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The PDZ Protein TIP-1 Interacts with the Rho Effector Rhotekin and Is Involved in Rho Signaling to the Serum Response Element*

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The human T-cell lymphotrophic virus, type 1 Tax protein can interact via its C terminus with various proteins including a PDZ domain. In this work, one of them, TIP-1, is characterized as a cytoplasmic 14-kDa protein mainly corresponding to one PDZ domain. A two-hybrid screen performed with TIP-1 as bait showed that it interacts with the human homologue of rhotekin that was previously identified in mice as a Rho effector. Both human and mouse rhotekins exhibit at their C termini the sequence QSPV-COOH that matches the *X*(S/T)*X*V-COOH consensus known for proteins recognizing PDZ domains. Mutation of the serine and valine residues to alanine impairs interaction of rhotekin with TIP-1. Transient expression experiments with a reporter construct including the c-Fos serum response element (SRE) showed that coexpression of TIP-1 with the constitutively active RhoA.V14 mutant and human rhotekin caused a strong activation of the SRE. A negative mutant of Rho, RhoA.N19, was unable to cooperate with TIP-1 and rhotekin. The positive effect of TIP-1 was also lost when the C terminus of rhotekin was mutated. These data show that the complex of active Rho with its effector rhotekin bound to TIP-1 produces in the cytoplasm a signal that triggers strong activation of the SRE.

A wealth of studies has established that proteins encoded by various types of viruses establish specific contacts with key regulatory cellular proteins. Recently, a novel class of cellular proteins characterized by a specific protein motif, the PDZ domain, has been shown to be targeted by viral transforming proteins. The PDZ domain, the name of which corresponds to the first letter of PSD-95 (a post-synaptic density protein), Discs-large (a *Drosophila* tumor suppressor), and ZO-1 (a tight junction protein), is known to be present in a rapidly increasing number of proteins exhibiting diverse functions (1, 2). The proteins E4 open reading frame 1 of adenovirus type 9, E6 of human papilloma virus type 16 or 18, and Tax of human T-cell leukemia virus type 1 have been shown to interact with hDlg, the human homologue of the *Drosophila* Discs-large tumor suppressor via its PDZ domains (3–5). The HTLV-1[1] Tax protein has also been shown to interact with a series of cellular proteins characterized by the presence of a PDZ domain (6). Two-hybrid studies have shown that the PDZ domain recognizes the *X*-(S/T)-*X*-V-COOH motif, *X* being any amino acid, at the C-terminal ends of proteins (7). This model of interaction stems from study of the interaction of PSD-95 with subunits of the glutamate receptor, as well as with shaker type potassium channels (8, 9). These original observations have been extensively confirmed with various PDZ proteins and have also received support from resolution of the structure of the third domain of PSD-95 in complex with a 9-amino acid peptide (10). This showed that the peptide forms an antiparallel β-sheet with strand β-B of the PDZ domain that includes six β strands interrupted by two α helices (10). The C-terminal amino acids of the peptide exchange several hydrogen bonds with a loop between strands β-A and β-B. A systematic study of peptides able to interact with particular PDZ domains has shown that many variations were possible on the basis of the *X*-(S/T)-*X*-V-COOH consensus (11). The C-terminal valine can be substituted with other hydrophobic or aromatic residues in the case of particular PDZ domains. This suggests that a specific relationship exists between a given PDZ domain and the C terminus of the associated proteins. If a number of PDZ proteins are cytoplasmic or localized at the inner side of the membrane, where they can interact with transmembrane receptors, playing an important role in receptor clustering and signal transduction (12, 13), this is not a general feature. Indeed PDZ domains have been identified in nuclear proteins (14), as in Bridge-1, which acts as a coactivator for the E12 basic helix-loop-helix factor (15). From what is currently known about PDZ proteins, it is clear that the presence of a PDZ domain correlates with association with a protein partner, but not with a specific function.

In a previous study, several PDZ proteins were identified as able to bind to the C-terminal end of the HTLV-1 Tax protein (6). Here we report cloning of the complete cDNA of one of them, TIP-1. The protein corresponding to this cDNA has been characterized, and a two-hybrid study showed that TIP-1 binds to human rhotekin (h-rhotekin), an effector of the Rho GTPases. An effect of RhoA, h-rhotekin, and TIP-1 on activation of the c-Fos serum response element is characterized.

EXPERIMENTAL PROCEDURES

Plasmids—Full-length TIP-1 cDNA was obtained by rapid amplification of cDNA ends-PCR using the Human Leukocyte Marathon-Ready cDNA library (CLONTECH). To generate pGB-TIP-1, TIP-1 cDNA was amplified using *Pfu* polymerase, digested with *Sal*I restriction enzyme, and inserted between the *Sma*I and *Sal*I restriction sites of pGBgly, which is a derivative of pGB-T9 (CLONTECH). The pTL-TIP-1 plasmid was made by subcloning the TIP-1 cDNA into the *Eco*RI

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[1] The abbreviations used are: HTLV-1, T-cell lymphotrophic virus, type 1; SRE, serum response element; FCS, fetal calf serum; h, human; m, mouse; GEF, guanine nucleotide exchange factor; LIMK-1, LIM kinase 1; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; BS, binding site.

