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Interaction of the PDZ Domain of Human PICK1 with Class I ADP-Ribosylation Factors

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We have cloned the cDNA encoding human PICK1 (protein interacting with C kinase 1), a PDZ domain-containing protein of 415 amino acids, and also identified the *Drosophila* homologue by search of the databank. Northern blot analysis shows a single mRNA of about 2.0 kb ubiquitously expressed in human tissues. Although PICK1 proteins harbor a region homologous to arfaptin1 and arfaptin2, two proteins that bind to the ARF (ADP-ribosylation factor), this region of PICK1 does not interact with ARFs in the yeast two-hybrid system. On the other hand, the PDZ domain of PICK1 is capable of interacting with constitutively active, GTP-bound forms of ARF1 and ARF3, but neither with those of ARF5/6 nor with the GDP-bound ARFs. The PICK1–ARF interaction is abrogated by introduction of mutations in the PDZ domain or by deletion of the extreme C-terminus of ARF1. Thus, PICK1 specifically interacts with ARF1/3 in the GTP-bound state, suggesting that PICK1 participates in ARF1/3-mediated cellular processes.

Mouse PICK1 (protein interacting with C kinase 1) is a PDZ domain-containing protein, which was cloned by the yeast two-hybrid screening using the catalytic domain in the α isoform of protein kinase C (PKCα) as a bait (1). PDZ domains occur in a variety of proteins that are often found in complexes at membranes, and mediate homotypic and/or heterotypic interactions, in the latter of which the domains usually recognize and bind to the C-terminus of their target proteins (2–7). Although PICK1 has been shown to be localized in the perinuclear region, probably in the Golgi complex (1), its functional role in the cell is presently unclear.

Here we have cloned the cDNA encoding a human homologue of PICK1 by some chance, and also identified its *Drosophila* homologue. In addition to the PDZ domain, PICK1 contains a region homologous to arfaptin1 and arfaptin2, designated AH (arfaptin homology) domain. The arfaptins are proteins that bind to the small GTPase ADP ribosylation factor (ARF) (8, 9). ARF was originally discovered as a cofactor for the ADP-ribosylation of the trimeric G protein Gs by cholera toxin (10, 11), and also known as an activator of phospholipase D (PLD) (12). Six mammalian ARFs have been thus far identified, and divided into three classes: class I, ARF1, -2, and -3; class II, ARF4 and -5; and class III, ARF6 (13). Class I ARFs exist at the Golgi apparatus and play an essential role for vesicle formation in this organelle (14, 15). The present results in the yeast two-hybrid system reveal that, unexpectedly, the AH domain of PICK1 is not able to bind to ARFs. Instead, the PDZ domain of PICK1 interacts with the GTP-liganded form of class I ARFs (ARF1 and -3) via binding to the C-terminus, but does not bind to ARF5 or ARF6, raising the possibility that PICK1 participates in ARF1/3-mediated cellular processes in the Golgi complex.

MATERIALS AND METHODS

Two-hybrid screening. Two-hybrid screening was performed using a Matchmaker Two-Hybrid System (Clontech) according to the instructions provided by the manufacturer. As a bait we used the N-terminal SH3 domain of $p67^{phox}$, a cytosolic factor of the phagocyte NADPH oxidase [amino acid residues 238–301; p67-SH3(N)] (16). The yeast reporter strain Y190 was transformed simultaneously with pGBT9-p67-SH3(N) and a plasmid of a human B cell cDNA library (Clontech) using a lithium acetate-based method (17). The double transformants were grown on SD agar plates lacking Trp, Leu, and His, and supplemented with 25 mM 3-aminotriazole for 10 days at 30°C. Positive colonies were picked, restreaked onto the plates, and assayed for the *LacZ* phenotype (18). One of these plas-

The novel nucleotide sequence data published here have been submitted to the DDBJ/EMBL/GenBank DNA databases and are available under Accession No. AB026491.

