pICln Binds to a Mammalian Homolog of a Yeast Protein Involved in Regulation of Cell Morphology*

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Since its cloning and tentative identification as a chloride channel, the function of the pICln protein has been debated. Although there is no consensus regarding the specific function of pICln, it was suggested to play a role, directly or indirectly, in the function of a swelling-induced chloride conductance. Previously, the protein was shown to exist in several discrete protein complexes. To determine the function of the protein, we have begun the systematic identification of all proteins to which it binds. Here we show that four proteins firmly bind to pICln and identify the 72-kDa pICln-binding protein by affinity purification and peptide microsequencing. The interaction between this protein and pICln was verified several ways, including the extraction of several pICln clones from a cDNA library using the 72-kDa protein as a bait in a yeast two-hybrid screen. The protein is homologous to the yeast Skb1 protein. Skb1 interacts with Shk1, a homolog of the p21Cd42Rac-activated protein kinases (PAKs). The known involvement of PAKs in cytoskeletal rearrangement suggests that pICln may be linked to a system regulating cell morphology.

Expression of the pICln cDNA in Xenopus laevis oocytes was correlated with the appearance of a nucleotide-sensitive chloride current (I = current, Cl = chloride, n = nucleotide-sensitive) (1–4). Although pICln was tentatively identified as an integral component of the chloride channel (1), several observations were inconsistent with the channel hypothesis for pICln. First, pICln lacks predicted hydrophobic membrane-spanning domains and structural homology to known channel proteins (1). Second, in mammalian cells and Xenopus oocytes, pICln was abundant and exhibited a predominantly cytoplasmic and nuclear localization, whereas a small fraction (n < 5%) was associated with the cytoskeleton (5). No pICln was detected in the plasma membrane.

The chloride conductance associated with expression of pICln was similar to an endogenous Xenopus oocyte chloride current elicited by hypotonic challenge (6). An anti-pICln antibody specifically ablated the swelling-induced chloride current in Xenopus oocytes (5), a finding supported by antisense experiments in mammalian cells (7). For the reasons stated above, we proposed that pICln was a cytosolic regulator of a swelling-induced chloride channel rather than a channel itself (5). In contrast, Paulmichl and co-workers (8) maintain that pICln is the swelling-induced chloride channel itself. Recently, data were presented suggesting that the chloride channel evoked by pICln expression has properties different from the swelling-induced chloride current, including a higher permeability to NO3, stronger outward rectification, and voltage-dependent nucleotide block (3). The molecular identification of the swelling-induced chloride channel has proven difficult, and several proteins including P-glycoprotein, pICln, CIC-2, and CIC-3 have been proposed to constitute this channel (9, 10). Although it seems unlikely that either pICln or P-glycoprotein are themselves chloride channels, both CIC-2 and CIC-3 are well established members of a family of chloride channel proteins. In contrast, pICln exhibits no significant homology to any known mammalian protein and contains no domains that suggest a specific function.

Although work from several laboratories supports a link between pICln expression and activation of a chloride current, the nature of this link is not clear. Currently there are no data to suggest that pICln directly regulates a chloride channel. Indeed, pICln may act far upstream from any plasma membrane-associated event and participate in such diverse functions as transcriptional or translational regulation, cytoskeletal rearrangement, or any one of several signal transduction cascades. pICln was shown previously to exist in several discrete complexes with other cytosolic proteins (5). We reasoned that the identification of proteins interacting with pICln might reveal functional connections to signaling pathways or known cellular mechanisms. Here we report the identification of one such pICln-interacting protein, a 72-kDa protein that appears to be the human homolog of Skb1. Skb1 is a yeast protein that interacts with Shk1, a homolog of the p21Cd42Rac-activated protein kinases (PAKs). Although the function of PAKs are only beginning to be understood, they appear to affect cell morphology through interactions with the cytoskeleton (11).