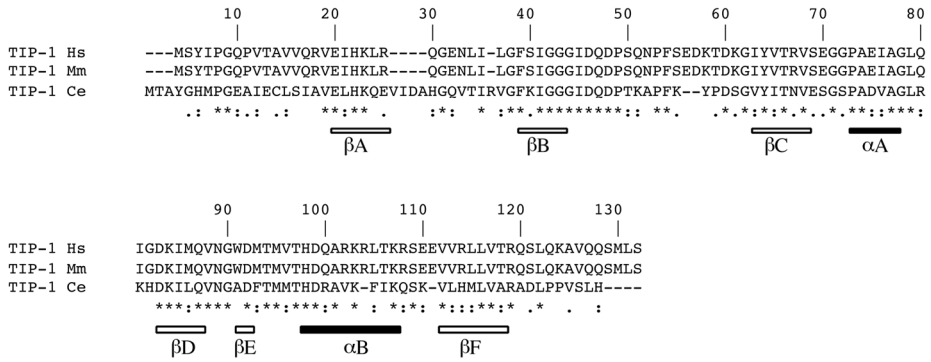

FIG. 1. **Sequence of TIP-1 protein.** Amino acid sequence alignment of human, mouse, and *C. elegans* (C45G9.7) TIP-1 is shown. These sequences were aligned using the ClustalW program (24) available on the Pole Bio-informatique Lyonnais web site. Alignment gaps are shown by *dashes*. Perfect matches are indicated by *asterisks* below the alignment. Conservative substitutions of high similarity and low similarity are marked by *two dots* and *one dot*, respectively. The six β sheets (*white bars*, βA–βF) and the two α helices (*black bars*, αA and αB) present in the structure of the PDZ3 motif of PSD95 (10) are indicated as they can be deduced from alignment of TIP-1 with different PDZ domains including PDZ3 of PSD-95 (6). The GenBank™ accession numbers of the proteins are as follows: TIP-1 Hs, AF028823; TIP-1 Mm, W59126, TIP-1 Ce (C45G9.7), U21323.

restriction site of pTL1, which is a pSG5 derivative (16). The bacterial expression vector pET1 was constructed by inserting the TIP-1 cDNA, amplified by PCR with an oligonucleotide creating an *Nde*I restriction site at the 5′ end, between the *Nde*I and *Bam*HI restrictions sites of pET11d (Novagen). The pSGF-h-rhotekin was constructed in two steps. h-rhotekin cDNA was amplified by PCR from the pACT yeast clone and inserted into pGEM-T (Promega). The *Sal*I restriction fragment obtained from this plasmid was inserted into the *Xho*I restriction site of pSG-Flag vector (17). Plasmid pSGF-h-rhotekin Cm in which the C-terminal serine and valine residues were changed into alanine was obtained by amplification of the h-rhotekin c-DNA with a 3′ oligonucleotide creating the mutation and insertion of the resulting DNA fragment in pSGF. The presence of the mutation was verified by sequencing. The following plasmids have been described previously: EXV-RhoA.V14 (18), SRE, and SRE-APm (mSRE in original publication; Ref. 19). The SRE and SRE-APm reporter constructs are derivatives of pBL-CAT4 bearing the SRE motif inserted in the *Sal*I restriction site in the sense orientation. The sequences inserted in these constructs are 5′-tcgACAGGATGTCCATATTAGGACATCTGCGTCAGC-3′ and 5′-tcgACAGGATGTCCATATTAGGACATCTGCcTtAGC-3′, for SRE and SRE-APm, respectively. Plasmid pCEV29-RhoAN19 was kindly provided by Silvio Gutkind through Marc Billaud.

Generation of TIP-1-specific Antibodies—TIP-1 protein was produced in *E. coli* from the pET1 expression vector. In induced bacteria TIP-1 protein was present within inclusion bodies. After lysis by sonication, these inclusion bodies were purified as described previously (20). After solubilization in SDS loading buffer, TIP-1 was purified by electrophoresis through a 14% SDS-polyacrylamide gel. The 14-kDa band corresponding to TIP-1 was lyophilized and, after mixing with adjuvant, injected into a rabbit.

Two-hybrid Screen in Yeast—Two-hybrid screen was carried out using the Matchmaker II kit (CLONTECH), according to manufacturer's instructions. HF7c yeast strain including pGB-TIP-1 was further transformed with a cDNA library of Epstein-Barr virus-transformed human lymphocytes (21). Yeast cells were plated on SD medium complemented with Ura, Lys, and Ade, along with 5 mM aminotrizole. Isolation of transformants and β-galactosidase assays on filters were performed as described previously (6). From an initial screen of 7.4×10^6 transformants, 265 clones grew on minimal medium lacking His and were positive for β-galactosidase expression. Plasmids from these colonies were recovered and transformed in *Escherichia coli* XL1-blue by electroporation as described previously (22).

Cell Culture, Immunoblot, and Immunoprecipitation—COS7 and HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) for regular culture and 5% FCS for transfection, which was performed by the calcium phosphate precipitation method. COS7 cells were grown in 10-cm diameter Petri dishes and were transfected with 1 μg of pTL-TIP-1 and 1.5 μg of vectors expressing RhoA.V14 or F-h-rhotekin. The total amount of plasmid DNA was adjusted to 15 μg with pBSK+. HeLa cells were grown in 6-cm diameter Petri dishes and were transfected with the following amounts of plasmids: 0.4 μg of SRE or SRE-APm reporter plasmids, 1 μg of pTL-TIP-1, 1.5 μg of vectors expressing RhoA.V14, RhoA.N19, or F-h-rhotekin. The total amount of transfected DNA was

