MOLECULAR AND CELLULAR BIOLOGY, Nov. 2002, p. 7953–7966 Vol. 22, No. 22
0270-7306/02/$04.00+0 DOI: 10.1128/MCB.22.22.7953–7966.2002

The Cysteine-Rich Sprouty Translocation Domain Targets Mitogen-Activated Protein Kinase Inhibitory Proteins to Phosphatidylinositol 4,5-Bisphosphate in Plasma Membranes

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Received 11 March 2002/Returned for modification 22 April 2002/Accepted 12 August 2002

Sprouty (Spry) proteins have been revealed as inhibitors of the Ras/mitogen-activated protein kinase (MAPK) cascade, a pathway crucial for developmental processes initiated by activation of various receptor tyrosine kinases. In COS-1 and Swiss 3T3 cells, all Spry isoforms translocate to the plasma membrane, notably ruffles, following activation. Here we show that microinjection of active Rac induced the translocation of Spry isoforms, indicating that the target of the Spry translocation domain (SpryTD) is downstream of active Rac. Targeted disruption of actin polymerization revealed that the SpryTD target appeared upstream of cytoskeletal rearrangements. Accumulated evidence indicated that phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] is the likely SpryTD target. Human Spry2TD (hSpry2TD) binds to PtdIns(4,5)P_2 in vesicle-binding assays. hSpry2TD colocalizes with the pleckstrin homology domain of phospholipase Cδ, which binds PtdIns(4,5)P_2. The plasma membrane localization of hSpry2TD was abolished in ionomycin-treated MDCK cells or when PtdIns(4,5)P_2 was specifically dephosphorylated by overexpression of an engineered, green fluorescent protein-tagged inositol 5-phosphatase. Similarly, Spred, a novel Ras/MAPK inhibitor recently found to contain the conserved cysteine-rich SpryTD, also translocated to peripheral membranes and bound to PtdIns(4,5)P_2. Alignment of the Spry and Spred proteins led us to identify a translocation-defective point mutant, hSpry2 D252. Targeting of hSpry2 to PtdIns(4,5)P_2 was shown to be essential for the down-regulation of Ras/MAPK signaling.

Receptor tyrosine kinase (RTK)-induced Ras/mitogen-activated protein kinase (MAPK) activation has been reiterated in various developmental processes. Sprouty (Spry) proteins play a role as inhibitors of the Ras/MAPK cascade, which is conserved in *Drosophila melanogaster* (5), zebra fish (4), chickens (13), and mice (12). All Spry proteins share a conserved, C-terminal cysteine-rich region that has been defined as a novel translocation domain (Sprouty Translocation Domain [SpryTD]) in a previous study based on transient overexpression of various Spry constructs (11). Translocation of endogenous Spry1 from the cytosol to the membrane has also been observed in vascular endothelial growth factor-activated endothelial cells, indicating that the translocation is of physiological relevance (7).

Spry isoforms specifically translocate to membrane ruffles upon RTK stimulation (11). Ruffles are cell peripheral-membrane protrusions enriched with a meshwork of filamentous actin (24). Rac1 is a key regulator in reorganizing actin cytoskeletal structures for membrane ruffle formation, while Cdc42 and RhoA activation results in the formation of microspikes and RhoA stress fibers, respectively (16).

There has been a paucity of details pertaining to the biochemistry of ruffle formation. Recently, the synergistic activation of phosphatidylinositol 4-phosphate 5-kinase [PI(4)P5K] by phosphatidic acid (PA) and Arf6 was reported to be important for membrane ruffling (6). The authors proposed a pathway whereby Rac1 activation leads to actin reorganization, in which the up-regulation of PI(4)P5K and resultant production of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] are necessary intermediate stages. In other studies PI(4)P5K was demonstrated to be the target of Rac1 in both pollen tube formation (10) and actin polymerization in platelets (27). The hydrolysis of PtdIns(4,5)P_2 by phospholipase Cγ (PLCγ), the removal of phosphate by inositol 5-phosphatase (5P), phosphorylation at the 3 position by phosphatidylinositol 3-kinase (PI3K), and the reversible sequestration of the lipid by various membrane-located proteins keep the level of free PtdIns(4,5)P_2 in the cells tightly regulated (26).

Several protein domains have been shown to target inositol phospholipids. FYVE (Fab1p, YOTB, Vac1p, and EEA1) and PX (Phox homology) domains play important roles in membrane trafficking of endosomes and lysosomes and generally bind to PtdIns lipids with a phosphate in the 3 position of the inositol ring (31). Pleckstrin homology (PH) domains, which are found mostly in signaling molecules, bind variably to inositol lipids with a wide range of affinity and specificity (1, 9). On the other hand, FERM (protein 4.1, ezrin, radixin, and moesin) and ENTH (epsin N-terminal homology) domains, which are involved in cytoskeletal organization and/or endocytosis, are believed to specifically bind PtdIns(4,5)P_2 (8).

