Mitogen-stimulated TIS21 protein interacts with a protein-kinase-Cα-binding protein rPICK1

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TIS21 is induced transiently by PMA and a number of extracellular stimuli. Yeast two-hybrid screening has identified three TIS21 interacting clones from a rat cDNA library [Lin, Gary, Yang, Clarke and Herschman (1996) J. Biol. Chem **271,** 15034–15044]. The amino acid sequence deduced from clone 5A shows 96.9% identity with the murine PICK1, a protein kinase Cα (PKCα)-binding protein postulated to act as an intracellular receptor for PKC. A fusion protein of glutathione S-transferase and rPICK1 associates with the TIS21 translated *in vitro*, suggesting a direct physical interaction between these two proteins. TIS21 and rPICK1 are co-immunoprecipitated from NIH 3T3 cells overexpressing these two proteins. This indicates that the interaction also occurs in mammalian cells. Deletion of the PDZ domain at the N-terminus of rPICK1 abolishes its interaction with TIS21. A putative carboxylate-binding loop required for PICK1 to bind PKCα [Staudinger, Lu and Olson (1997) J. Biol. Chem **272,** 32019–32024] is within this deleted region. Our results suggest a potential competition between TIS21 and PKC for binding to PICK1. We show that recombinant TIS21 is phosphorylated by PKC *in vitro*. The catalytic activity of PKC towards TIS21 is significantly decreased in the presence of rPICK1, whereas phosphorylation of histone by PKC is not affected. rPICK1 seems to modulate the phosphorylation of TIS21 through specific interactions between these two proteins. TIS21 might have a role in PKC-mediated extracellular signal transduction through its interaction with rPICK1.

Key words: protein interaction, signal transduction, yeast two-hybrid screening.

INTRODUCTION

The TIS21/PC3/BTG2 gene was initially cloned as a PMA-inducible sequence in murine Swiss 3T3 cells [1] and as a nerve-growth-factor-inducible sequence in rat PC12 phaeochromocytoma cells [2]. It was subsequently shown to be induced by several stimuli, including serum, epidermal growth factor, nerve growth factor, forskolin and KCl, in a variety of cell types [3]. On stimulation, the TIS21/PC3 message accumulates rapidly, reaches its peak within 30–60 min and returns to the undetectable level in approx. 2 h [3]. The TIS21 gene encodes a polypeptide of 158 amino acid residues with a half-life of less than 15 min [4]. TIS21/PC3 has also been shown to be induced during programmed cell death on the withdrawal of trophic factor/serum [5,6] and in stress responses induced by ionizing radiation and DNA-damaging agents [7]. It seems that the expression of TIS21 is an early event involved in mitogenic responses, differentiation, stress and cell death.

The protein sequence of BTG2, the human counterpart of TIS21, shows 66.4% identity with the human BTG1 (B-cell translocation gene 1) sequence [8]. The major difference between TIS21/PC3/BTG2 and BTG1 proteins is a ten-residue insertion in the BTG1 sequence towards its C-terminus [8]. The TIS21/BTG2$^{-/-}$ homozygous embryonic stem cells demonstrate an increased sensitivity to the killing effects of adriamycin, whereas the wild-type cells are arrested at G_2/M phase under the same conditions [7]. The presence of wild-type p53 is required for the expression of TIS21/BTG2 and for cell cycle arrest induced by DNA damage [7,9]. Overexpression of TIS21 or BTG1 in murine 3T3 cells results in decreased cell growth [8,10]. These observations led to the suggestion that TIS21 and BTG1 belong to a new antiproliferative gene family. So far, there are at least seven distinct genes in vertebrates identified as members of this new gene family, as indicated by sequence similarity [11,12].

The speculation that TIS21 might interact with other proteins and, through them, modulate their activities or functions led to screening for TIS21 interaction partners by using the yeast two-hybrid system. Three distinct genes were identified in the screen [13]. PRMT1 (protein arginine methyltransferase 1) interacts not only with TIS21 but also with BTG1 [13]. Both TIS21 and BTG1 modulate the activity of PRMT1 towards endogenous polypeptide substrates including hnRNP A1, which is involved in alternative RNA splicing and is extensively methylated on arginine residues in cells [14]. It is speculated that transient expression of the gene encoding TIS21 might mediate signals in modulation of splicing after stimulation with ligand [13]. In addition, both TIS21/BTG2 and BTG1 are shown to interact with CAF-1, the carbon catabolite repressor protein (CCR4)-associative factor 1 [15,16]. This result suggests that TIS21 and BTG1 might participate in transcriptional regulation.

The reversible phosphorylation of proteins regulates most aspects of cellular processes. The targeting subunits (or intracellular receptors) of protein kinases and phosphatases specify the location, catalytic and regulatory properties of these enzymes and thereby have a key role in ensuring the fidelity of protein phosphorylation [17,18]. Protein kinase C (PKC) consists of a widespread family of kinases responsible for many diverse and critical cellular functions [19]. Activation of PKC isoenzymes is associated with translocation to distinct subcellular sites [18,20], which is mediated by their binding to isoenzyme-specific receptors

Abbreviations used: CMV, cytomegalovirus; GST, glutathione S-transferase; hnRNP, heterogeneous nuclear ribonucleoprotein particle; PKC, protein kinase C; PRMT1, protein arginine methyltransferase 1.

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for activated C-kinases [18]. PICKs (for 'proteins that interact with C-kinase'), have been identified by yeast two-hybrid screening with the use of activated PKC as a bait [21]. PICK1 binds specifically to PKCα; mutation in the carboxylate-binding loop in the PDZ domain of PICK1 abolishes its binding to PKCα [22]. PICK1 has been shown to induce clustering of EphB2 receptor, a tyrosine kinase receptor enriched in synaptic membrane [23], as well as clustering of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors, which are known to mediate rapid synaptic transmission [24]. PICK1 is therefore proposed to have a role in the ephrin and AMPA receptor signalling mediated by PKC pathway.