Abbreviations used: PICK1, protein interacting with C kinase 1; PKCα, the α isoform of protein kinase C; ARF(s), ADP-ribosylation factor(s); PLD, phospholipase D; AH, arfaptin homology; ER, endoplasmic reticulum.

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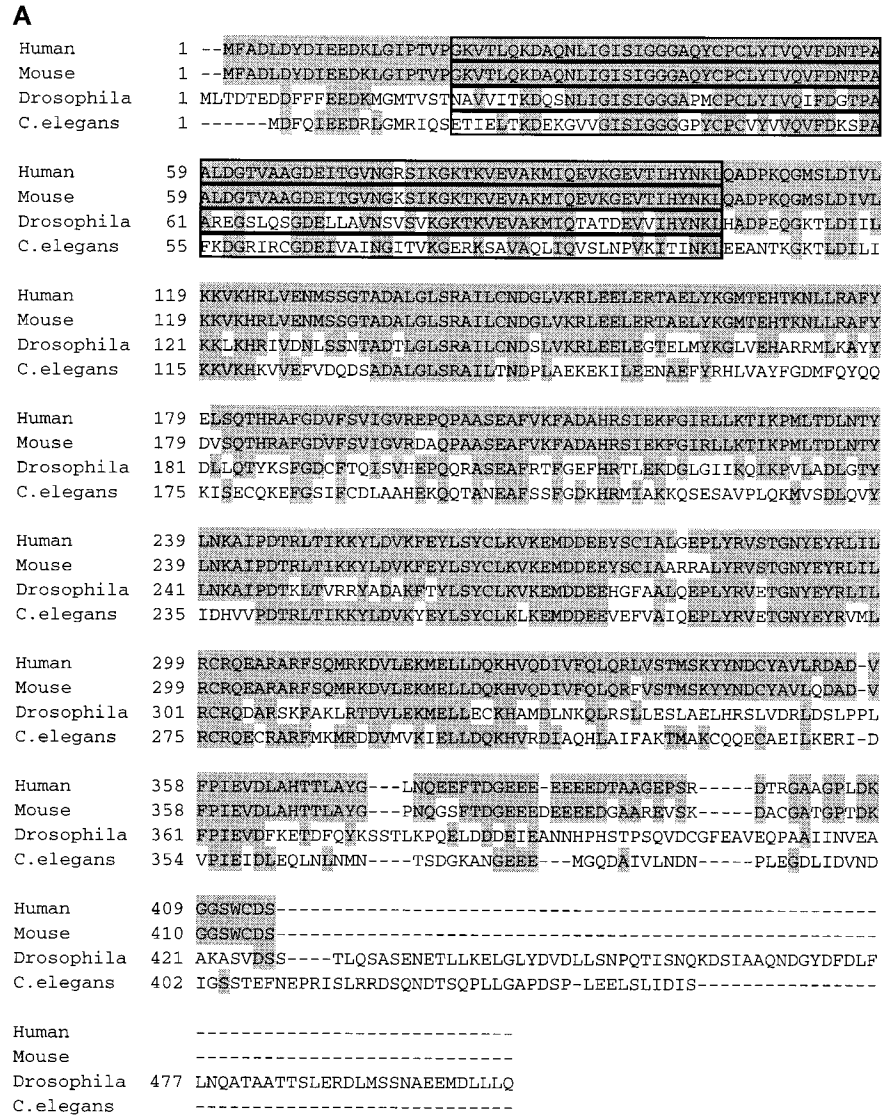
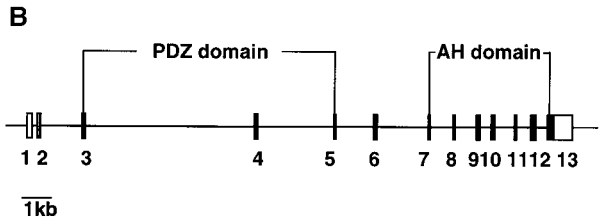