Recently, a novel class of Ras/MAPK inhibitor proteins

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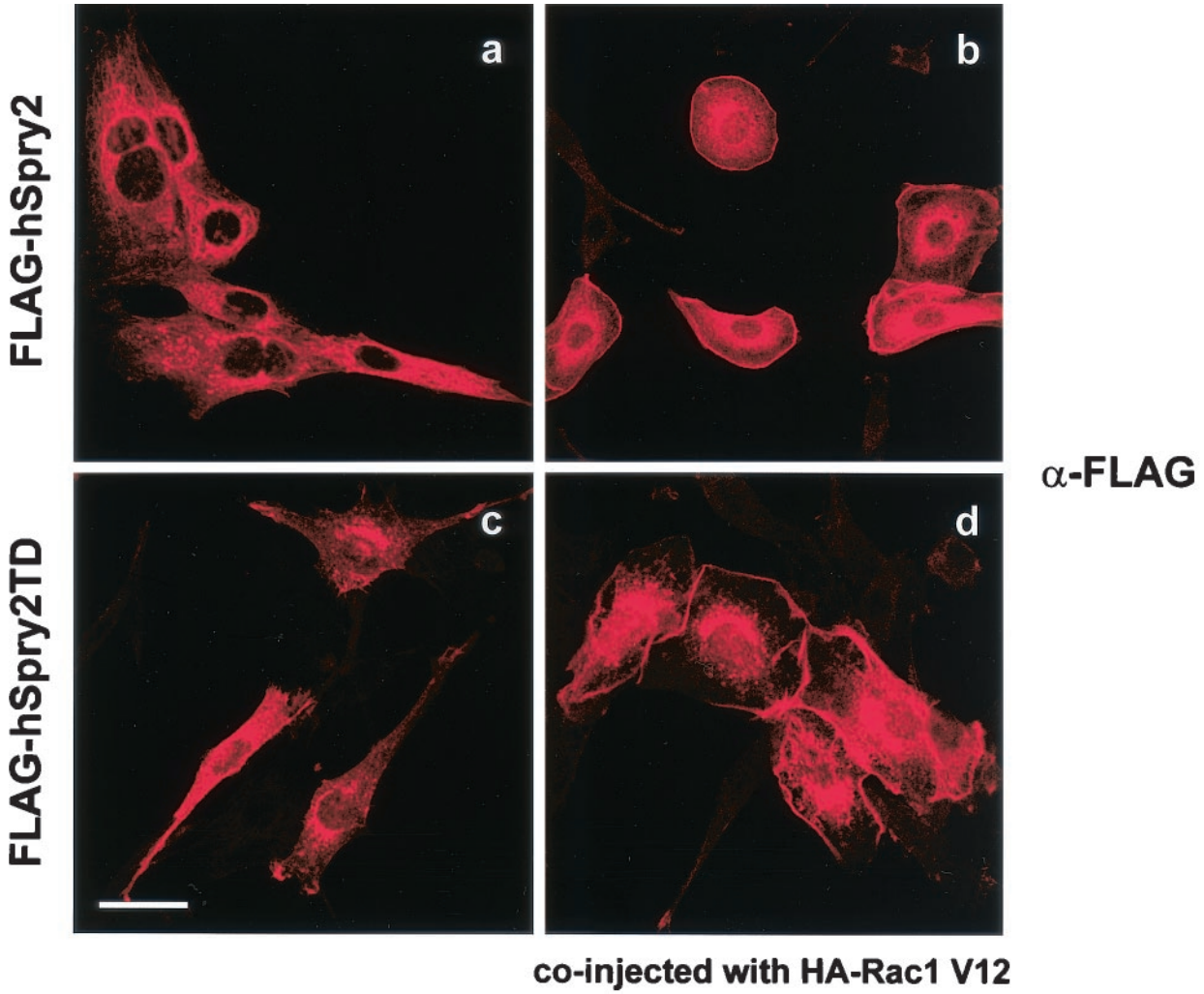


FIG. 1. hSpry2 translocation is stimulated by constitutively active Rac1. Swiss 3T3 cells were microinjected with either FLAG-hSpry2 (a and b) or FLAG-hSpry2TD (amino acids 178 to 282) (c and d) either alone (a and c) or in combination with HA-Rac1 V12 (b and d). The proteins expressed were visualized by confocal microscopy using monoclonal anti-FLAG to detect hSpry2 and hSpry2TD. The cell images portrayed are typical of more than 85% of the injected cells. Bar, 20 μm.

named Spred (Sprouty-related EVH1 domain-containing protein) was identified (29). Both Spred-1 and Spred-2 contain a cysteine-rich domain related to the SpryTD. This domain most likely serves as a targeting domain in these proteins, as it appears to do with Spry isoforms. This finding indicates that the sequence has been conserved to perform a specific function in signaling modification, most likely the precise targeting of a select group of Ras/extracellular signal-related kinase (ERK)-inhibiting proteins.

Our aim was to identify the cellular target of the SpryTD. Previous evidence had indicated that the target "appeared" during membrane ruffle formation. We reasoned that the cellular target could be (i) a modified protein, as seen in the recruitment of Src homology 2 (SH2) domains or phosphotyrosine binding (PTB) domains to the phosphotyrosine sites of RTKs or (b) a newly synthesized phospholipid, e.g., PtdIns(4,5)P_2, which is responsible for targeting of PH and FERM domain-containing proteins (14, 21), or that (c) SpryTD could bind to a domain on an as yet unspecified protein that is involved in translocation to the membrane.

In this study we show that the plasma membrane localization of SpryTD is due to direct PtdIns(4,5)P_2 binding and that this interaction is essential for the full function of human Spry2 (hSpry2) as an inhibitor of Ras/MAPK signaling.

MATERIALS AND METHODS

DNA constructs. Rac and Cdc42 constructs were subcloned from DNA provided by Ed Manser (Glaxo-IMCB, Singapore) and Alan Hall (University College, London, United Kingdom). Myc-tagged profilin1 constructs were generous gifts from Shiro Suetsugu (University of Tokyo, Tokyo, Japan). The glutathione *S*-transferase (GST)-PH domain of Akt was constructed by PCR amplification from full-length Akt cDNA provided by Philip Cohen (University of Dundee, Dundee, United Kingdom) into the pGEX4T1 vector. The green fluorescent protein (GFP)-PH and GST-D40-PH domains of PLCδ and GFP-tagged 5P were obtained from Hitoshi Yagisawa (Himeji Institute of Technology, Himeji, Japan) and Tobias Meyer (Stanford University, Palo Alto, Calif.), respectively. Various constructs of Spry1, Spry2, and Spry4 were generated as described previously (11) by using pGEX4T1 as a cloning vector for GST fusion protein production.