Many ligands that stimulate the expression of TIS21 also activate the PKC pathway. Clone 5A was obtained in a yeast two-hybrid screen with TIS21 as a bait and was shown to interact with TIS21 but not BTG1 [13]. A partial sequence of 5A cDNA showed great similarity to the mouse PICK1 gene [13]. In this article, we confirm that 5A is a rat counterpart of PICK1 and demonstrate that the protein product of the immediate-early gene TIS21 interacts with rPICK1 not only in the yeast two-hybrid system but also in mammalian cells. The interaction is through a direct physical contact, as shown by a binding assay *in vitro*. The PDZ domain of rPICK1 is required for interaction with TIS21; phosphorylation of TIS21 by PKC is significantly decreased by rPICK1. The evidence suggests that TIS21 might have a role in mediating PKC activity through interaction with rPICK1.

EXPERIMENTAL

Yeast two-hybrid analysis

The pLexA(L) plasmid [13] was used as the DNA-binding-domain fusion vector. The pPC86 plasmid containing the GAL4 transcription activation domain was used as the activation-domain fusion vector. The pLexA(L) plasmid, containing wild-type TIS21, wild-type BTG1, mutant TIS21 or mutant BTG1 cDNA, was introduced together with pPC86rPICK1(wild-type) or pPC86rPICK1 mutants into yeast strain L40 as described [13]. In brief, the L40 yeasts were grown to a density of $(0.5–1) \times 10^7$ cells/ml, incubated in transformation buffer [1 M sorbitol/0.1 M lithium acetate/10 mM Tris/HCl (pH 7.5)/0.1 mM EDTA] then tranformed with pPC86 and pLexA(L) plasmids. SC (synthetic complete) medium without leucine and tryptophan (SC-leu-trp) was used for the selection of transformants harbouring both the pPC86 and pLexA(L) plasmids. At 2–3 days after transformation, the yeasts that grew in the SC-leu-trp medium were pooled and streaked on a fresh SC-leu-trp plate. The filter-paper β-galactosidase activity assay was conducted on the next day, with 5-bromo-4-chloro-3-indolyl β-D-galactoside as a substrate.

Construction of plasmids

The pLexA(L)-TIS21 plasmid was used as a template for PCR to obtain TIS21 deletion mutants. The sense primers P1 (5′-GCAGAATTCGGTTCTGGTTCTGGGAG-3′), P3 (5′-GATGAATTCGGTTCTGGTTCTGGGCATTACAAACACCACTGG-3′) and P5 (5′-GATGAATTCGGTTCTGGTTCTGGGTTCCTCTCCAGTCTCCTG-3′) and the anti-sense primers P2 (5′-CGAGTCGACGGGCATTACAAACACCACTGG-3′) and P4 (5′-GTAGTCGACCTACAGGACACAGATGGAGCC-3′) were used in pairs. For the purpose of cloning, an *Eco*RI site (underlined) was included in the sense primers and a *Sal*I site (underlined) was included in the anti-sense primers. The pair of primers P1 and P4 were used to obtain the $TIS21_{M1–L122}$ fragment, primers P5 and P4 for the $TIS21_{F19–L122}$ fragment, primers P5 and P2 for the $TIS21_{F19–S158}$ fragment, primers P3 and P2 for the $TIS21_{H49–S158}$ fragment and primers P3 and P4 for the $TIS21_{H49–L122}$ fragment. The rPICK1 deletion mutants were obtained by PCR with pPC86-rPICK1 as a template. The sense primers used were RP1 (5′-GGATGTTTAATACCACT-3′), RP2 (5′-CTGGTCGACGGTATTTGATAACACGCCTG-3′) and RP3 (5′-CTGGTCGACCGCCATCCTGTGTAACGATT-3′); the anti-sense primers used were RP4 (5′-CTGGCGGCCGCTCACAGCACTGCATAGCAGTC-3′) and RP5 (5′-GTCGCGGCCGCCGAGCCAGCAGGCTTTTATG-3′). The pair of primers RP1 and RP4 were used to obtain the $rPICK1_{M1–L352}$ fragment, primers RP1 and RP5 for the $rPICK1_{M1–S416}$ fragment, primers RP2 and RP4 for the $rPICK1_{V52–L352}$ fragment, primers RP2 and RP5 for the $rPICK1_{V52–S416}$ fragment, primers RP3 and RP4 for the $rPICK1_{A142–L352}$ fragment, and primers RP3 and RP5 for the $rPICK1_{A142–S416}$ fragment. For the purpose of cloning, a *Sal*I site (underlined) was included in the RP2 and RP3 primers and a *Not*I site (underlined) in the RP4 and RP5 primers.

The TIS21 cDNA fragments were then ligated to the pLexA(L) plasmids, and the rPICK1 cDNA fragments to the pPC86 plasmids.

Expression and purification of glutathione S-transferase (GST) fusion proteins

Expression of GST fusion proteins was induced with isopropyl β-D-thiogalactoside (0.5 mM) for 2–3 h at 37 °C. Bacteria were harvested, resuspended in extraction buffer [PBS (pH 7.4)/5 % (v/v) glycerol/1 mM EDTA/1 mM EGTA/10 mM 2-mercaptoethanol/10 μg/ml leupeptin/10 μg/ml aprotinin/10 μg/ml pepstatin/1 mM PMSF/0.5 % (v/v) Triton X-100] and disrupted by sonication. After the removal of debris by centrifugation (16000 ***g***, 20 min, 4 °C), the GST fusion proteins in supernatant were purified with glutathione–Sepharose 4B beads (Amersham Pharmacia Biotech) in accordance with the manufacturer's instruction. To release TIS21 and rPICK1 from GST protein, the purified, immobilized GST–TIS21 and GST–rPICK1 were digested with thrombin for 2 h at room temperature. After centrifugation, TIS21 and rPICK1 were recovered in the supernatant fractions, whereas the GST remained bound to the glutathione–Sepharose 4B beads (results not shown).

Binding assay *in vitro*

To obtain [^{35}S]methionine-labelled TIS21 and [^{35}S]methionine-labelled BTG1, the plasmids pSP64XβSNTIS21 and pSP64XβSNBTG1 [13] were used as templates for transcription and translation *in vitro*. The reactions were performed with the TNT SP6 coupled reticulocyte lysate system (Promega), in accordance with the manufacturer's instructions. [^{35}S]Methionine (1000 Ci/mmol; Amersham) was used to label the protein products.