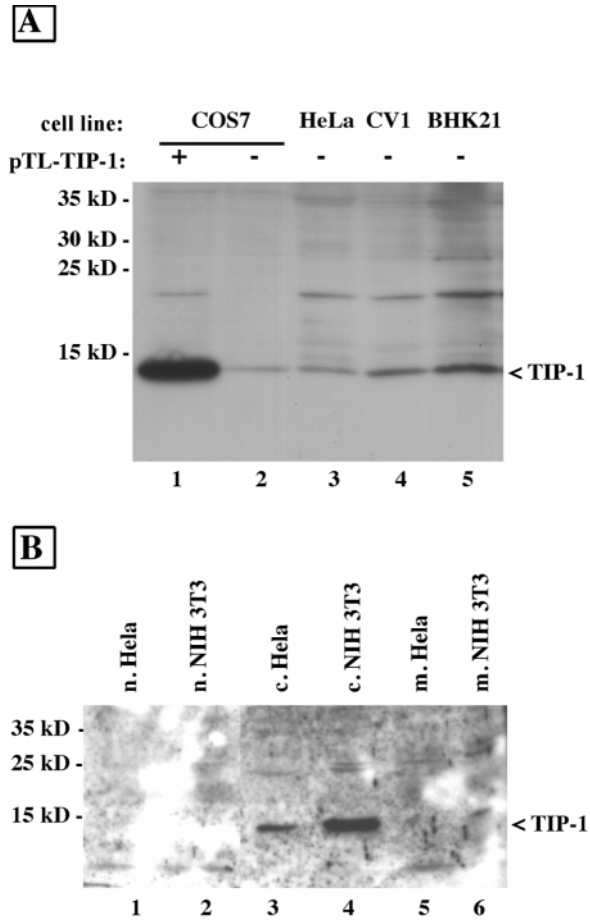

FIG. 2. **Expression and subcellular localization of TIP-1.** *A*, immunoblot analysis of TIP-1 in untransfected or transfected cells. Total cell lysates from pTL-TIP-1-transfected COS7 cells (*lane 1*), untransfected COS7 cells (*lane 2*), HeLa cells (*lane 3*), CV1 cells (*lane 4*), and BHK21 cells (*lane 5*) were loaded on a 14% polyacrylamide protein gel. After blotting, proteins were revealed using the rabbit polyclonal antibody to TIP-1 (1:500 dilution). Positions of the bands of a molecular size marker run in parallel along with that of the 14-kDa band corresponding to TIP-1 are indicated. *B*, immunoblot analysis of cytoplasmic (*c.*), nuclear (*n.*), and membrane (*m.*) extracts of HeLa and NIH3T3 cells. These subcellular fractions were prepared as described under "Experimental Procedures." After migration through a 14% polyacrylamide protein gel, an immunoblot was performed with the antibody to TIP-1. Positions of the bands of a molecular size marker run in parallel along with that of TIP-1 are indicated.

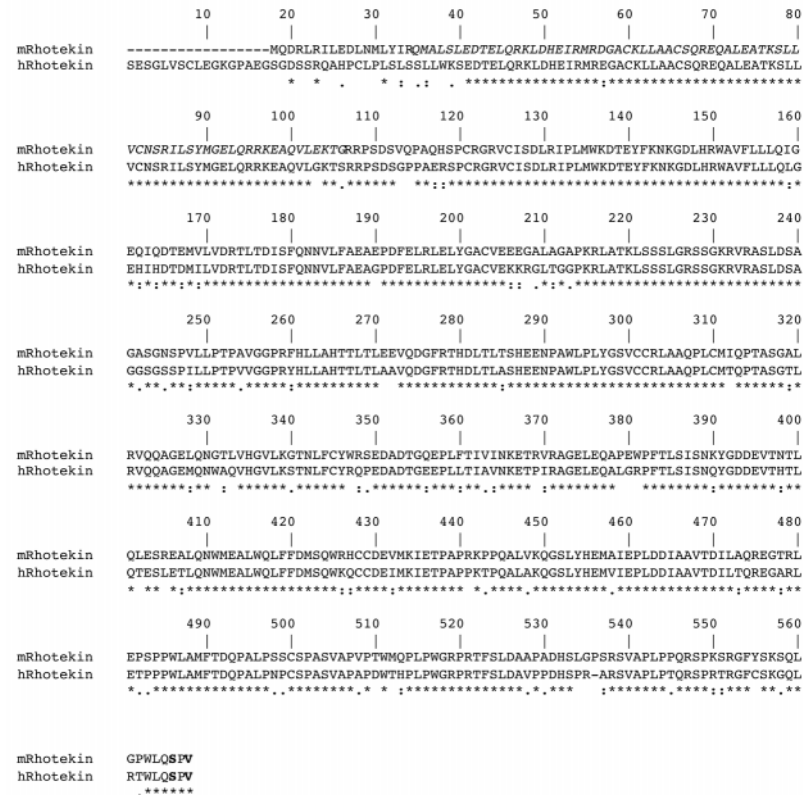

FIG. 3. **Sequence of h-rhotekin.** The amino acid sequence of human rhotekin translated from the longest cDNA isolated by two-hybrid screening with TIP-1 as bait, was aligned with that of mouse rhotekin as described in legend to Fig. 1. Symbols used to mark alignment gaps, perfect matches, and high and low similarity are described in the legend to Fig. 1. Amino acids of the Rho binding domain are in *italics* in the m-rhotekin sequence. The GenBank™ accession numbers of m-rhotekin and h-rhotekin are U54638 and AF290512, respectively.

adjusted to 7.5 μg, and the amount of SV40 promoter containing expression vectors was adjusted to 4.5 μg with pSG5.