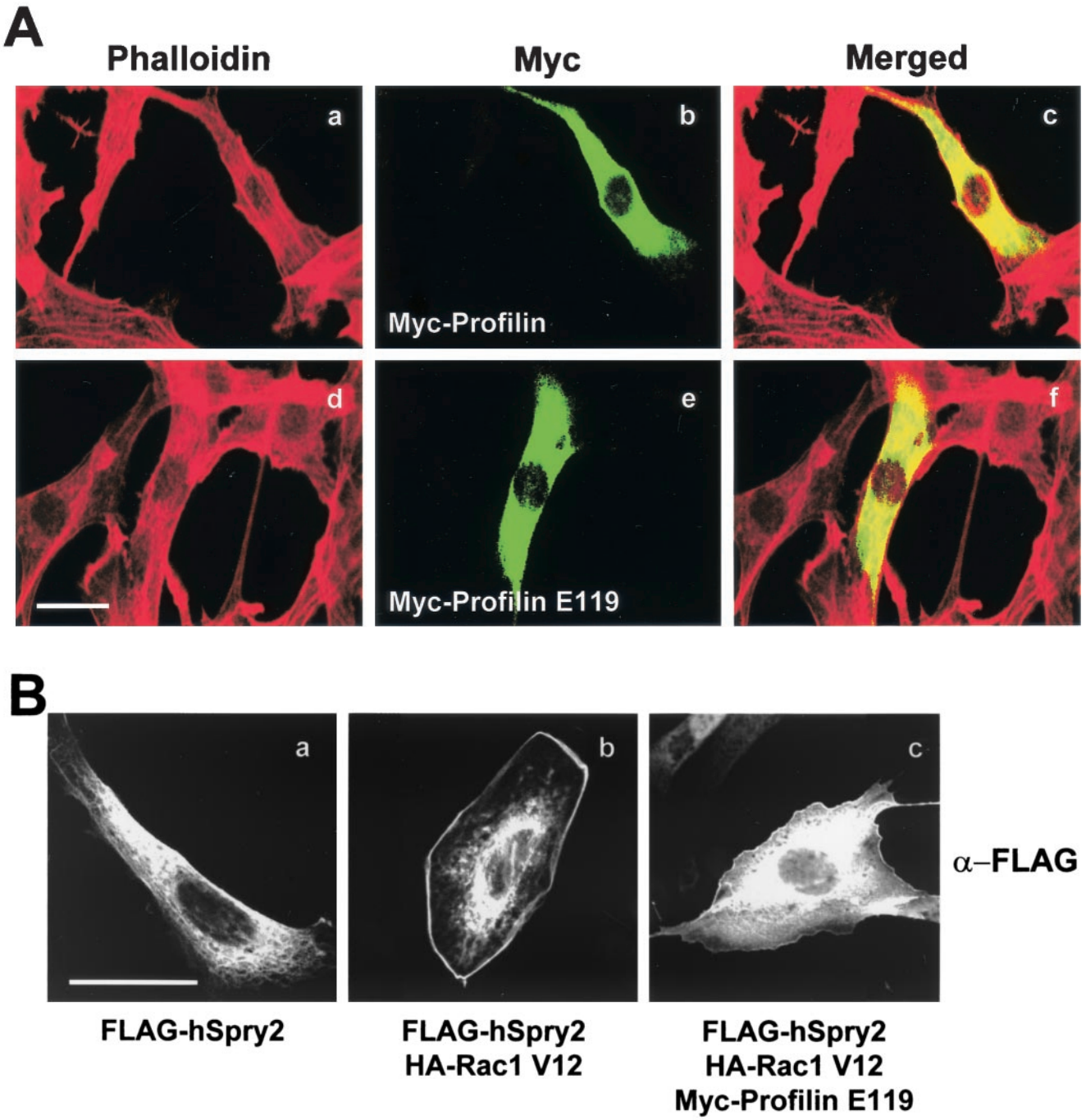


FIG. 2. hSpry2 translocation occurs despite inhibition of actin polymerization. (A) Swiss 3T3 cells were microinjected with Myc-tagged profilin1 (a, b, and c) or the dominant-negative profilin1 E119 construct (d, e, and f). Cells were probed with either rhodamine phalloidin (a and d) or a monoclonal anti-Myc antibody, followed by a fluorescein isothiocyanate-labeled anti-mouse antibody (b and e), and the images were merged (c and f). (B) Swiss 3T3 cells were microinjected with combinations of FLAG-hSpry2, HA-Rac1V12, and Myc-profilin1 E119 constructs, as indicated. The distribution of hSpry2 was revealed in singly microinjected cells (a), cells coinjected with active Rac V12 (b), and cells coinjected with active Rac V12 and dominant-negative profilin1 E119 (c) by using an anti-FLAG antibody. Bar, 20 μm.

Point mutations were engineered with a site-directed mutagenesis kit (Stratagene, La Jolla, Calif.) according to the manufacturer's instructions.

Cell culture and transfection. PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (4.5 g of glucose/ml) supplemented with 10% fetal bovine serum, 5% horse serum, 2 mM L-glutamine, 10 mM HEPES (pH 7.4), 100 U of penicillin/ml, and 100 U of streptomycin/ml. COS-1 monkey kidney cells and Madin-Darby canine kidney (MDCK) cells were grown and maintained in DMEM with 1 g of glucose/ml; Swiss 3T3 fibroblast cells were grown and maintained in DMEM with 4.5 g of glucose/ml; and 293T human kidney epithelial cells were grown and maintained in RPMI medium. Culture medium was supplemented with 10% fetal bovine serum (HyClone Laboratories), 2 mM L-glutamine, 10 mM HEPES (pH 7.4), 100 U of penicillin/ml, and 100 U of streptomycin/ml. Cells were seeded in six-well plates containing glass coverslips for immunofluorescence studies or in 60-mm-diameter culture dishes for Western blot analyses. Cells at 80% confluency were transfected with various expression plasmids using LipofectAMINE 2000 reagent (Life Technologies Inc.,