FIG. 1. (A) Comparison of the amino acid sequences of human, mouse and C. elegans PICK1 as well as a hypothetical protein in Drosophila genome. Amino acid residues identical to those of human PICK1 are shaded, and the PDZ domains are boxed. (B) Exon–intron distribution. Translated sequences are shown as gray boxes and untranslated sequences as open boxes. (C) Sequence around splice sites of the human PICK1 gene. Intron sequences are shown in lowercase and exon sequences in uppercase. The 3′- and 5′-splice consensus sequences of introns and the poly-A signal are underlined. Nucleotide position defined according to the cDNA sequence that was determined in this study (AB026491).

mids contains an approximately 2.0-kb cDNA with a continuous open reading frame of 1245 nucleotides encoding a 415-amino-acid peptide. Since the amino acid sequence is 94% identical to mouse PICK1, we concluded that the cDNA encodes human PICK1. In the process of characterization of the interaction between human PICK1 and p67-SH3(N), we noticed that this interaction is mediated via binding of the PDZ domain of PICK1 to the C-terminal residues of p67-SH3(N) containing Leu^{299}-Arg^{300}-Ile^{301}-COOH: the interaction was completely abolished not only by truncation of the three amino acids, but also by extension of five amino acids from the C-terminus of p67-SH3(N) (data not shown). The findings indicated that, as generally do PDZ domains (2–7), the PICK1 PDZ domain recognizes the carboxyl group of the C-terminal residue Ile^{301} in p67-SH3(N), that does not occur in endogenous p67^{phox}. Hence we concluded that the interaction between human PICK1 and p67-SH3(N) is an artificial one.

Two-hybrid experiments. The multiple cloning sites of pACT2 and pGBT9 were modified so that the cloned cDNAs can be readily subcloned into their correct orientation and reading frames (18). Human cDNA encoding the full-length PICK1 was cloned into the BamHI and EcoRI sites of the modified GAL4 DNA-binding domain vector pGBT9g, to obtain pGBT-PICK1. The PDZ domain of PICK1 (amino acid residues 1–105), PICK1-ΔN (residues 99–415), and PICK1-AH (residues 99–378) were constructed using PCR and cloned into pGBT9g. To obtain cDNAs for human ARF1, -3, -5, and -6, we synthesized oligonucleotide primers, derived from the reported human sequences of ARF1, 3, 5, and 6 (10, 11). PCR amplification was performed with the oligonucleotides, carrying a BamHI or EcoRI restriction site to facilitate cloning of the PCR fragment, using the human B cell cDNA library. The PCR products were subcloned into the BamHI and EcoRI sites of the modified GAL4 activation domain vector pACT2g, and confirmed by DNA

B



C

Exon	Size (*bp*)	5' end	3' Splice acceptor	5' Splice donor	Intron size (*kb*)
1	146	1		TTTGTACCTAgtaagaatca	0.3
2	98	147	ccggatccagTTCCCCATTC	AGGATAAACTgtgagtattt	1.5
3	112	245	tccttttctagCGGAATCCCG	TATCGTCCAGgtattgggcg	5.8
4	129	357	gcacccacagGTATTTGACA	GGAGGTGAAGgtaagggctg	2.7
5	67	486	gctcttccagGGGGAGGTGA	CTGGACATTGgtaagctggt	1.3
6	90	553	ccccgctcagTGTTGAAGAA	CTGTGCAATGgtgagtccct	1.8
7	54	643	atcccgacagATGGGCTTGT	CTATACAAAGgtgggtgggg	0.8
8	63	697	tctcccacagGGATGACGGA	ACTCACCGGGgtaatggcat	0.8
9	134	760	tgtcctgcagCCTTTGGGGA	CATCAAGCCGgtaggtccta	0.5
10	93	894	cctcttccagATGCTGACGG	TGAGTACCTGgtgagtagtc	0.8
11	51	987	tcccacacagTCGTACTGCC	CAGCTGCATTgtgagtgttg	0.5
12	145	1038	cccgccccagGCCCTAGGCG	CAGAAGCACGgtgagcgccg	0.6
13	836	1183	cccaccccagTCCAGGACAT	poly(A) CGAATAAAAC	