To examine protein–protein interactions, the bacterial extracts containing GST–rPICK1, GST–PRMT1 or GST proteins were first incubated with glutathione–Sepharose 4B beads at 4 °C for 1 h. The unbound proteins were removed by centrifugation and the beads were washed twice with binding buffer [50 mM Tris/HCl (pH 7.4)/100 mM KCl/0.05 % Tween 20/1 mM dithiothreitol/1 mM PMSF]. The resulting GST–rPICK1–Sepharose bead complex was then resuspended in binding buffer containing 1 % (v/v) non-fat milk and incubated with 5 μl of the *in vitro* translation reaction mixture containing [^{35}S]TIS21 or [^{35}S]BTG1. After incubation for 1 h at 4 °C, the Sepharose 4B beads were collected by centrifugation, washed five times with binding buffer and resuspended in SDS/PAGE sample

buffer. The entire mixture was subjected to SDS/PAGE [15% (w/v) gel]. The gel was stained with Coomassie Blue, destained, dried and autoradiographed at −80 °C.

Antibody preparation

Purified recombinant rPICK1 (200 μg) was mixed with an equal quantity of complete adjuvant and used to immunize rabbits (New Zealand). Immunization was boosted three more times at intervals of 3–4 weeks. The antisera were collected a week after the last immunization and antibody titres were determined by Western blot analysis with recombinant rPICK1 as an antigen (results not shown).

Transfection and immunoprecipitation

Transfection was performed with LIPOFECTAMINE Plus Reagent (Life Biotechnologies) in accordance with the manufacturer's instruction. At 24 h after transfection, the cells were disrupted in 500 μl of lysis buffer [50 mM Tris/HCl (pH 7.4)/150 mM NaCl/1 mM EDTA/1 mM PMSF/20 μg/ml leupeptin/20 μg/ml aprotinin/20 μg/ml pepstatin/1% (v/v) Triton X-100] by pipetting. After centrifugation at 13000 rev./min in a Microfuge for 15 min, the supernatants were collected, preincubated with Protein A–Sepharose beads (Amersham Pharmacia Biotech) to remove proteins that bound non-specifically to the beads. The clarified cell lysates were then incubated for 2 h at 4 °C with Protein A–Sepharose beads bound by a polyclonal antibody directed against rPICK1 or directed against TIS21 (a gift from Dr Harvey Herschman, UCLA, Los Angeles, CA, U.S.A.). After centrifugation, the pellets were washed four times with PBS, pH 7.4, containing 0.05% (v/v) Tween 20, and resuspended in SDS sample buffer for the analysis of proteins that associated with the antibody–beads complex by SDS-PAGE followed by Western blot analysis with either a monoclonal antibody against FLAG (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) (M5; Kodak) or a polyclonal anti-rPICK1 antibody. The enhanced chemiluminescence (ECL®, Amersham Pharmacia Biotech) reagent was used for detection.

Kinase assays

Recombinant TIS21 and rPICK1 were purified as described above; histone III was purchased from Sigma. Phosphorylation by PKC was conducted in 20 mM Tris/HCl (pH 7.4)/10 mM $MgCl_2$/0.12 mM ATP/[γ-^{32}P]ATP (specific radioactivity 1200 d.p.m./pmol) (30 Ci/mmol; Amersham Pharmacia Biotech) with TIS21, rPICK1 or histone III as substrates. After the addition of PKC (1 μl, active form; Calbiochem-Novabiochem), the reaction mixtures were incubated for 30 min at 30 °C before being stopped with equal amounts of 2 × SDS sample buffer. The entire reaction mixture was subjected to SDS/PAGE. The gel was stained with Coomassie Blue, destained, dried and subjected to autoradiography at −80 °C to reveal ^{32}P incorporation. To test whether rPICK1 affected the phosphorylation of TIS21 by PKC, the TIS21 protein or histone was incubated with an approximately equal molecular mass of rPICK1 for 10 min at 30 °C before the addition of PKC. To quantify ^{32}P incorporation, the corresponding phosphorylated proteins were excised from the gel and radioactivity was counted in a β-scintillation counter. Alternatively, the autoradiogram was analysed by densitometry (Personal Densitometer SI; Molecular Dynamics).

RESULTS

Clone 5A interacts specifically with TIS21 in the yeast two-hybrid system

The LexA fusion TIS21 was previously used as a bait to screen a Gal4-activation-domain fusion rat cDNA library in the yeast two-hybrid system. Three interacting clones including 5A were identified in the screening [13]. Clone 5A interacted with TIS21 specifically in the yeast two-hybrid system (Figure 1). The blue colour started to develop 15 min after the reaction had been initiated and reached maximum in approx. 40 min. In spite of a high sequence similarity to TIS21, BTG1 did not interact with 5A (Figure 1). This pattern was different from PRMT1, which was shown to interact with TIS21 as well as BTG1 [13]. PRMT1 was used as a positive control in this study. The colour development in yeasts expressing TIS21 and PRMT1 was slightly, however consistently, faster than in yeasts expressing TIS21 and 5A.

Clone 5A is a rat counterpart of PICK1

The sequence of approx. 300 nt at the 5′ end of the clone 5A cDNA was shown to have great similarity to the mouse gene encoding PICK1 [13]. The nucleotide sequence of the longest uninterrupted open reading frame of the 5A cDNA encodes a polypeptide of 416 residues that showed 96.9% identity with the mouse PICK1 sequence (Figure 2). We confirmed that clone 5A is a rat counterpart of PICK1 and termed it rPICK1. A putative PDZ domain was identified in PICK1 [22]. The corresponding region in rPICK1 (residues 21–109) was also similar to the PDZ domains of various proteins (results not shown).

rPICK1 protein interacts with the TIS21 protein in a binding assay *in vitro*

To investigate whether rPICK1 associates with TIS21 in a system other than yeasts, we performed a binding assay *in vitro*. GST–rPICK1 protein, GST–PRMT1 protein and GST protein were expressed in *Escherichia coli* and purified with