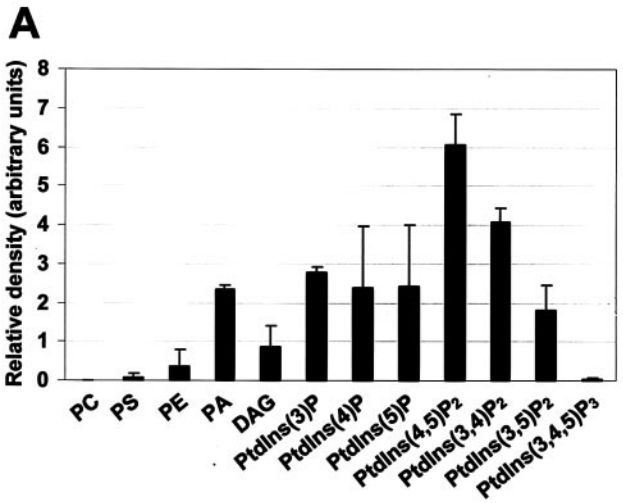
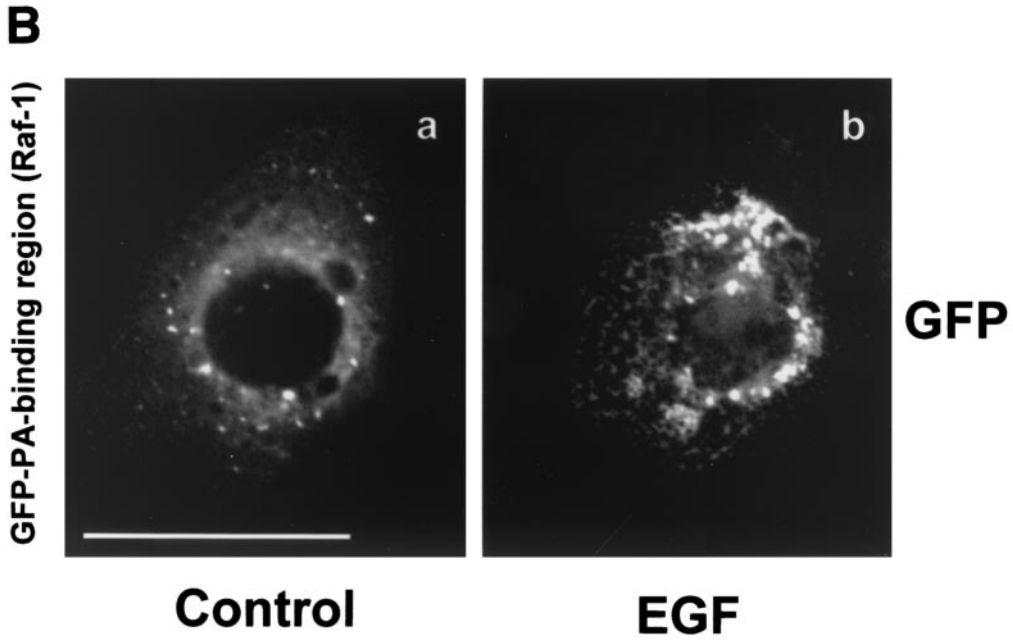


FIG. 3. hSpry2 and hSpry2TD bind to PtdIns(4,5)P_2 in in vitro assays. (A) The ability of hSpry2 to bind to phospholipid vesicles was tested. Lipid vesicles loaded with sucrose, containing 95% PC and 5% of the indicated phospholipids, were mixed with lysates of COS-1 cells expressing FLAG-hSpry2, followed by ultracentrifugation. Vesicle pellets were analyzed by SDS-PAGE and Western blotting. The intensity of the FLAG signal was determined by densitometry. Data from two independent experiments ± standard deviations are presented. PE, phosphatidylethanolamine. (B) To determine the cellular location of PA, COS-1 cells were transiently transfected with the GFP-PA binding region of Raf-1, serum deprived overnight, and then either left untreated or induced with EGF (50 ng/ml). The GFP signal was visualized by confocal microscopy. Bar, 20 μm. (C) A lipid vesicle-binding assay was performed with lysates from COS-1 cells expressing GFP-PH (PLCδ), the PtdIns(4,5)P_2 binding-defective mutant GFP-PH D40, FLAG-hSpry2, FLAG-hSpry2TD, or FLAG-annexin II. The distributions of various tagged proteins in the pellet (P) and the supernatant (S) were assessed by SDS-PAGE and Western blot analysis. Membranes were probed with either polyclonal anti-GFP or monoclonal anti-FLAG. (a and b) Comparison of the relative distributions of the binding domains in the pellet and soluble fractions. (c) Comparative dose-response analysis of hSpry2/TD binding to vesicles containing increasing amounts of PtdIns(4,5)P_2 or PS. (d) Binding profiles of the PH (PLCδ) domain. The label "hSpry2/TD" indicates that similar results were obtained with full-length hSpry2 and hSpry2TD.



Rockville, Md.) according to the manufacturer's recommendations. Four to six hours later, the transfection medium was aspirated, and the cells were washed twice with warm phosphate-buffered saline and incubated in 10% serum-containing medium for 24 h. The cells were then washed and maintained in serum-free medium overnight. Prior to being fixed or lysed, cells were either left untreated or stimulated with 50 ng of epidermal growth factor (EGF; Sigma Aldrich, St. Louis, Mo.)/ml for 10 min.