FIG. 1—*Continued*

sequencing. Constitutively active forms of ARFs with a Glu → Leu substitution (Glu-71 in ARF1, -3, and -5; Glu-67 in ARF6) (19) and dominant negative ARFs with a Thr → Asn substitution (Thr-31 in ARF1, -3, and -5; Thr-27 in ARF6) were prepared by PCR-mediated site-directed mutagenesis, and cloned into pACT2g. A deletion mutant, ARF1ΔC (residues 1–178), was constructed by PCR to remove amino acids 179–181 and cloned into pACT2g. All the plasmids were subjected to DNA sequencing for the confirmation of correct construction. Various pairs of the pACTg and pGBTg plasmids were cotransformed into competent yeast Y190 cells, as previously described (16). Following the selection for Leu^+ and Trp^+ phenotypes, transformants were tested for their ability to grow on plates lacking histidine. These indicator plates were supplemented with 25 mM 3-aminotriazole to suppress the background growth due to leaky expression of the HIS3 gene in Y190 cells.

Northern blot analysis. Human Multiple Tissue Northern blots (Clontech) were hybridized with ^{32}P-labeled human PICK1 cDNA fragments (corresponding to amino acids 99–267) under high-stringency conditions using ExpressHyb (Clontech).

RESULTS AND DISCUSSION

Primary structures of human and Drosophila PICK1. In the process of the yeast two-hybrid screening with a human B cell cDNA library (for detail, see Materials and Methods), we cloned a cDNA of approximately 2.0 kb with an open reading frame of 1245 nucleotides encoding a 415-amino-acid peptide, in which the first methionine codon was surrounded by a consensus Kozak sequence (20). Since the amino acid sequence was 92 and 44% identical to those of mouse and *C. elegans* PICK1 (1, 21), respectively (Fig. 1A), we concluded that the cDNA encodes human PICK1. Searching the sequence database revealed that a region identical to human PICK1 cDNA occurs in the human genomic DNA sequence from the clone 1039K5 (DDBJ/EMBL/GenBank Accession No. AL031587), which is localized to chromosome 22q12.3–13.2. Alignment with the genomic DNA sequence revealed that PICK1 protein is encoded by 13 exons with exon-intron boundaries (Figs. 1B and 1C), covering a minimum of 18 kb. Northern blot analysis revealed that human PICK1 was expressed as one major transcript of approximately 2.0 kb ubiquitously expressed in human tissues (Fig. 2). The size is close to that of the cloned cDNA, supporting that the clone is derived from the full-length mRNA.

We also found a hypothetical protein in the genome of *Drosophila melanogaster*, as a PICK1 homologue. Searching the sequence database revealed that a region homologous to human PICK1 occurs in the region 33F3–34A2 of chromosome 2L in *Drosophila melanogaster* (AC005891). Application of the program Genie (22), a Hidden Markov model-based genefinder, led to prediction of the coding regions from the genomic sequence of the insect chromosome: the initial (11,344–11,390), internal (12,495–12,606) and final (14,087–15,046) exons. These exon-intron boundaries are completely consistent with the second and third ones of human PICK1 gene, supporting that the Drosophila gene encodes a homologue of PICK1. The *Drosophila* protein contains 504 amino acid residues that shares 35–45% identity with PICK1 proteins of other species (Fig. 1A). Thus PICK1 seems evolutionarily well conserved, suggestive of its biological significance.

PICK1 proteins contain not only a PDZ domain but also a region homologous to arfaptins. In the N-terminus, human and Drosophila PICK1 proteins harbor a PDZ domain, as does mouse PICK1 (Fig. 1A).

