indicate the position of introns.

**FIG. 4.** PASKIN expression in mouse tissues. Total RNA was isolated from several adult and embryonic mouse tissues and analyzed by Northern blotting. The
blot was subsequently hybridized to a mouse PASKIN probe and a ribosomal protein L28 probe. The positions of ribosomal 28S (4712 nt) and 18S (1869 nt) RNA were used as size markers.

**FIG. 5.** Ubiquitous PASKIN expression in human tissues. Dot blot analysis of normalized amounts of polyA⁺ RNA derived from the indicated tissues. The blot was hybridized to a human PASKIN probe under high stringency conditions.

**FIG. 6.** PASKIN protein analysis. Immunoblotting of His-tagged, recombinant human PASKIN produced by stably transfected CHO cells (CHO, maternal cell line; PKCHO, transfected cells) or by *in vitro* transcription/translation (IVTT).
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