Lipid vesicle-binding assay. Phosphatidylcholine (PC; Sigma Aldrich) and the indicated lipids were solubilized in chloroform and dried under a stream of nitrogen. Lipids were then suspended in buffer A (170 mM sucrose buffered with 50 mM Tris to pH 7.3–100 mM sodium chloride) with PtdIns(4,5)P_2 (Sigma) to a final concentration of 5% (vol/vol). Aliquots (0.5 ml) of 10 to 50 mM lipid were subjected to five freeze-thaw cycles and filtered repeatedly through 0.2-μm-pore-size filters. For "dose-dependent" binding studies, vesicles were made to contain increasing concentrations of PtdIns(4,5)P_2 or phosphatidylserine (PS), as indicated. Vesicles were mixed with the sonicated COS-1 lysates in buffer A (with a cocktail of protease inhibitors [Roche Applied Science, Indianapolis, Ind.]), which had been precleared by centrifugation. The mixtures were immediately subjected to ultracentrifugation at 100,000 × *g* for 30 min at 4°C. One-tenth of the supernatant or the entire pellet of each sample was mixed with 2× Laemmli buffer before separation by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis (SDS–12.5% PAGE) followed by Western-blotting with rabbit polyclonal anti-GFP (Santa Cruz, Santa Cruz, Calif.) or monoclonal anti-FLAG M2 (Sigma Aldrich). Western blots were developed by ECL (Amersham Biosciences Inc., Little Chalfont, Buckinghamshire, United Kingdom).

Amino acid sequence alignment. Partial amino acid sequences were extracted from the National Center for Biotechnology Information protein database. The GenBank accession numbers are as follows: hSpry2, NP_005833; hSpry1, XP_036349; hSpry4, XP_096029; hSpry3, O43610; mSpred1, BAB62848; mSpred2, BAB62849. Alignment was performed by using the DNASTAR Clustal method with a structural residue weight table.

Phospho-MAPK assay and immunoprecipitation. The antibody against phospho-ERK was from New England Biolabs (Beverly, Mass.), and monoclonal antibodies against horseradish peroxidase-conjugated phosphotyrosine (PY20), pan-ERK, and ERK2 were from Transduction Laboratories (Lexington, Ky.). Cells were harvested at 40 h posttransfection and lysed in 1 ml of lysis buffer

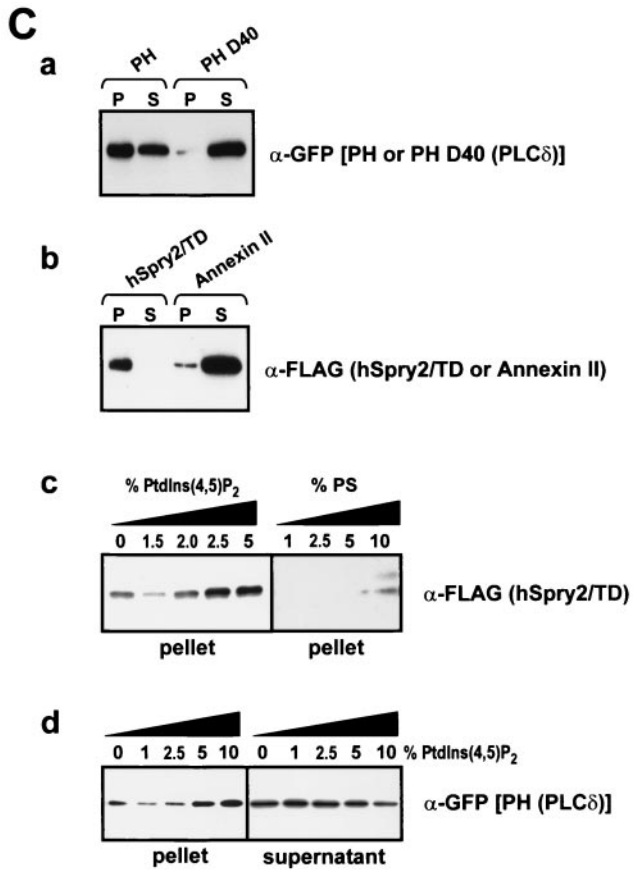


FIG. 3—*Continued.*

(20 mM HEPES [pH 7.4], 137 mM sodium chloride, 1.5 mM magnesium chloride, 1 mM EGTA, 10% [vol/vol] glycerol, 1% Triton X-100, a mixture of protease inhibitors, and 0.2 mM sodium orthovanadate). Protein concentrations of cell lysates were normalized by use of a bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.) before incubation with various antibodies. FLAG-tagged proteins were immunoprecipitated by using mouse monoclonal anti-FLAG M2 agarose-conjugated beads for 2 h at 4°C. Immunoprecipitates were collected by centrifugation and washed three times with lysis buffer. Eluted proteins were resolved on SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. After being blocked in phosphate-buffered saline containing 1% bovine serum albumin and 0.1% Tween 20 for 1 h at room temperature, the membranes were probed with 1 μg of the primary antibody/ml followed by 0.5 μg of the horseradish peroxidase-conjugated secondary antibody/ml. Immunoreactive protein bands were visualized by ECL.