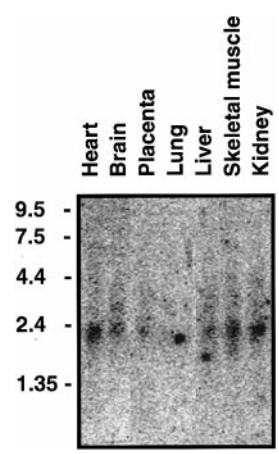
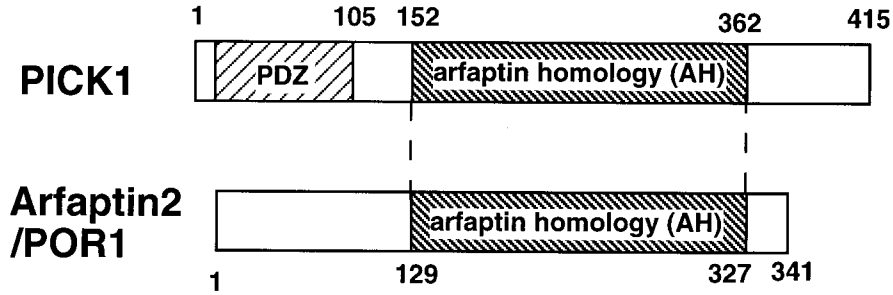


FIG. 2. Northern blot analysis of the human PICK1 mRNA. Human Multiple Tissue Northern blots (Clontech) were hybridized with ^{32}P-labeled human PICK1 cDNA fragments (corresponding to amino acids 99–267) under high-stringency conditions using ExpressHyb (Clontech). Positions of RNA molecular size markers are shown in kilo bases.