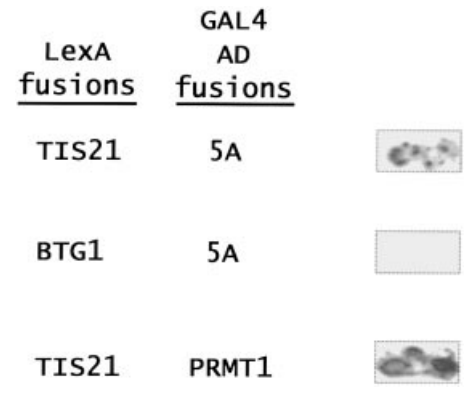

Figure 1 Clone 5A interacts with TIS21 but not BTG1 in the yeast two-hybrid analysis

The plasmids expressing LexA fusion proteins [pLexA(L)TIS21 and pLexA(L)BTG1] were introduced into yeast strain L40 together with plasmids expressing GAL4-activation-domain (AD) fusion proteins (pPC865A and pPC86PRMT1). The transformed yeasts were analysed for β-galactosidase activity with a yeast colony filter assay. Blue colour development indicates an interaction between two fusion proteins.

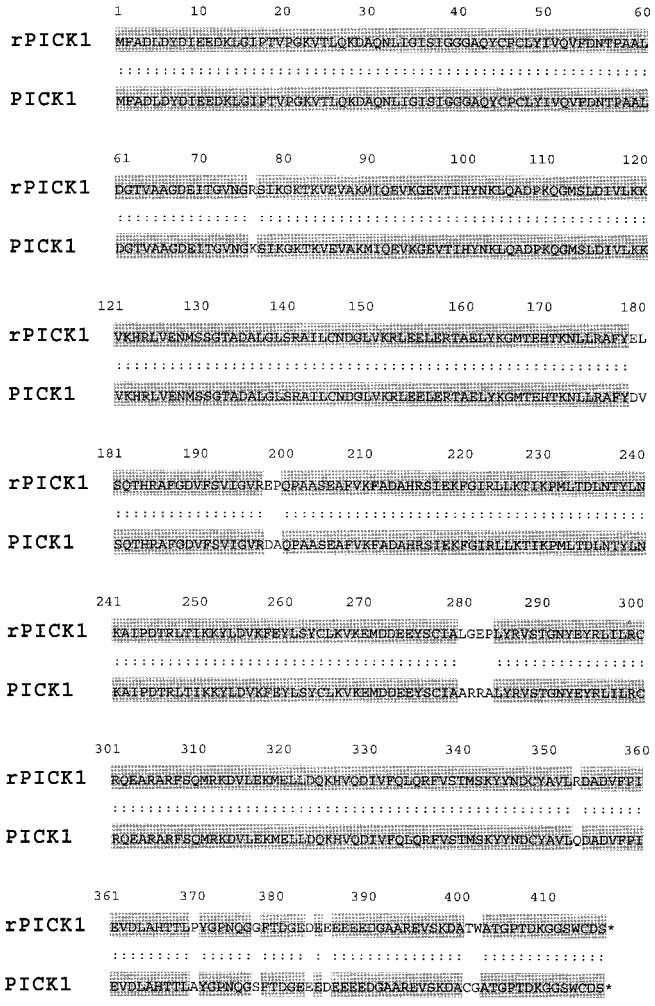

Figure 2 Comparison of the deduced amino acid sequences of clone 5A and PICK1

The longest open reading frame of 5A cDNA encodes a polypeptide of 416 residues with 96.9% identity to the mouse PICK1 protein.

glutathione–Sepharose 4B beads. The ^{35}S-labelled TIS21 and BTG1 were transcribed and translated *in vitro*. Only one ^{35}S-labelled band with a molecular mass corresponding to TIS21 (or BTG1) from each reaction was observed in the autoradiogram (Figure 3A). To test for association of rPICK1 and TIS21, the immobilized GST–rPICK1 protein was incubated with ^{35}S-labelled TIS21 (or BTG1). After being washed, the beads and its associated proteins were analysed by SDS/PAGE (Figures 3B and 3C, left panels) followed by autoradiography (Figures 3B and 3C, right panels). The ^{35}S-labelled TIS21 was recovered in the immobilized fraction, suggesting an association between TIS21 and GST–rPICK1 (Figure 3B). Only a background level of TIS21 was recovered in the immobilized fraction when incubated with GST protein (Figure 3B). These results indicated that TIS21 associated with the rPICK1 but not GST and that TIS21 physically associated with rPICK1. The possibility that interactions between TIS21 and rPICK1 were mediated by a third party present in the yeasts and also in the reticulocyte lysates was not completely ruled out. The ^{35}S-labelled BTG1 protein was not recovered in the immobilized fraction after incubation with GST–rPICK1 (Figure 3C). This was consistent with the observation in the yeast two-hybrid analysis (Figure 1). PRMT1, which was previously shown to interact with TIS21 and BTG1 in the binding assay *in vitro* as well as in the yeast two-hybrid system [13], was used as a positive control. These results indicated a specific and direct interaction between TIS21 and rPICK1.

rPICK1 and TIS21 are co-immunoprecipitated from NIH 3T3 cells

To show that rPICK1 and TIS21 associate in mammalian cells, NIH 3T3 cells were transfected with the pFlagCMV-TIS21 (in which CMV stands for cytomegalovirus) and pFlagCMV-rPICK1 plasmids and the expression of FLAG-rPICK1 and FLAG-TIS21 was examined by Western blot analysis. Only two proteins, one with an apparent molecular mass of approx. 53 kDa and the other of approx. 24 kDa, were detected with anti-FLAG antibody (Figure 4A). The identities of rPICK1 and TIS21 were further confirmed with anti-rPICK1 antibodies (Figure 4A) and anti-TIS21 antibodies (results not shown). When the FLAG-rPICK1 protein was immunoprecipitated from NIH 3T3 cells with anti-rPICK1 antibody, the FLAG-TIS21 protein was also recovered in the precipitates, suggesting an association of these two proteins (Figure 4B). In cells transfected with either pFlagCMV-rPICK1 or pFlagCMV-TIS21 plasmids, only the corresponding protein was immunoprecipitaed (Figure 4B). Only a portion of TIS21 was co-immunoprecipitated by rPICK1 antibody in comparison with the total amount of FLAG-TIS21 expressed. This agreed with the notion that both TIS21 and rPICK1 have several interacting partners.