For COS7 cells, the precipitate was washed 16 h after its addition and cells were further incubated in Dulbecco's modified Eagle's medium plus 5% FCS for 36 h before lysis in radioimmunoprecipitation assay buffer (23). Lysates were centrifuged for 10 min at 12,000 rpm, and supernatants were used for immunoblot or for immunoprecipitation, which were performed as described (23). All antibodies were diluted 1:250 for immunoprecipitation and 1:500 for immunoblotting. After transfection, HeLa cells were grown 16 h in medium with 5% FCS, and 24 h in medium containing 0.5% FCS. Induction by serum was made by incubating cells for 8 h in medium supplemented with 15% FCS. CAT enzyme-linked immunosorbent assays were performed according to the manufacturer's instructions (Roche).

Subcellular Fractionation—5 × 10^7 HeLa or NIH3T3 cells were harvested and washed twice in PBS. The cellular pellet was resuspended in five volumes of buffer A: 10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride, and incubated 10 min on ice. The suspension was centrifuged 10 min at 1000 rpm, and pellet was resuspended in three volumes of buffer A. 0.05% Nonidet P-40 was added and the suspension was homogenized with 20 strokes of a tight-fitting Dounce homogenizer to release the nuclei. After a centrifugation of 10 min at 1000 rpm, the supernatant was kept (solution 1) and the pellet was resuspended in 0.3 ml of buffer B (5 mM Hepes (pH 7.9), 26% glycerol (v/v), 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). Total volume was measured and NaCl was added to a final concentration of 300 mM. After incubation for 30 min on ice and centrifugation for 20 min at 20,000 rpm, the supernatant containing the nuclear fraction was snap-frozen in dry ice and stored at −80 °C. Solution 1 was then centrifuged for 30 min at 40,000 rpm. Pellet containing membranes, resuspended in TE, pH 8.0, and supernatant corresponding to the cytoplasmic fraction were stored at −80 °C.

RESULTS

Characterization of TIP-1 Protein—Screening by the two-hybrid method with HTLV-1 Tax protein as bait led to isolation of six different proteins, including one or several PDZ domains (6). Three of them were related to previously characterized proteins, PSD-95, β1-syntrophin, and lin-7. As a first step to characterize TIP-1 encoded by one of the clones previously identified, its full-length cDNA was isolated from a leukocyte library (see "Experimental Procedures"). The cDNA included an in-frame stop codon 25 codons upstream from an AUG, which hence was considered as the initiation codon. The reading frame starting at this position encodes a 124-amino acid protein, which mainly corresponds to a PDZ domain flanked by 14 and 12 amino acids at the N-terminal and C-terminal ends, respectively. The calculated molecular mass is 13.7 kDa. Data bank searches identified expressed sequence tags encoding a murine homologue and analysis of the *Caenorhabditis elegans* genome showed the existence of a homologue in this distant organism. In these three species, the number of amino acids is conserved and an alignment with the ClustalW program (24) shows a good conservation (Fig. 1). In particular, the high number of amino acids between strand βB and βC of TIP-1, as compared with other PDZ domains (6), is observed in these three species. This part is likely to confer some specificity to the PDZ domain of TIP-1.

In order to characterize the product of the human cDNA, a rabbit polyclonal antibody was raised against TIP-1 protein produced in bacteria. Immunoblot analysis of a COS7 cells extract with this antibody gave some background, but a band was clearly revealed at 14 kDa (Fig. 2*A*, *lanes 2–5*). Transfection of the COS7 cells with a mammalian expression vector including the entire coding sequence increased the intensity of this 14-kDa band (Fig. 2*A*, *lane 1*). These observations confirmed the proposed reading frame for the cDNA. Expression of TIP-1 was detected in various cell lines as COS7 (Fig. 2*A*, *lane 2*), HeLa (Fig. 2*A*, *lane 3*), CV1 (Fig. 2*A*, *lane 4*), and BHK21 (Fig. 2*A*, *lane 5*). However, by comparison with these cell lines, TIP-1 appeared weakly expressed in PBLs as well as in T-cell lines expressing Tax.[2]

To determine the subcellular localization of the protein, fractionation experiments were performed. HeLa and NIH 3T3 cells were fractionated giving membrane, cytoplasmic, and nu-

[2] C. Reynaud, unpublished data.

clear extracts (see "Experimental Procedures"). Immunoblot analysis of these extracts unambiguously showed that the endogenous protein is in the cytoplasmic soluble fraction (Fig. 1*B*), its presence being only detected in this fraction.

TIP-1 Interacts with Rhotekin—It is now well established that PDZ domains bind to the C-terminal end of their partner proteins. In order to identify such partner proteins, a two-hybrid screen was performed with a bait corresponding to the entire TIP-1 coding sequence fused to that encoding the GAL4 DNA binding domain. A total of 7.4 million transformants were analyzed. Analysis of 265 positive clones showed that many corresponded to the GAL4 DNA binding domain associated out of frame with a cDNA, the resulting fusion protein ending by a motif matching the consensus *X*-(S/T)-*X*-V-COOH known for proteins able to bind PDZ domains.[2] Besides these artifactual constructs, two different clones corresponded to a cDNA encoding the human homologue of rhotekin, which was originally identified as a protein preferentially associating with a mutated form of RhoC that mimicked the GTP-bound form of this small GTPase (25). The authors showed that rhotekin can associate with the activated form of RhoA and RhoC, but not with that of Rac or Cdc42, and they described the existence of three different splice variants of rhotekin (25). The two cDNAs obtained in the two-hybrid screen performed with TIP-1 correspond to a different form, one that lacks part of the sequence at the N-terminal end. Alignment of the human and mouse forms shows a very good conservation between these two species, in particular in the Rho binding domain (Fig. 3). This latter domain is likely to fold into an antiparallel coiled-coil finger, which binds to Rho, as was recently shown for the homologous Rho binding domain of PKN (26). Interestingly, a 6-amino acid sequence at the C-terminal end, WLQSPV-COOH, is perfectly conserved and fits with the *X*-(S/T)-*X*-V-COOH consensus. This strongly suggested that rhotekin binds to the PDZ domain of TIP-1 by its C-terminal end.