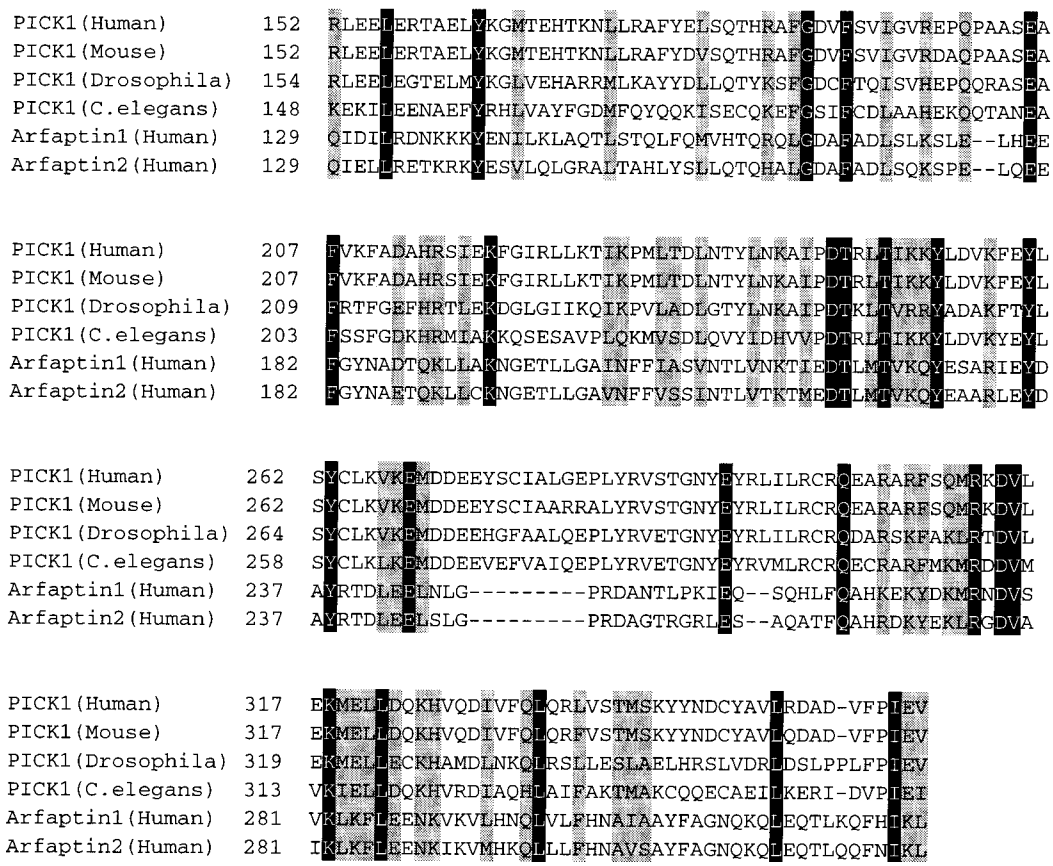


FIG. 3. Sequence alignment of AH (arfaptin homology) domains of PICK1 from human, mouse, *Drosophila*, and *C. elegans*, and those of arfaptin1 and arfaptin2. Residues identical among PICK1 from various species and arfaptin1/2 are shown with black boxes. Highly but not completely conserved residues are shaded.

During a search of the database for proteins related to PICK1, we found that PICK1 also contains a region homologous to arfaptin1 and arfaptin2 (8), the latter being also known as POR1 (partner of Rac1) (23) (Fig. 3A). The arfaptins, which are 60% identical in the overall sequences, were originally cloned as putative target proteins of the ARF small GTPases using the two-hybrid screening (8). The homologous domain, designated AH (arfaptin homology), contains the most conserved region of amino acids 244–327 (in human PICK1) among the PICK1 proteins.

It has been reported that the arfaptins interact with ARF1 and -2 in the yeast two-hybrid system (8). To clarify the role for the AH domain of arfaptins in the interaction with ARFs, we constructed the cDNA encoding the C-terminal region of arfaptin2 (amino acids 108–341), comprising the AH domain, and tested its ability to bind to ARF1. As shown in Fig. 4, this region

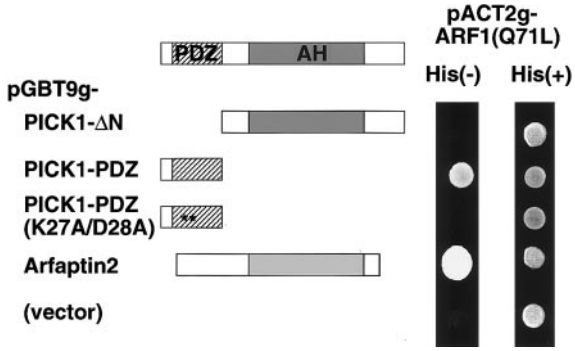


FIG. 4. Interaction of various regions of human PICK1 with ARF1 in the yeast two-hybrid system. The yeast reporter strain Y190 was cotransformed with pairs of recombinant plasmids pACT2g and pGBT9g, the former encoding ARF1 (Q71L), a constitutively active, GTP-bound form of ARF1, and the latter encoding arfaptin2 (amino acid residues 39–341) or various regions of PICK1: PICK1-F (amino acid residues 1–415), PICK1-ΔN (residues 105–415), PICK1-PDZ (residues 1–105), or the mutated domain (residues 1–105) carrying both Lys27 → Ala and Asp28 → Ala substitutions, namely PICK1-PDZ (K27A/D28A). The transformants were cultured in the presence or absence of histidine as described under Materials and Methods.

did interact with a constitutively active form of ARF1 in the two-hybrid system. It was thus expected that the AH domain of PICK1 also can interact with ARFs. To test this possibility, we used an AH domain-containing region of human PICK1 (amino acids 99–415) under the same conditions. This region was, however, incapable of binding to ARF1 (Fig. 4).