RESULTS

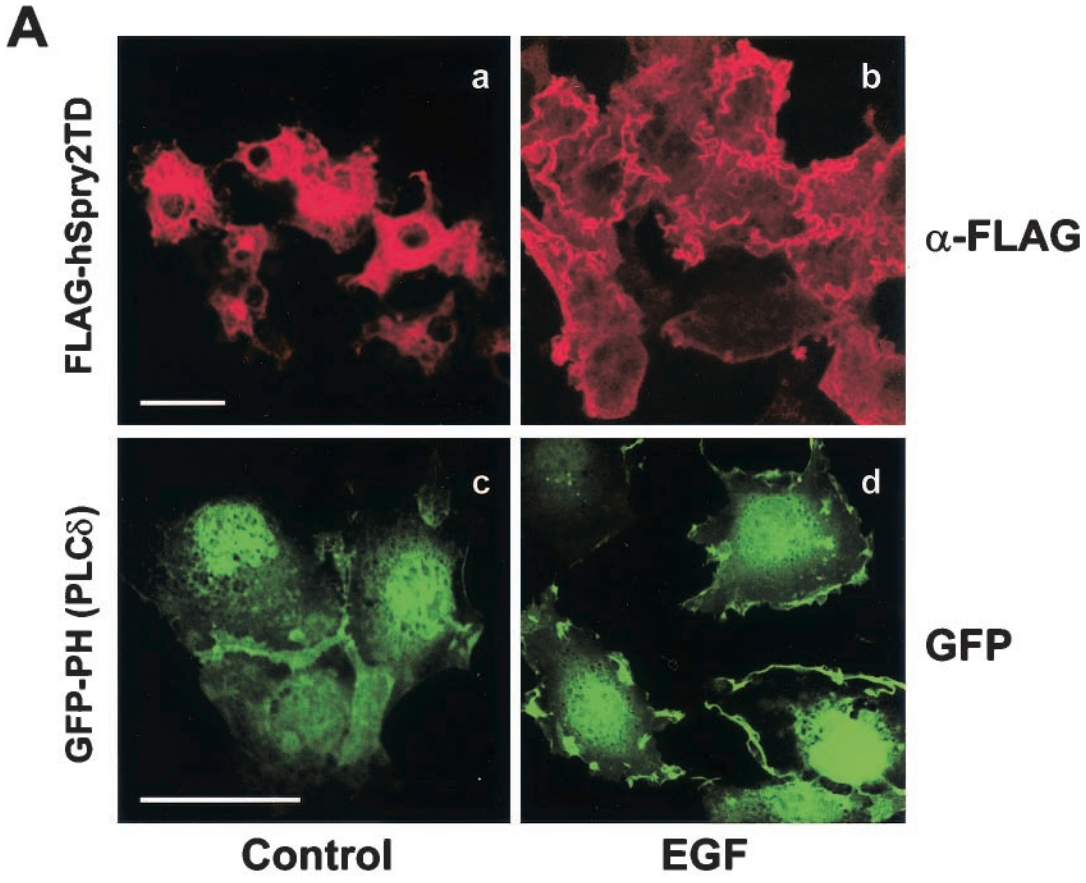
The SpryTD target is downstream of active Rac. hSpry2 is distributed along the microtubules in nonstimulated COS-1 cells and translocates rapidly to the membrane ruffles upon stimulation with EGF (11). The signaling pathways elicited by the EGF receptor include Ras/MAPK, PI3K, signal transducers and activators of transcription (STAT), and the Rho family of small G-protein-induced cytoskeletal reorganization. It was previously found that dominant-negative Rac1 N17 blocked the translocation of hSpry2, indicating that the hSpry2 translocation target "appeared" downstream of activated Rac1 (11). In order to isolate the target of the SpryTD from the various pathways initiated by EGF activation, we asked whether active Rac1 V12 alone could induce the relocation of hSpry2. To this end, we employed microinjection to coexpress active Rac1 with hSpry2 or hSpry2TD in Swiss 3T3 cells. Active Rac1 V12 was able to induce the translocation of hSpry2 or hSpryTD to membrane ruffles (Fig. 1b and d) from the microtubule location of hSpry2 (Fig. 1a) or the cytosolic disposition of hSpry2TD (Fig. 1c) in the respective untreated cells. Similar results were obtained using HeLa cells (data not shown). These results indicate that the target of the SpryTD in activated cells lies downstream of active Rac.

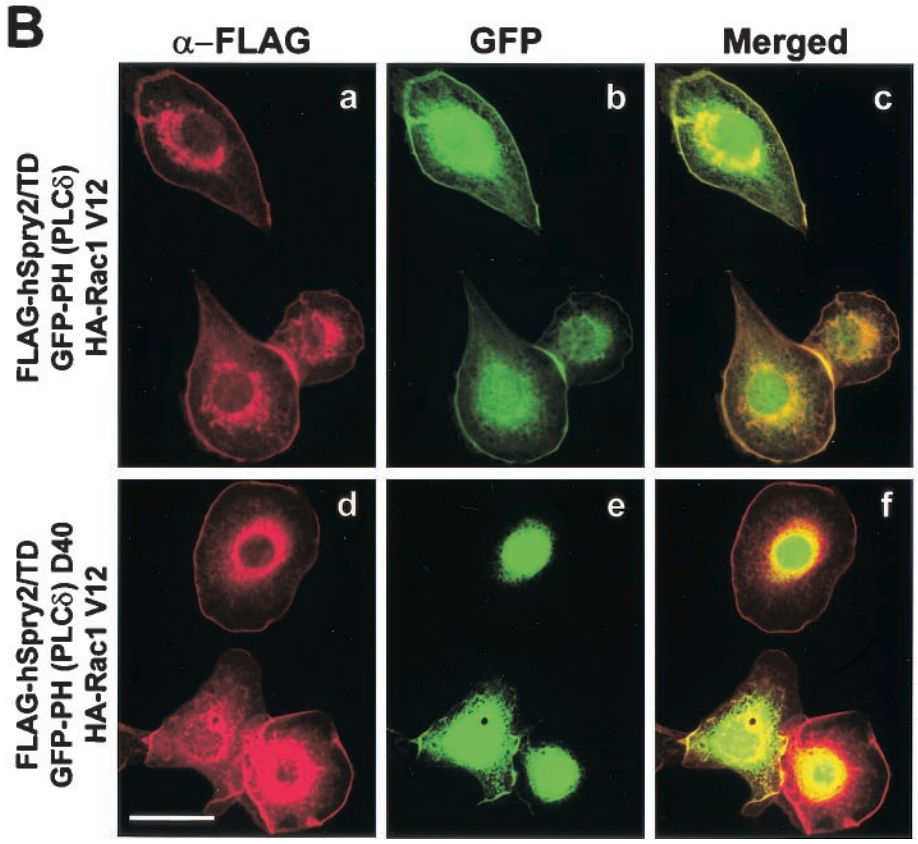
The SpryTD target is upstream of actin polymerization. Two major steps in the proposed pathway(s) from the activation of Rac1 to the formation of ruffles are the synthesis of PtdIns(4,5)P_2 and the polymerization of actin. Profilin1, the ubiquitous actin monomer-binding protein, plays a key role in the latter step (23). Dominant negative Profilin1 E119 has been shown to suppress the formation of Rac-induced membrane ruffles (22). Figure 2Aa through Ac and Ad through Af compare the disposition of actin filaments in cells microinjected with wild-type profilin1 (Fig. 2Aa) versus dominant-negative profilin1 E119 (Fig. 2Ad), respectively. The cellular locations of wild-type profilin1 and profilin1 E119 are shown in Fig. 2Ab and Ae, respectively. The merged images are portrayed in Fig. 2Ac and Af. These data demonstrate that profilin1 E119 abolishes the polymerization of actin, as seen from the lack of actin fibers in the cell microinjected with this dominant-negative mutant compared with the cell injected with wild-type profilin1, which clearly contains actin filaments.