N-terminal putative PDZ domain of rPICK1 is required for interaction with TIS21

To investigate the sequences of rPICK1 required for interaction with TIS21, we made a series of deletion constructs of rPICK1 fused to the Gal4 activation domain. Yeast two-hybrid analysis was performed with LexA-TIS21 and various Gal4AD–rPICK1 fusion proteins (Figure 5). The mutant rPICK1$_{A142–S416}$ lacking the N-terminal 141 residues, which cover the entire putative PDZ domain, failed to interact with TIS21. Deletion of only the first 51 residues of rPICK1 did not restore its interaction with TIS21 (Figure 5, rPICK1$_{V52–S416}$). These results suggested that a complete PDZ domain was required for rPICK1 to interact with TIS21. The carboxylate-binding loop of the putative PDZ domain (residues 27–35) was shown to be involved in the binding of rPICK1 to PKCα [22]. Our results raise the possibility that TIS21 might compete with PKCα for binding to rPICK1. The C-terminal 64 residues of rPICK1s were not required for the interaction with TIS21, as the deletion of residues 353–416 did not affect the interaction (Figure 5, rPICK1$_{M1–L352}$).

Deletion at the N-terminus of TIS21 abolishes interaction but the middle region alone is capable of interacting with rPICK1

To map the region in TIS21 required for interactions with rPICK1, various deletion constructs of TIS21 were made for the expression of LexA fusion proteins (Figure 6A). Each of these plasmids was introduced into the yeast L40 together with pPC86-rPICK1 to analyse their interactions. Deletion of the N-terminal 48 residues of TIS21 abolished its interaction with rPICK1 (Figure 6A, TIS21$_{H49–S158}$). Shortening of this deletion region

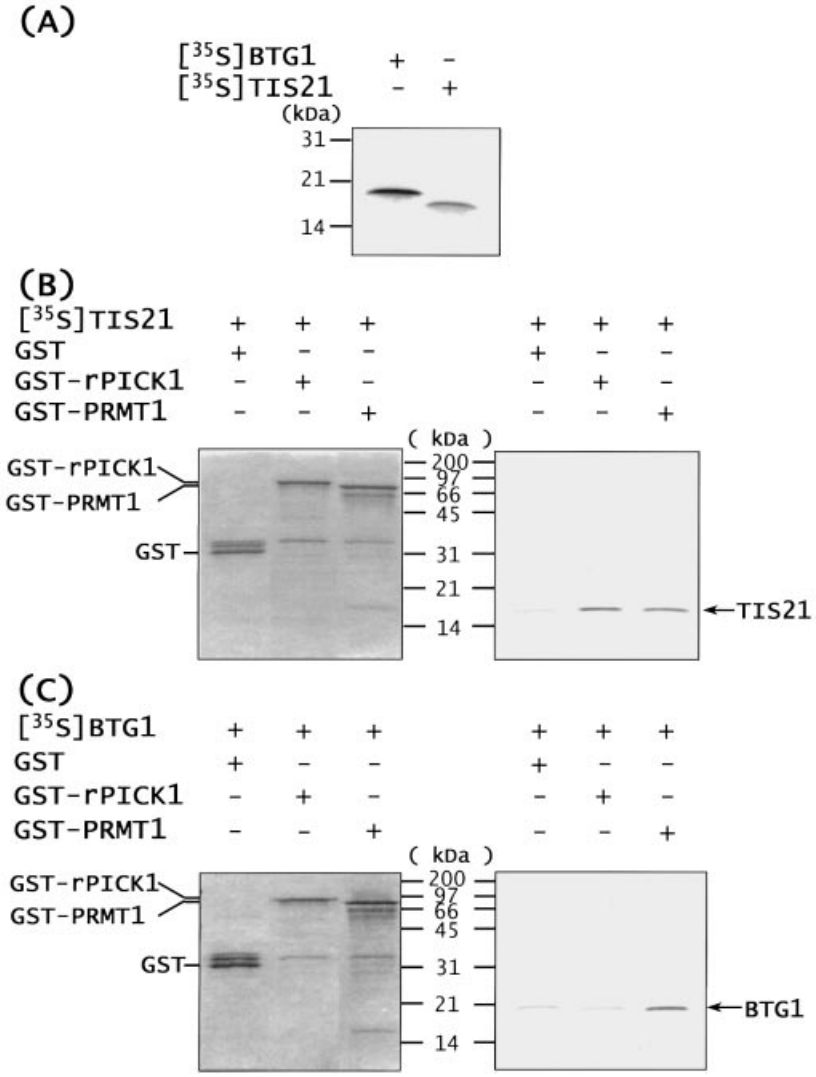

Figure 3 TIS21 translated *in vitro* associates with recombinant GST–rPICK1

[^{35}S]Methionine-labelled TIS21 and [^{35}S]methionine-labelled BTG1 were obtained by transcription and translation *in vitro* (**A**). The [^{35}S]TIS21 (**B**) and [^{35}S]BTG1 (**C**) proteins were incubated for 1 h with GST, GST–rPICK1 or GST–PRMT1 immobilized on glutathione-Sepharose 4B beads. After being washed, proteins associated with beads were analysed by SDS/PAGE. (**B**, **C**) The gels were stained, destained, dried (left panels) and subjected to autoradiography at −80 °C (right panels).

to the first 18 residues did not restore the ability for TIS21 to interact (Figure 6A, $TIS21_{F19–S158}$). These suggested that the existence of the N-terminus was crucial for wild-type TIS21 to interact with the rPICK1. The possibility that expression levels of the various TIS21 mutants in yeasts might not be the same was not ruled out. The mutant $TIS21_{M1–L122}$, lacking the C-terminal 36 residues, interacted with rPICK1 (Figure 6A). No significant difference in β-galactosidase activity was observed between full-length TIS21 and the C-terminus-deleted TIS21, suggesting that the C-terminal 36 residues of TIS21 were not required for its interaction with rPICK1. Interestingly, the mutant $TIS21_{H49–L122}$, lacking both the N-terminus and the C-terminus, was able to turn on the expression of reporter gene β-galactosidase when introduced into yeasts together with rPICK1 (Figures 6A and 6B). The mutant $TIS21_{F19–L122}$ showed very weak activity. These suggested that the H^{49}–L^{122} fragment contained an interacting region that was probably masked in wild-type TIS21. To exclude the possibility that the mutant $TIS21_{H49–L122}$ had become sticky and interacted non-specifically with proteins, yeast two-hybrid analysis with $TIS21_{H49–L122}$ and PRMT1 was conducted. Unlike the wild-type protein, the mutant $TIS21_{H49–L122}$ did not interact with PRMT1 (Figure 6B), suggesting a specific nature of the interaction between $TIS21_{H49–L122}$ and rPICK1 proteins.