The PDZ domain of human PICK1 interacts with the GTP-bound form of the small GTPases ARF1. During the study on the binding of ARF1 to individual regions of PICK1, we found that the PDZ domain of PICK1 interacted with a constitutively active, GTP-bound form of ARF1 (Q71L) in the assay system (Fig. 4). When the wild-type or a GTP-binding defective form (T31N) of ARF1 was used, we observed a negligible or no interaction, respectively (Fig. 5A). These observations suggest that the PICK1 PDZ domain binds to the GTP-bound ARF1.

The C-terminus of ARF1 is required for the interaction with the PICK1 PDZ domain. PDZ domains usually interact, via the carboxylate-binding loop, with the C-terminus of target proteins (24). We substituted alanines for both Lys-27 and Asp-28 in the loop of the PICK1 PDZ domain to obtain PICK1 (K27A/D28A), a mutation which has been reported to abolish the interaction with the C-terminus of PKCα (21). The mutated PDZ domain (K27A/D28A) failed to interact with ARF1 (Fig. 4), confirming the involvement of the PDZ domain in the interaction with ARF1.

Although PDZ domains bind preferentially to peptides that terminate in a hydrophobic amino acid (usually Val or Ile) (24), it has been reported that the fifth PDZ domain of the protein phosphatase PTPbas/FAP-1 binds to the peptide KNXXXEYYKK-COOH with significant selectivity using the oriented peptide library technique (7). To determine whether the PICK1 PDZ domain recognizes the ARF1 C-terminal sequence Asn-Gln-Lys-COOH, we tested the ability of a deletion mutant that lacks the three amino acids of the extreme C-terminus, ARF1(Q71L)ΔC, to interact with the PDZ domain. As shown in Fig. 5A, this mutant protein failed to interact with the PDZ domain, whereas it could bind to arfaptin2. This result indicates that the extreme C-terminal sequence in ARF1 is required for interaction with PICK1, which is consistent with that the carboxylate-binding loop of the PICK1 PDZ domain is involved in the interaction (Fig. 4).

The PICK1 PDZ domain specifically interacts with class I ARFs. Mammalian ARF isoforms can be divided into three classes based on size, amino acid se-