COS-1 cells were next microinjected with combinations of FLAG-hSpry2, hemagglutinin (HA)-tagged Rac1 V12, and Myc-profilin1 E119, as indicated in Fig. 2B. Despite the lack of ruffle formation, hSpry2 was translocated to the cell periphery in the Rac1 V12-coinjected cell (Fig. 2Bb). The signal at the cell periphery appears weaker than that expected in membrane ruffles. This could be due to one of at least two reasons: (i) there is a greater area (general cell periphery versus small, ruffling protrusions) over which the probe is dissipated, and (ii) translocation of hSpry2TD may have some dependence on actin cytoskeletal dynamics. A similar result was observed when actin polymerization was suppressed by use of the chemical inhibitor latrunculin A or cytochalasin A (data not shown).

We reasoned that the target for the SpryTD was therefore upstream of actin polymerization and downstream of Rac1 activation. Assessment of collated data and current models led to the suggestion that PtdIns(4,5)P_2 is a likely target for the SpryTD in activated cells. PtdIns(4,5)P_2 has also been shown to be a key factor in the formation of ruffles by Rac1 and is concentrated in actin-rich protrusions (25).

hSpry2 binds to PtdIns(4,5)P_2. The likely binding of Spry proteins to PtdIns(4,5)P_2 was assessed by using a lipid vesicle-binding assay coupled with ultracentrifugation. In the first experiment the ability of hSpry2 to bind to a range of phospholipids was assessed, and the binding to vesicles containing 5% of the indicated phospholipid is shown in Fig. 3A. There is essentially no binding to PC, PS, diacyl glycerol (DAG), or phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3]. There is differential binding to PtdIns(3)P, PtdIns(4)P, PtdIns(5)P, PtdIns(4,5)P_2, PtdIns(3,4)P_2, PtdIns(3,5)P_2, and PA. The highest level of binding is to PtdIns(4,5)P_2. In the physiological context it has been shown previously (11) that PtdIns(3)P-substituted


A
FLAG-hSpry2TD
GFP-PH (PLCδ)
a
b
c
d
α-FLAG
GFP
Control
EGF


B
α–FLAG
GFP
Merged
FLAG-hSpry2/TD
GFP-PH (PLCδ)
HA-Rac1 V12
FLAG-hSpry2/TD
GFP-PH (PLCδ) D40
HA-Rac1 V12
a
b
c
d
e
f

lipids are unlikely to play a major role in the translocation, because there is no prominent inhibition of membrane targeting when cells are treated with wortmannin (a PI3K inhibitor). Experiments utilizing an in vivo PA-binding probe have previously shown that PA is enriched in vesicles in stimulated cells and has scant presence on the cell membrane (19). We performed a similar experiment with the same probe and verified that PA is essentially localized to endosome-like structures (Fig. 3B). PA was therefore deemed not to be the target of hSpry2TD.

To further verify the authenticity of the vesicle-binding assay, we performed "dose-response" binding experiments that employed GFP-PH (PLCδ) as a positive control and FLAG-tagged annexin II and the nonbinding GFP-PH mutant D40 as negative controls. GFP-PH (PLCδ) was enriched in the pellet after ultracentrifugation, while the GFP-PH D40 mutant and annexin II remained in the supernatant, confirming a lack of specific phospholipid binding (Fig. 3Ca and Cb). Full-length hSpry2 or hSpry2TD (hSpry2/TD) was detected in the pellet (Fig. 3Cb), whereas the N-terminal nonconserved region of hSpry2 was almost entirely present in the supernatant (data not shown). In dose-response experiments, increasing the amount of PtdIns(4,5)P_2 in vesicles increased the abundance of hSpry2/TD in the pellet, whereas comparative increases in PS barely evoked any protein in the pellet fraction (Fig. 3Cc). As expected, a dose-dependent increase in the abundance of hSpry2/TD in the pellet and a concomitant decrease in the supernatant were apparent with the PH domain of PLCδ (Fig. 3Cd).

The SpryTD colocalizes with the PtdIns(4,5)P_2-binding PH domain (PLCδ) following various experimental procedures in different cell types. (i) Cytosol to membrane translocation. We next investigated whether hSpry2/TD and the PH domain of PLCδ colocalize in cells. Free PtdIns(4,5)P_2 has been variously reported to appear in the membranes of stimulated cells and sometimes in nonstimulated cells. We set up experiments to test the location of hSpry2/TD in various cells and with various agonists in comparison to the disposition of the PH domain of PLCδ. First, COS-1 cells were transfected with FLAG-hSpry2TD or GFP-PH (PLCδ) and the cells were either left unstimulated or stimulated with EGF (50 ng/ml) for 10 min. From the images shown in Fig. 4A, it is apparent that both hSpry2TD (a and b) and GFP-PH (PLCδ) (c and d) were translocated to ruffles at the cell periphery upon stimulation. Next, the cellular localizations of PtdIns(4,5)P_2, GFP-PH, and the D40 mutant of PLCδ were assessed following their microinjection into Swiss 3T3 cells with combinations of Rac1 and hSpry2/TD. As previously shown, hSpry2/TD localized to membrane ruffles (Fig. 4Ba and Bd). GFP-PH (PLCδ) (Fig. 4Bb) but not GFP-PH (PLCδ) D40 (Fig. 4Be) was localized to membrane ruffles induced by active Rac1. In all of the many experiments performed, hSpry2/TD colocalized with GFP-PH (PLCδ), as exemplified by the image shown in Fig. 4Bc.