To investigate whether the deletion mutants of TIS21 and rPICK1 acted similarly to the wild-type proteins, we performed yeast two-hybrid analysis with mutant TIS21 proteins and mutant rPICK1 proteins. Only the TIS21 mutants ($TIS21_{M1–L122}$ and $TIS21_{H49–L122}$) that interacted with wild-type rPICK1 were able to activate the expression of the reporter gene *lacZ* when introduced into yeasts together with $rPICK1_{M1–L352}$ (results not shown). These mutants were unable to activate the transcription of β-galactosidase when expressed in yeasts together with the N-terminus-deleted rPICK1 proteins ($rPICK1_{V52–S416}$ and $rPICK1_{A142–S416}$) (results not shown). The results suggest that,

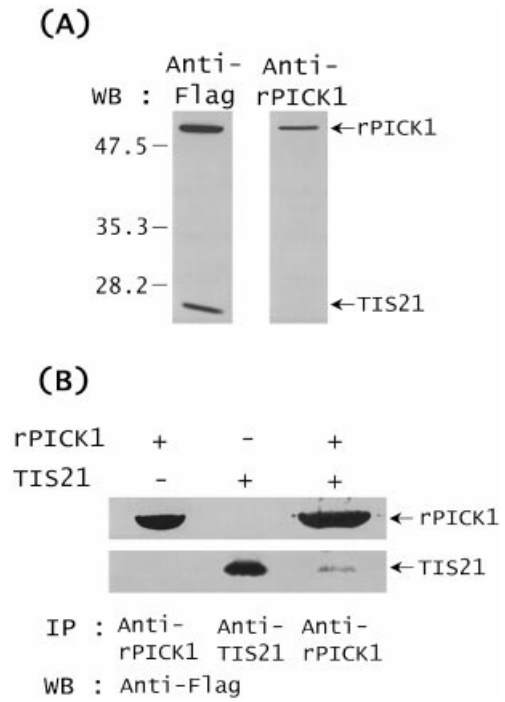

Figure 4 TIS21 protein is co-immunoprecipitated with rPICK1 from NIH 3T3 cells

NIH 3T3 cells were transfected with either pFlagCMV-TIS21 or pFlagCMV-rPICK1, or both. (**A**) After being transfected with both plasmids, the cells were lysed and the proteins were separated by SDS/PAGE and transferred to a nitrocellulose membrane. Expression of FLAG-tagged proteins was detected with anti-FLAG M5 antibody. The identity of FLAG-rPICK1 was confirmed with polyclonal anti-rPICK1 antibody. Abbreviation: WB, Western blot. (**B**) The soluble fraction of cell lysates collected after transfection with the indicated plasmids was incubated with Protein A–agarose bound with anti-rPICK1 antibody or anti-TIS21 antibody. The proteins associated with agarose beads were separated by SDS/PAGE and subjected to Western blot analysis (WB) using anti-FLAG M5 antibody. Abbreviation: IP, immunoprecipitation.

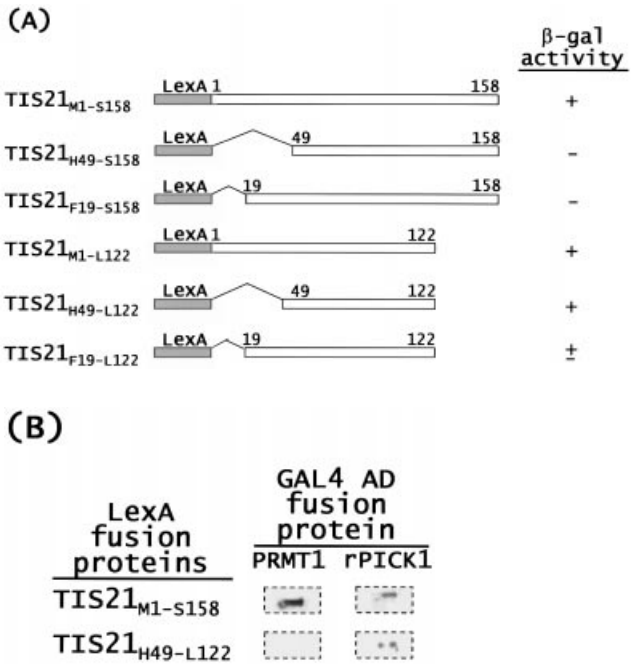

Figure 6 Mapping of interaction regions on TIS21

(**A**) A schematic representation of TIS21 deletion mutants expressed as LexA fusion proteins. The yeasts L40 were co-transformed with a pPC86rPICK1 plasmid and various pLexA(L)TIS21 constructs to examine the regions required for interactions. The transformed yeasts were analysed for the expression of β-galactosidase activity by colony filter assay with 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside ('X-Gal') as a substrate. Key: +, expression of β-galactosidase activity; —, no detectable β-galactosidase activity. (**B**) The yeasts L40 were co-transformed with a pPC86rPICK1 (or pPC86PRMT1) plasmid and a pLexA(L)TIS21 wild-type (or $TIS_{H49–L122}$ mutant) plasmid and analysed for β-galactosidase activity. The development of a blue colour indicates an association between the LexA fusion protein and the GAL4 activation domain (AD) fusion protein.