To further validate the interaction between TIP-1 and h-rhotekin, immunoprecipitation experiments were performed. COS7 cells were transfected with vectors expressing TIP-1 and h-rhotekin fused at its N-terminal end to the Flag epitope. Proteins were immunoprecipitated with an anti-Flag antibody and analyzed with the antibody to TIP-1. This experiment showed that h-rhotekin associates with TIP-1 in these cells (Fig. 4*A*, *lane 3*). To establish that this interaction indeed relies on the C terminus of rhotekin, a double point mutation was introduced to change the natural sequence QSPV-COOH to QAPA-COOH. This C-terminal mutant of Rhotekin was unable to precipitate TIP-1 (Fig. 4*A*, *lane 4*), which was, however, clearly present in the extract (Fig. 4*A*, *lane 2*). Analysis of the blot with the anti-Flag antibody showed that equal amounts of wild type and mutated h-rhotekin were precipitated in this experiment.[2] It was also verified that h-rhotekin indeed binds to the active form of Rho. COS7 cells were transfected with vectors expressing the Flag-tagged h-rhotekin and the active RhoA.V14 mutant fused to the Myc epitope. Immunoblot analysis with the antibody to Flag of proteins immunoprecipitated with the antibody to the Myc epitope clearly revealed presence of h-rhotekin (Fig. 4*B*). Collectively, these results show that h-rhotekin binds to the activated form of RhoA and to TIP-1. The former interaction involves the Rho binding domain at the N-terminal end of h-rhotekin (25), and the latter the C-terminal extremity of this protein as demonstrated here. Given the small size of Rho and TIP-1 proteins and that the domains involved are completely different, these interactions are likely not to be mutually exclusive.

Activation of the SRE Enhancer Activity by h-Rhotekin and TIP-1—Rho GTPases are involved in many important cellular

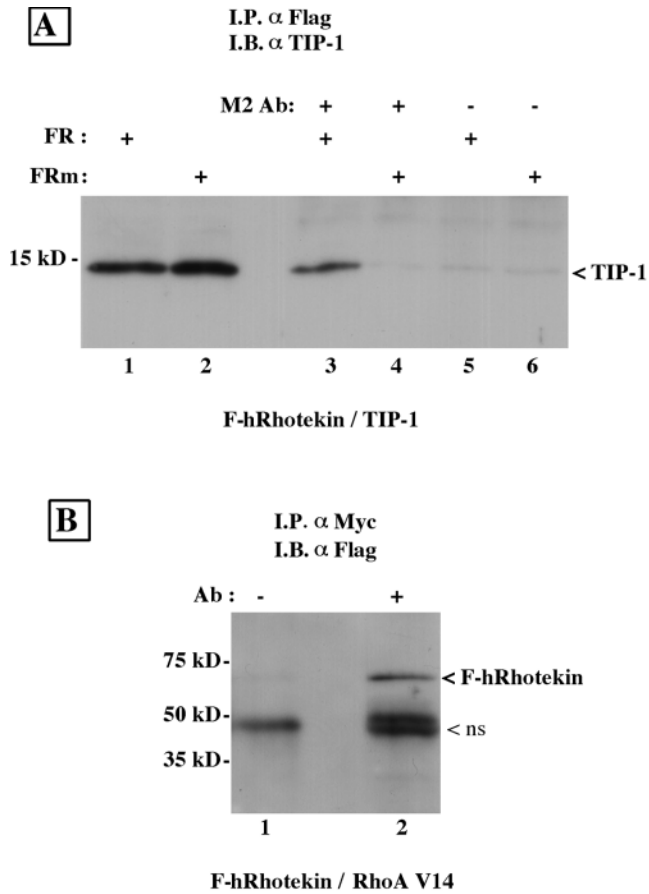

FIG. 4. **Interaction of F-h-rhotekin with TIP-1 and RhoA.V14.** *A*, COS7 cells were transfected with plasmid pTL-TIP-1 and either pSGF-h-rhotekin (*FR*) or pSGF-h-rhotekin Cm (*FRm*), which expresses a mutant form of rhotekin of which C terminus is changed into QAPA-COOH. After immunoprecipitation with the M2 antibody to Flag (*lanes 3* and *4*), immunoblotting was performed with the anti-TIP-1 antibody. In *lanes 5* and *6*, the M2 antibody was omitted, as a control for nonspecific binding to protein A beads. In *lanes 1* and *2*, 10 μl of extracts of transfected cells were loaded on the gel. Positions of the 15-kDa band of the marker as well as of TIP-1 are indicated. *B*, COS7 cells were transfected with pSGF-h-rhotekin and EXV-RhoA.V14. After immunoprecipitation with the anti-Myc 9E10 monoclonal antibody (*lane 2*), immunoblotting was performed with the M2 antibody to Flag. In *lane 1* the 9E10 antibody was omitted. Positions of the bands of a molecular size marker run in parallel along with that of F-h-rhotekin are indicated. *ns* marks nonspecific bands corresponding to protein A and heavy immunoglobulin chains.