EXPERIMENTAL PROCEDURES

IBP72 Affinity Purification—Rat pICln coding sequence was subcloned into the pGEX-2T plasmid (Amersham Pharmacia Biotech). The GST-pICln fusion protein was expressed in BL-21 bacteria and purified over glutathione-Sepharose according to the manufacturer’s protocols. GST-pICln was immobilized using ActiGel ALD (Stereogene) at 2 mg of protein/ml of gel. Bovine ventricular tissue was minced and homogenized by Polytron (setting 7) for 3 × 30 s in MB buffer (10 mM NaHEPES [pH 7.5], 20 mM KCl, 1 mM EGTA, 3 mM MgCl2, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 2 mg/ml each of aprotinin, leupeptin, and pepstatin). Following centrifugation at 100,000 × g, the supernatant (2.6 g of protein) was loaded onto a 2 × 25 cm DEAE-Sepacel (Amersham Pharmacia Biotech) column and

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† The abbreviations used are: PAK, p21Cd42Rac-activated kinase; IBP, pICln-binding protein; MDCK, Madin-Darby canine kidney; GST, glutathione S-transferase; EST, expressed sequence tag; PCR, polymerase chain reaction.
pICln specifically (Fig. 1, lane 1). pICln was immunoprecipitated from [35S]methionine-labeled Madin Darby canine kidney (MDCK) total cell lysates using a polyclonal antibody generated to a GST-pICln fusion protein. Several proteins consistently co-immunoprecipitated with pICln (pICln-binding proteins (IBP)) with electrophoretic mobilities corresponding to molecular masses of 72, 43, 29, and 17 kDa (Fig. 1, lane 1). Since the same set of associated proteins was co-immunoprecipitated from the water-soluble cell fraction, we concluded that the IBPs are not membrane-associated proteins. Their association with pICln was judged to be specific because the same set of proteins was co-immunoprecipitated with a different anti-pICln antibody (data not shown).

IBP72 was purified by affinity to pICln. Cytosolic extracts from human heart were used as the source of IBP72. Initial experiments indicated that it was relatively abundant in this tissue. IBP72 was enriched significantly in eluates from the GST-pICln resin (Fig. 1, lane 2). Immobilized GST did not bind this protein, indicating that the 72 kDa protein interacted with GST-pICln specifically (Fig. 1, lane 3). The purified 72-kDa protein was digested with trypsin and cyanogen bromide, and five different peptides were sequenced. The peptide sequences obtained from the 72-kDa protein were used to screen the expressed sequence tag (EST) database. Several overlapping clones were identified that predicted a single open reading frame (ORF) of 1911 base pairs and whose translation contained sequences identical with the 72-kDa protein-derived peptides. Subsequently, two EST clones containing an identical 2.4-kb insert were identified (GenBank accession numbers R13970 and AA099674) that spanned the ORF. Using 5′-rapid amplification of cDNA ends with a human fetal brain library, an additional 60 bases of 5′-untranslated sequence were identified.

The ORF predicts a protein of 637 amino acids with a molecular mass of 72.6 kDa (Fig. 2A). Consistent with this prediction, in vitro translation of the cDNA yielded a protein with an apparent molecular mass of 72 kDa (Fig. 2B). Only two residues are not conserved between the bovine and human proteins within the sequence specified by the five fragments. A 2.4-kb transcript for IBP72 was identified in a wide range of human tissues including skeletal muscle, brain, heart, placenta, kidney, pancreas, lung, and liver (Fig. 2C). Although the cloned 72-kDa protein has no significant homology with other cloned mammalian proteins and contains no consensus structural motifs, it does exhibit moderate homology to putative proteins encoded in the Caenorhabditis elegans and Saccharomyces cerevisiae genomes, as well as significant homology to the skb1 gene product (12) from Schizosaccharomyces pombe (52% homology; Fig. 2A). Recently, a human cDNA identical with our IBP72 was cloned by homology to Skb1 and submitted to GenBank (accession number AF015913).