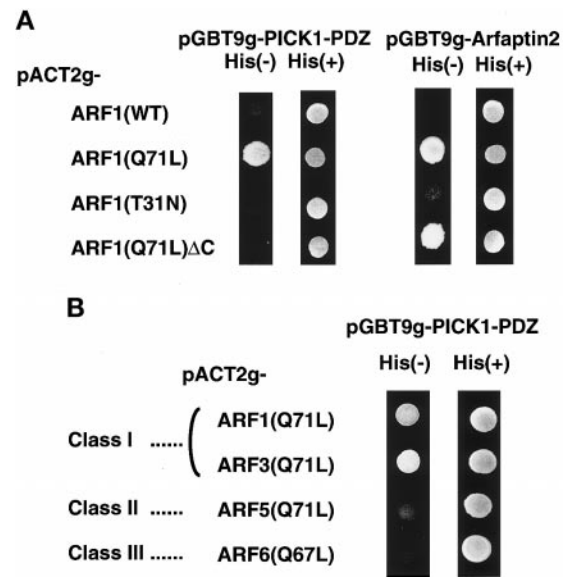


FIG. 5. Interaction of the PDZ domain of human PICK1 with class I ARFs in the GTP-bound state. (A) Interaction of the PDZ domain of PICK1 with various forms of ARF1. The yeast reporter strain Y190 was cotransformed with pairs of recombinant plasmids pGBT9g and pACT2g, the former encoding PICK1-PDZ (amino acid residues 1–105) or arfaptin2 (residues 39–341), and the latter encoding wild-type (WT) ARF1 or a mutant one carrying the following mutation: Q71L, a constitutively active, GTP-bound form; T17N, a GTP-binding defective, dominant negative form; or ΔC, a deletion mutant lacking the three amino acid residues of the extreme C-terminus. The transformants were cultured in the presence or absence of histidine as described under Materials and Methods. (B) Specificity of PICK1-PDZ for interaction with various isoforms of ARFs. The yeast reporter strain Y190 was cotransformed with pairs of recombinant plasmids pGBT9g and pACT2g, the former encoding PICK1-PDZ (residues 1–105), and the latter encoding various isoforms of ARFs carrying a Glu → Leu substitution (Glu-71 for ARF1, -3, and -5; Glu-67 for ARF6). The transformants were cultured in the presence or absence of histidine as described under Materials and Methods.

quence, gene structure, and phylogenetic analysis: class I, ARF1, -2, and, -3; class II, ARF4 and -5; and class III, ARF6 (13). To clarify the specificity of the binding of PICK1, we examined two-hybrid interactions with a variety of human ARFs (Fig. 5B). The PICK1 PDZ domain interacted with ARF3, a member of class I ARF. ARF3 is 96% identical to ARF1 in amino acid sequence and harbors the extreme C-terminal residues Asn-Lys-Lys-COOH, which similar to that of ARF1, Asn-Gln-Lys-COOH (13).

On the other hand, the PDZ domain bound much less to class II ARF (ARF5) and very little to class III ARF (ARF6). The extreme C-terminal sequences of these ARFs are completely different from those of type I ARFs, i.e., Ser-Lys-Arg-COOH in ARF5 and Tyr-Lys-Ser-COOH in ARF6 (10), which is consistent with the crucial role for the C-terminus of ARF1 in interaction with the PICK1 PDZ domain (Fig. 5A). When any of GTP-binding defective ARFs with a Thr → Asn substitution (Thr-31 in ARF1, -3, and -5; Thr-27 in ARF6) was used, interaction with the PDZ domain was not observed (Fig. 5A; and data not shown). Thus the PICK1 PDZ domain specifically interacts with class I ARFs in the GTP-bound state.

CONCLUDING REMARKS

The findings in this study show that the PDZ domain of PICK1 specifically interacts with class I ARFs, such as ARF1 and ARF3, in the GTP-bound state. To date a variety of PDZ domain-containing proteins have been shown to interact with small GTPases: rhophilin with Rho (25), AF-6 with Ras (26), and Tiam1 with Rac (27, 28). There is, however, no indication that the PDZ domain mediates the binding of these proteins to the small GTPases. The interaction between PICK1 and ARF requires both the carboxylate-binding loop of the PICK1 PDZ domain (Fig. 2) and the C-terminus of ARF that terminates in lysine (Fig. 3A). On the other hand, the ARF C-terminus is not involved in the GTP-dependent binding to arfaptin2 (Fig. 3A) or in the GTP-dependent activation of phospholipase D (PLD) (29, 30). It is conceivable that the interactions of ARF with PICK1 and with arfaptin or PLD may not be mutually exclusive, which raises the possibility that a ternary complex containing ARF, PICK1, and arfaptin or PLD may be formed, when ARF is activated to become the GTP-bound state. The role of the interaction between PICK1 and class I ARFs is presently unknown. The ARF GTPases are multifunctional proteins with roles in vesicle transport and PLD activation (12). Class I ARFs are mainly localized in the Golgi (31), and play a crucial role in retrograde vesicle transport from Golgi to rough endoplasmic reticulum (ER) (14, 15). It has been proposed that activation of PLD in the Golgi is important for ARF-dependent vesicle trafficking in this organelle (32–34). In this context it should be noted that PICK1 expressed in COS cells is concentrated to one side of the nucleus, probably in the rough ER and/or Golgi (1), suggesting that PICK1 is colocalized with class I ARFs at these organelles. It is thus tempting to speculate that the interaction between PICK1 and ARF1/3 may regulate Golgi to ER vesicle transport. Future studies should be designed to test this possibility.

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