functions such as cytoskeleton reorganization, cell growth control, development, and transcriptional regulation (27). Various effectors binding to the GTP-bound active form of Rho have been identified (25, 28). In agreement with previous observations (29), a detailed analysis relying on mutants in the RhoA effector loop has clearly established the involvement of the ROCK kinase in stress fiber induction as well as cell transformation by Rho (30). By contrast, the effectors linking active Rho to activation of the SRE/CArG box are still uncharacterized. Therefore, the effect of h-rhotekin and TIP-1 in this pathway was investigated. HeLa cells were transfected with a CAT reporter construct including the wild type c-Fos SRE upstream of the TK promoter (19). After transfection, cells were cultured in low amounts (0.5%) of fetal calf serum. Under these conditions, treatment of the cells by serum led to a clear activation of transcription (Fig. 5, *lane 1*). Expression of RhoA.V14 in cells kept in low serum also stimulated transcription (Fig. 5, *lane 2*). Transfection of the cells with the vector expressing h-rhotekin did not cause a significant activation, nor did it modify the

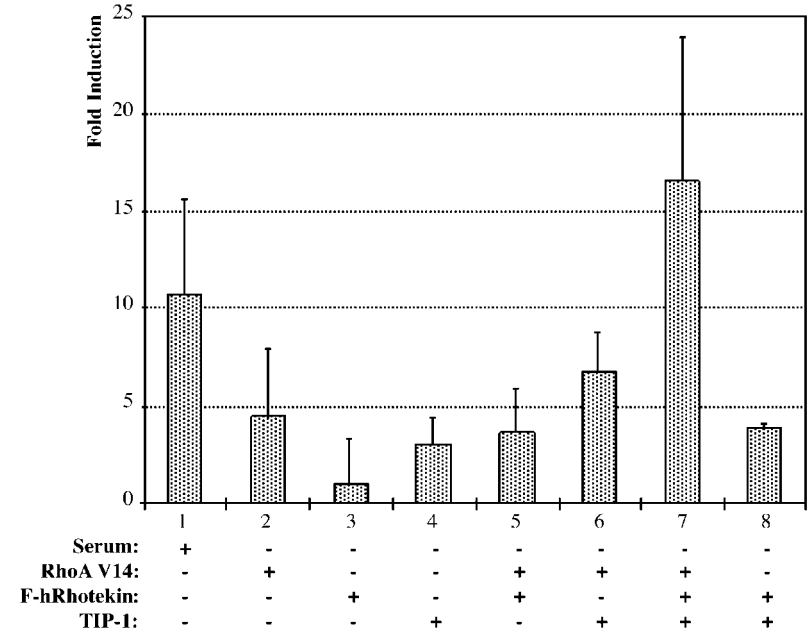

FIG. 5. **RhoA.V14, F-h-rhotekin, and TIP-1 proteins activate the SRE.** HeLa Cells were cotransfected with the SRE reporter plasmid and expression vectors EXV-RhoA.V14, pSGF-h-rhotekin, and pTL-TIP-1, as indicated. The total amount of expression vector including the SV40 early promoter was adjusted to a constant amount using the pSG5 parental vector. CAT enzyme concentrations were measured by enzyme-linked immunosorbent assay (Roche) according to manufacturer's instructions. -Fold induction was determined with respect to activity of the reporter construct alone. Each point of transfection was performed in duplicate. The mean of two independent experiments is represented. *Error bars* indicate standard deviation.

effect exerted by RhoA.V14 (Fig. 5, *lanes 2* and *5*). Expression of TIP-1 led to an activation similar to that exerted by RhoA.V14 and did not significantly modify the effect of this latter protein (Fig. 5, *lanes 4* and *6*). The stimulatory effect of TIP-1 was also observed in COS7 cells.[2] Interestingly, coexpression of all three factors, RhoA.V14, h-rhotekin, and TIP-1 led to a strong activation, above that exerted by serum treatment (Fig. 5, *lane 7*). This effect was dependent on RhoA.V14 since h-rhotekin and TIP-1 in combination gave an effect similar to that exerted by TIP-1 alone (Fig. 5, *lane 8*). Expression of h-rhotekin, TIP-1, or both proteins together did not modify the amount of RhoA.V14 produced in the transfected cells,[2] indicating that activation did not result merely from an increase of the RhoA activator.

This effect was also tested with the reporter construct SRE-APm, which includes a SRE mutated in the flanking AP-1 site (19). Similarly to what was observed previously, TIP-1 was as active as RhoA.V14 (Fig. 6, *lanes 2* and *4*) and combination of RhoA.V14, h-rhotekin, and TIP-1 led to a marked increase (Fig. 6, *lane 5*). Under the same conditions, the parental plasmid pBL-CAT4 was unresponsive to expression of these proteins.[2] These data show that the combination RhoA.V14, h-rhotekin, and TIP-1 markedly enhances the activity of the SRE and that this effect does not involve the flanking AP-1 site.

It was checked whether this effect is indeed dependent on the active form of Rho. This was done by testing the effect of the overexpression of h-rhotekin and TIP-1 with RhoA.N19 that is a dominant negative mutant. These experiments were performed with the SRE-APm reporter construct. Whereas the strong activation with RhoA.V14 was clearly seen, a much lower effect was observed with RhoA.N19 (Fig. 7, compare *lanes 3* and *5*). This clearly shows that the effect observed with the combination of all three proteins depends on the active state of Rho.