The cloned 72-kDa protein appears to be IBP72 as indicated by several approaches. First, in vitro translated protein exhibited specific binding to the GST-pICln fusion protein but not to GST alone (Fig. 3, left panel). Second, an affinity-purified polyclonal antibody raised against the recombinant 72-kDa protein recognized IBP72 co-immunoprecipitated with pICln (Fig. 3, right panel). Third, a LexA-IBP72 fusion protein specifically interacts with a GAL4 activation domain–ICln fusion protein.
protein in the yeast two-hybrid system (Table I). Moreover, when a human heart cDNA library was screened in the yeast two-hybrid system using the LexA-IBP72 fusion protein as bait, six independent ICln clones were obtained. These results argue strongly that our cloned protein is IBP72.

To identify the domain(s) of pICln critical for interaction with IBP72, we generated several epitope-tagged (FLAG) human pICln deletion constructs and examined their ability to bind native IBP72. The full-length FLAG-pICln protein and endogenous pICln interacted with the same set of proteins in human embryonic kidney (HEK293) cells (data not shown). All deletion constructs were expressed at levels equivalent to or higher than the full-length FLAG-pICln protein, as assessed by immunocytochemical analysis and immunoprecipitation of [35S]methionine-labeled proteins (data not shown). Based on this approach, we conclude that the extreme carboxyl terminus of human pICln, specifically the last 37 amino acids, is critical for interaction with IBP72 (Fig. 4). Consistent with this result, one of the pICln clones identified by yeast two-hybrid selection was identical to the native protein interacting with pICln.
DISCUSSION

In an effort to identify the functional role of pICln, we are characterizing IBPs. Four major IBPs consistently co-purify with pICln in several tissues. We cloned the human cDNA for IBP72 based on microsequence data obtained from affinity-purified bovine IBP72. The interaction between the cloned human IBP72 and pICln was confirmed by several lines of evidence, including the extraction of pICln from a cDNA library using the full-length coding sequence for IBP72 as the bait in a yeast two-hybrid screen. IBP72 is ubiquitously expressed and has no identifiable mammalian homologs or recognizable structural motifs that would suggest a specific function. Currently we are cloning the other IBPs and will use similar approaches to verify the specificity of their interaction with pICln.

Although IBP72 has no known human homologs, sequence similarity suggests that IBP72 represents a human homolog of the Skb1 protein from S. pombe (12). Skb1 was identified by a yeast two-hybrid screen using the S. pombe protein kinase Skk1 as bait. The Skk1 kinase is linked to Ras- and Cdc42-dependent signaling cascades regulating cell viability, morphology, and mitogen-activated protein kinase-mediated pheromone responses (13). S. pombe lacking Skb1 are less elongated than wild-type yeast, emphasizing the role of this protein in the regulation of cell morphology. Skk1 is a homolog of the mammalian PAK, of which there are three cloned isoforms (14). PAK kinases are activated by GTP-bound forms of the small GTP-binding proteins Rho, Rac, and Cdc42 and have been implicated in control of cytoskeletal rearrangement and cell morphology (11, 14).

One consistent conclusion from previous studies of pICln function is that its overexpression induces, either directly or indirectly, the appearance of a chloride conductance (1, 2, 4). Given the biochemical characteristics of pICln, we favor the hypothesis that pICln is not a channel itself but rather part of a pathway either closely or remotely connected to a chloride current, possibly through cytoskeletal rearrangement. Indeed, actin co-immunoprecipitated with pICln, and a fraction of pICln associated with insoluble cytoskeletal elements (5). The protein identified in this report, IBP72, may provide a link between pICln and cytoskeletal rearrangement. Regulation of swelling-induced chloride channels is likely to involve cytoskeletal rearrangement (15, 16). Also, recent evidence links p21Rho-dependent cytoskeletal reorganization to a swelling-induced chloride conductance (17). Whether pICln and IBP72 are linked to a swelling-induced chloride current (5–7), a volume-insensitive chloride conductance (2, 3), or both will be determined only by understanding all elements of the pathway.

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