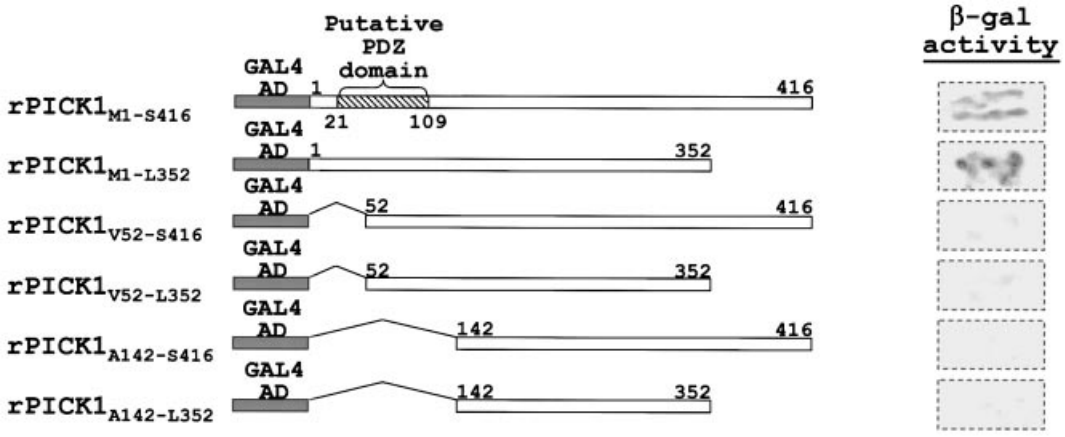

Figure 5 PDZ domain of rPICK1 is required for interaction with TIS21

A schematic representation of rPICK1 deletion mutants expressed as GAL4 fusion proteins is shown. The yeasts L40 were co-transformed with the pLexA(L)TIS21 plasmid and various pPC86rPICK1 constructs. The transformed yeasts were analysed for β-galactosidase activity by colony filter assay with 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside ('X-Gal') as a substrate. The development of a blue colour indicates an association between the TIS21 protein and the rPICK1 protein.

like the wild-type TIS21, these two mutant TIS21 proteins interacted with the putative PDZ domain of rPICK1. The non-interacting $TIS21_{H49–S158}$, as determined with the wild-type rPICK1, did not interact with any of the deletion mutants of rPICK1 (results not shown). These were in agreement with the observations with wild-type proteins, which suggests that no

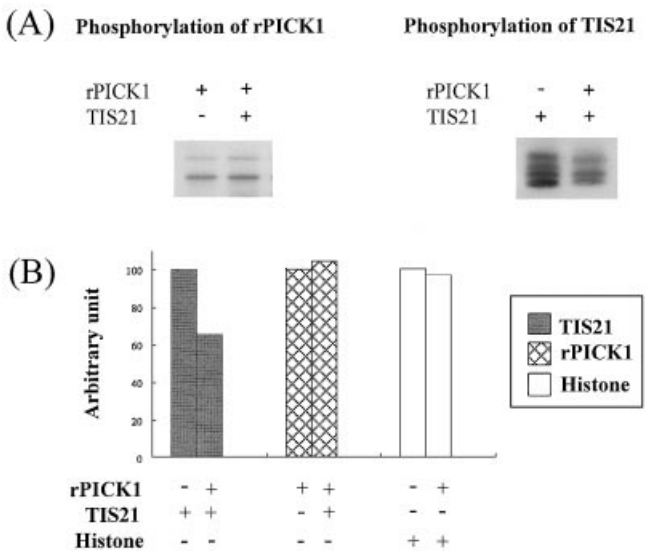

Figure 7 Phosphorylation of TIS21 by PKC is significantly decreased in the presence of rPICK1

(**A**) Phosphorylation by PKC was performed with either recombinant TIS21 protein or recombinant rPICK1 protein as a substrate and analysed by gel electrophoresis followed by autoradiography. To test whether rPICK1 affected phosphorylation by PKC, TIS21 or histone III was preincubated with or without rPICK1 for 10 min before the addition of PKC and [γ-^{32}P]ATP. (**B**) ^{32}P incorporation into TIS21, rPICK1 and histone was quantified by densitometry or scintillation counting of the gel slices.

apparent changes occurred in the deletion mutants that altered their behaviour in interaction.

Catalytic activity of PKC towards TIS21 is affected significantly in the presence of rPICK1

Among the stimuli that induce TIS21 expression, some (including PMA) are known to be potent activators of PKC. Several potential PKC phosphorylation sites were found in the TIS21 sequence. To investigate whether TIS21 could be phosphorylated by PKC, kinase assays were performed *in vitro* with purified recombinant TIS21 as a substrate. Both TIS21 and rPICK1 were phosphorylated by active PKC (Figure 7A). The two rPICK1 polypeptides (53 and 43 kDa) resulted from the digestion of GST–rPICK1 with thrombin. Both species of rPICK1 were phosphorylated by PKC. Phosphorylation of TIS21 by PKC caused a mobility change in SDS/PAGE (Figure 7A). The incorporation of ^{32}P into TIS21 was significantly more than that into rPICK1 when equimolar amounts of TIS21 and rPICK1 were used (results not shown), suggesting that TIS21 was a better substrate for PKC *in vitro*. No ^{32}P incorporation into either TIS21 or rPICK1 was observed in the absence of PKC (results not shown), suggesting that no kinase activity was associated with either TIS21 or rPICK1. After preincubation with equimolar amounts of rPICK1 before the addition of PKC, TIS21 phosphorylation was decreased by 35–40 % (Figures 7A and 7B). Phosphorylation of rPICK1 by PKC was not affected after preincubation with TIS21 (Figures 7A and 7B), indicating that TIS21 phosphorylation by PKC was selectively altered. Phosphorylation of histone III, a known PKC substrate, was not affected in the presence of rPICK1 (Figure 7B). It is unlikely that rPICK1 competed with TIS21 as a substrate for PKC, because TIS21 was more readily phosphorylated than rPICK1. The phosphorylation state of TIS21 in cells might be modulated through interactions with rPICK1.

DISCUSSION

With the goal of investigating the role of TIS21 in cellular responses to ligand stimulation, we used the yeast two-hybrid screen to search for genes whose protein products interacted with TIS21. Clone 5A is one of these genes identified in the screening [13].