It was further asked whether the observed activation depends on the binding of rhotekin to TIP-1. To analyze this point, the effect exerted by wild type rhotekin was compared with that of the C-terminal mutant, which is unable to bind TIP-1. These experiments showed that this mutation clearly reduces the activation (Fig. 8, compare *lanes 5* and *6*). These results show that the strong effect produced by the combination of RhoA, h-rhotekin, and TIP-1 depends on the active form of RhoA as well as on the binding of h-rhotekin to TIP-1. Taken

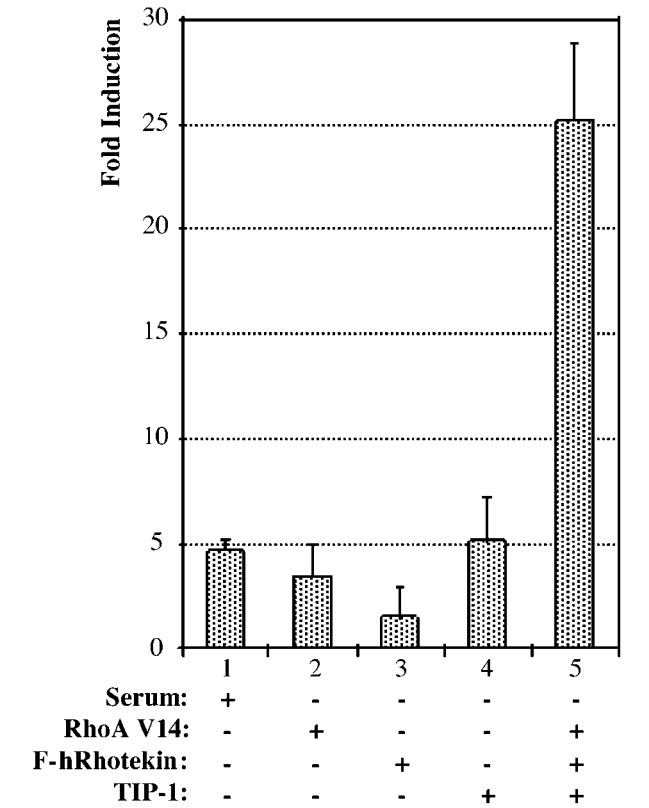

FIG. 6. **RhoA.V14, F-h-rhotekin, and TIP-1 proteins activate SRE mutated in the *fos* AP-1-like sequence site.** Transfection in HeLa cells was performed as described in legend to Fig. 5 with the reporter construct SREm, which is mutated in *fos* AP-1-like sequence site (19) and plasmids expressing RhoA.V14, F-h-rhotekin, and TIP-1 as indicated. -Fold induction was calculated and is represented with *error bars* corresponding to standard deviation.

together these data support the notion that activation of the enhancer activity of the SRE results from formation of a complex between active RhoA, h-rhotekin, and TIP-1.

DISCUSSION

The Rho small GTPases have multiple functions and affect key regulatory cellular processes (27, 28). In agreement with

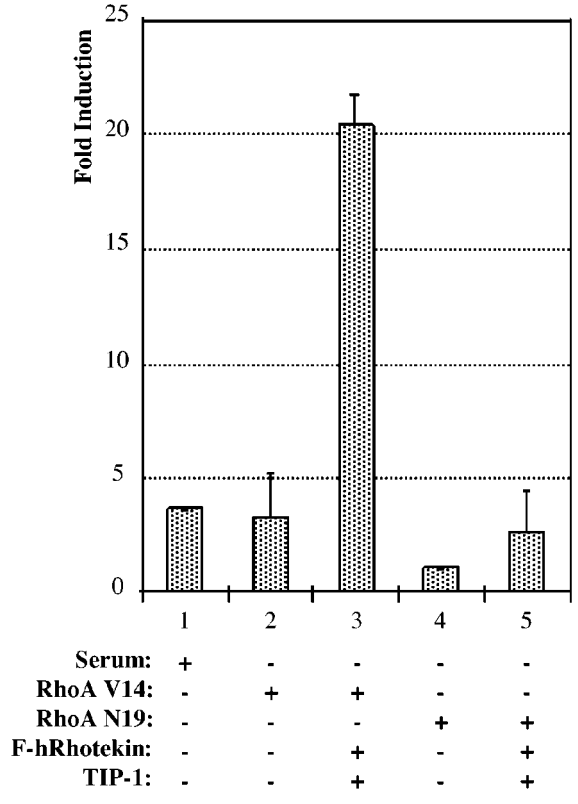

FIG. 7. **Activation of the SRE by F-h-rhotekin and TIP-1 requires the active form of Rho.** Transfection in HeLa cells was performed with the SRE-APm reporter construct and vectors expressing F-h-rhotekin, TIP-1, and either RhoA.V14 or the dominant negative mutant RhoA.N19 as indicated. Data are represented as described in legend to Fig. 5.

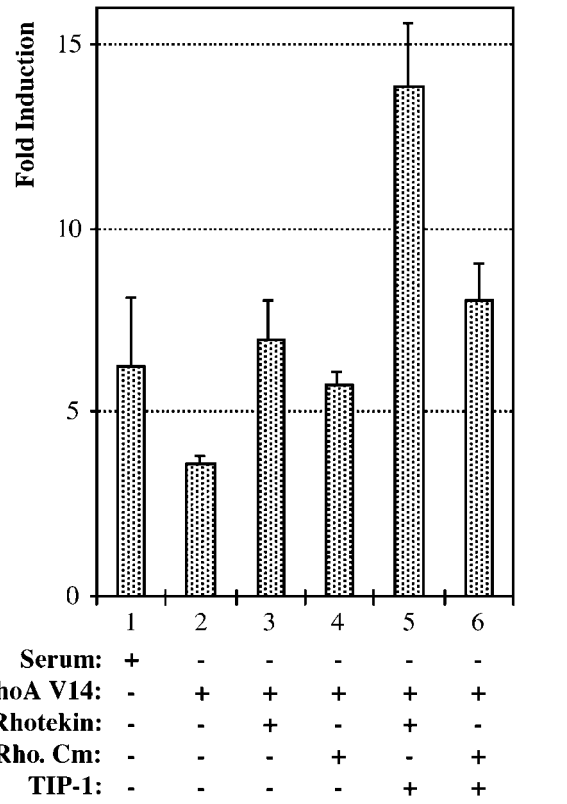

FIG. 8. **Activation of the SRE by F-h-rhotekin and TIP-1 depends on the serine and valine residues at the C terminus of h-rhotekin.** Transfection in HeLa cells was performed with the SRE-APm reporter construct and vectors expressing RhoA.V14, TIP-1, and F-h-rhotekin either wild type or bearing a double point mutation changing the QSPV-COOH wild type C-terminal end into QAPA-COOH as indicated. Data are represented as described in legend to Fig. 5.

these pleiotropic effects, numerous effectors able to bind these GTPases in their active state have been identified. Some of these effectors exhibit kinase activity, whereas the others have no known enzymatic activity. Previous studies have implicated the kinase ROCK in induction of stress fibers and also cell transformation (29, 30). Our results show that h-rhotekin and TIP-1 markedly increase transactivation of the c-Fos SRE by RhoA.V14. The PDZ protein TIP-1 can by itself produce a significant activation of the same intensity as that exerted by RhoA.V14. h-rhotekin alone showed no or weak effects. This indicates that TIP-1 is probably downstream of h-rhotekin in the pathway leading to SRE activation. However, it is clear that TIP-1 alone cannot produce the strong activation seen with all three components. This indicates that the complex of all three proteins is necessary to trigger maximal activation. The results of fractionation experiments show that TIP-1 is cytoplasmic. This observation is in agreement with immunofluorescence analyses, which showed that overexpressed RhoA.V14, h-rhotekin, and TIP-1 are co-localized in the cytoplasm.[3] The nature of the signal emitted by the association of RhoA.V14, h-rhotekin, and TIP1 that causes activation of the SRE within the nucleus remains to be understood.

Interestingly, Sotiropoulos *et al.* (31) have shown recently that LIM kinase-1 (LIMK-1) is able to activate SRF by acting on the actin dynamics. This kinase would exert its effect by increasing the amount of monomeric actin *versus* polymerized actin. How the increased monomeric actin modifies the activity of SRF is presently unknown. Interestingly, LIMK-1 includes a PDZ domain. One can wonder whether a specific effector might bridge activated Rho and LIMK-1 by interacting both with GTP-bound Rho and LIMK-1 via the PDZ domain. Rhotekin might exert such a function. When the C-terminal ends of the various Rho effectors are compared, it appears that rhotekin is not the only protein exhibiting a PDZ domain binding site (PDZ-BS). The C-terminal end of Citron also fits with the PDZ-BS consensus (Table I). In agreement with this observation, an interaction of Citron with PSD-95 in GABAergic neurons from the hippocampus has been reported (32). Rhophilin exhibits a threonine at position −2, but the C-terminal residue is a proline. In the study by Songyang *et al.* (11), a proline was never observed as C-terminal residue, raising doubts about the notion that this Rho effector C-terminal end can represent a functional PDZ-BS. The other kinase Rho effectors do not include a PDZ-BS. Additional studies will be required to determine whether or not rhotekin and Citron can bridge activated Rho with LIMK-1.

TABLE I

Comparison of the sequences of the C-terminal ends of Rho effectors

The residues matching the *X*-(S/T)-*X*-V-COOH consensus for binding to PDZ domains are in bold. The GenBank accession numbers are as follows: mouse rhotekin, U54638, mouse citron, U39904, mouse rhophilin, U43194, human PKN, D26181, mouse Rock-I, U58512, mouse Rock-II, U58513, mouse p140mDia, U96963.

Rhotekin Mm	...FYSKSQLGPWLQ**SPV**-c
Citron Mm	...TPLSQVNKVWDQ**SSV**-c
Rhophilin Mm	...SHPDPCTNRNCV**T**CP-c
PKN Hs	...EQAAFLDFDFVAGGC-c
Rock-1 Mm	...QSFRKVVKNTSGKTS-c
Rock-2 Mm	...SIRRPSRQLAPNKPS-c
P140mDia Mm	...PTILEEAKELVGRAS-c

A number of PDZ proteins are known to interact with the

[3] S. Fabre, unpublished data.

cytoskeleton (33–35). Despite its small size, it is possible that TIP-1 also has this property. Such a possibility could be related to an effect of TIP-1 on actin polymerization. It could also allow the anchoring of Rho-containing protein complexes at specific sites. A way to explain the multiplicity of the effects of Rho, as well as the high number of its effectors, could be that it is present at different specialized subcellular sites. A PDZ protein as TIP-1 could allow anchoring of the effector and, as a consequence, of active Rho at a particular position, leading to a given effect. A scaffold role for proteins with multiple PDZ domains is now well established (36–38). Precise studies of the subcellular localization of the Rho effectors and of the corresponding PDZ proteins, when appropriate, should allow the testing of such an hypothesis.

Our results establish that PDZ proteins could be important molecules in the Rho pathways, in particular activation of the SRE, and that an effector like rhotekin can act as a bridge between Rho and such PDZ proteins. Interestingly, another class of molecules participating in the regulation of the Rho activity exhibit PDZ domains: the guanine nucleotide exchange factors (GEF). Such a domain is indeed present in KIAA0380, a Rho-specific GEF (39). Conversely, another GEF, NET1 (40), includes a functional PDZ-BS.[4] These observations raise the possibility that PDZ domains play an important role in the organization of the Rho effects, both upstream and downstream of these important cellular regulators.

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