We have sequenced the entire cDNA of clone 5A. The longest open reading frame encodes a protein of 416 residues showing 96.9 % identity with the mouse PICK1. We confirm that clone 5A is a rat counterpart of PICK1. Mouse PICK1 was previously identified as a PKCα-binding protein and was proposed to act as an intracellular receptor of PKC [21]. rPICK1 associates with TIS21 through direct physical contact, as shown by the binding assay *in vitro*. In contrast with PRMT1 [13] and mCaf1 [16], which have been shown to be able to interact with both TIS21 and BTG1, rPICK1 interacts only with TIS21. These results suggest that TIS21 shares common partners such as PRMT1 and mCaf1 with BTG1 but also has distinct interacting partners such as rPICK1. TIS21 and BTG1 are the closest members of the TIS21/BTG1 gene family. These two highly similar proteins might exert distinct functions under certain conditions through interacting with different partners.

The N-terminal region of TIS21 is probably involved directly or indirectly in the interaction with rPICK1, because deletion of the N-terminus abolished interaction (Figure 6, TIS21$_{F19–S158}$ and TIS21$_{H49–S158}$). Failure in interaction is unlikely to be solely accounted for by a distortion in the tertiary structure caused by deletion, because TIS21$_{H49–L122}$ interacted well with rPICK1 (Figure 6). However, only very weak interaction was detected with TIS21$_{F19–L122}$ and no interaction was detected with TIS21$_{H49–S158}$ (Figure 6). Our results suggest that the interacting sequence in the H^{49}–L^{122} fragment might be masked by sequences in both the N-terminus and the C-terminus in wild-type TIS21. It is of particular interest that the H^{49}–L^{122} fragment of TIS21 interacts specifically with rPICK1. This region might participate in interactions when exposed under certain conditions. It has recently been shown that residues 50–68 of TIS21/PC3 are required for the anti-proliferative activity of TIS21/PC3 as well as for the down-regulation of cyclin D1 [25]. The essential role of this region might be mediated by its interaction with other cellular proteins.

We have demonstrated that the putative PDZ domain of rPICK1 is required for its interaction with TIS21. PDZ domains are protein-binding motifs identified in many proteins associated with the plasma membrane, cell–cell junctions, cytoskeletal proteins and signalling molecules [26]. The InaD protein from *Drosophila* contains five copies of PDZ domains, serving as a scaffold on which to assemble different components of the phototransduction cascade [27]. Many PDZ domains bind directly to the C-terminal ends of their target molecules [28]. Specificity is determined primarily by the C-terminal three to seven residues, as analysed with peptide libraries [29]. Because deletion at the C-terminus of TIS21 did not affect its interaction with rPICK1 (Figure 6), recognition of TIS21 by the PDZ domain of rPICK1 is not through the C-terminal motif. This is not surprising, because PDZ domains have also been shown to bind to non-C-terminal target sequences such as internal (T/S)XV motifs, other PDZ domains, LIM domains or ankyrin and spectrin repeats [26]. These observations reveal a complexity

in PDZ–target interactions. The molecular mechanisms for these types of recognition are unclear. Our results show that the N-terminal region of TIS21 is required for interaction with rPICK1. In addition, the H^{49}–L^{122} region of TIS21 contains sequences that alone can bind to rPICK1s carrying an intact PDZ domain. The interaction of TIS21 with the PDZ domain of rPICK1 might represent a still unidentified method of binding of PDZ proteins to their targets.

Increasing evidence suggests that the interaction of PDZ domains with partners can be a regulatory event. Binding of the inward rectifier K^+ channel Kir2.3 to the PDZ domain of PSD-95 is regulated by the phosphorylation of protein kinase A [30]. Interaction of the β_2-adrenergic receptor with the epithelial PDZ protein NHERF is dependent on agonist-mediated activation of the receptor [31]. It is of particular interest that the carboxylate-binding loop of the PDZ domain on PICK1 is required for its interaction with PKCα through the C-terminal motif of PKCα [22]. It remains to be determined whether TIS21 competes with PKCα for binding to rPICK1 on stimulation with ligand or whether TIS21 is recruited by rPICK1 to form a ternary complex together with PKCα for a unique cellular response.

We have demonstrated that TIS21 is phosphorylated by PKC *in vitro*. Phosphorylation of TIS21 is significantly decreased in the presence of rPICK1. This modulation in phosphorylation by PKC seems to be specific for TIS21 because the phosphorylation of histone is not affected. There are several potential PKC phosphorylation sites in TIS21. Association of TIS21 with rPICK1 might induce a conformational change and result in a restricted phosphorylation on a particular site, leading to a specific cellular response. Proteins with multivalent binding activity can serve as platform for the assembly of signalling molecules. Through this connection, modifications of substrates can occur specifically and efficiently in response to the appropriate signals. AKAP79 (for 'a kinase anchoring protein 79') co-ordinates the association of cAMP-dependent kinase, phosphatase 2B (calcineurin) and PKC in neurons [32]. The phosphatase activity of calcineurin and the kinase activity of PKC are inhibited by the binding of AKAP79 [32,33]. This suggests that the anchoring protein AKAP79 serves not only as a scaffold to recruit three enzymes but also has a role in regulation of the phosphorylation state of key substrates.

Our results indicate that, in addition to the recruitment of PKC to the proximity of its substrates, rPICK1 is likely to modulate substrate phosphorylation by PKC. Many ligands that stimulate the expression of TIS21 also activate the PKC pathway. rPICK1 might act as a scaffold to recruit essential components such as transiently expressed TIS21 and therefore modulate their phosphorylation states for a particular cellular response. Demonstration of the association of TIS21 with a PKC-binding protein, rPICK1, and the modulated phosphorylation of TIS21 by PKC supports the speculation that TIS21 might mediate PKC activity in cellular responses.

We thank Dr Harvey R. Herschman (UCLA, Los Angeles, CA, U.S.A.) for his critical reading of this manuscript and for helpful discussions; Dr Yu Su for his critical reading of this manuscript; and Mr L.-J. Wu and Ms S.-Y. Shiu for their technical assistance. This work was supported by a grant (NSC-87-2314-B-010-006) from the National Science Council, Taiwan, Republic of China, to W.-J.L.

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Received 3 October 2000/18 December 2000; accepted 11 January 2001