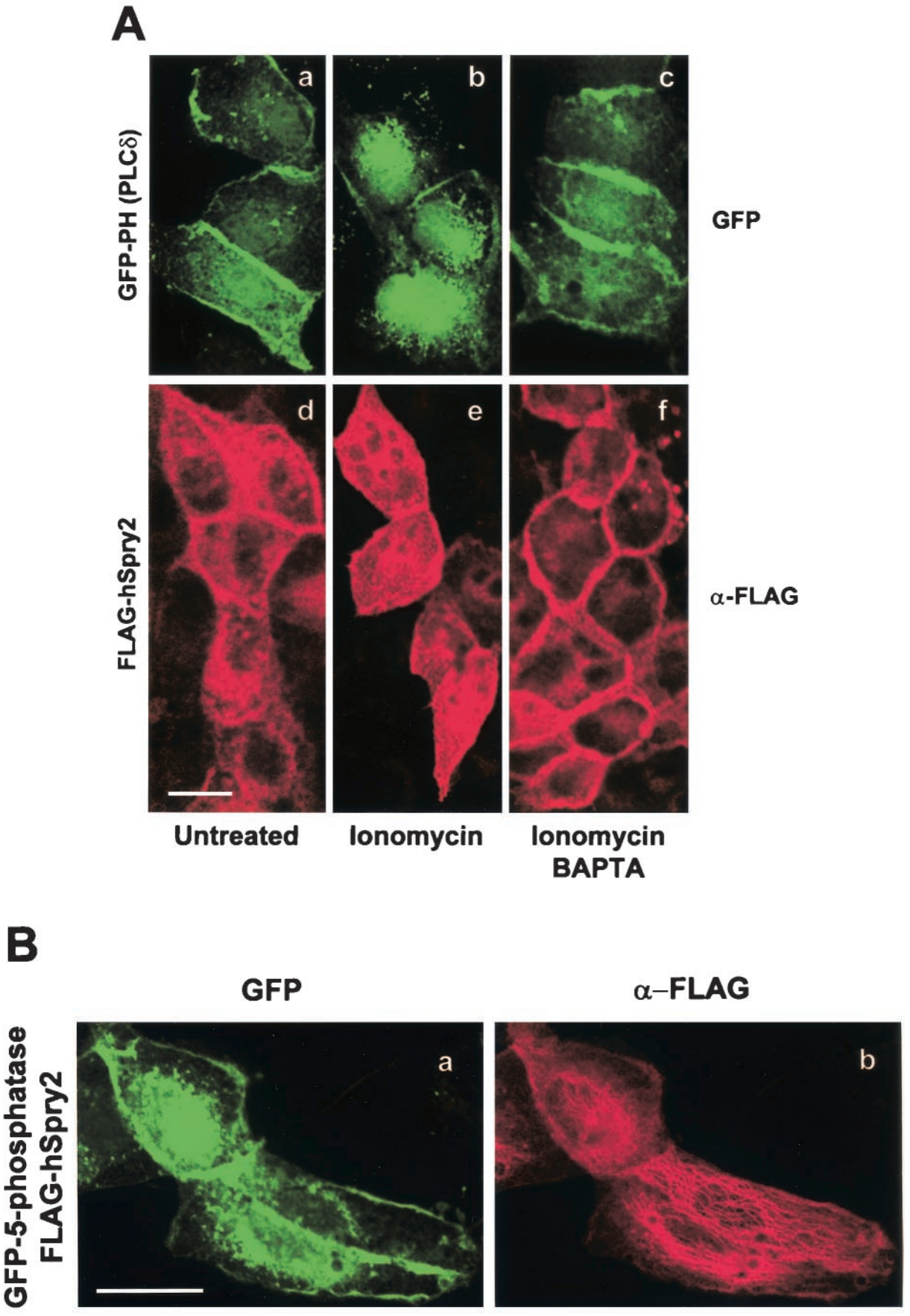
(ii) Membrane location in unstimulated cells. There has been some controversy regarding the disposition of PtdIns(4,5)P_2 before and after growth factor stimulation. We observed that Spry proteins and GFP-PH (PLCδ) translocate into membrane ruffles from the cytosol when resting COS-1 cells are stimulated with EGF. Conversely, Stauffer et al. (21) and Várnai and Balla (28), similarly using the PH domain of PLCδ to monitor the dynamics of PtdIns(4,5)P_2 levels, found that the probe moved from the plasma membrane to the cytosol in response to receptor activation. To test whether hSpry2 behaves similarly in similar circumstances, we overexpressed GFP-PH (PLCδ) or FLAG-tagged hSpry2 in MDCK cells, a line previously shown to exhibit plasma membrane localization of the PH domain of PLCδ in unstimulated cells (3). Indeed, both tagged molecules were observed to localize in the plasma membranes of unstimulated cells (Fig. 5Aa and Ad). It is therefore apparent that the disposition of PtdIns(4,5)P_2 that can be detected by these specific probes is cell type and stimulation dependent.

PLCγ is responsible for the hydrolysis of PtdIns(4,5)P_2 to Ins(1,4,5)P_3 and DAG in the plasma membrane in a calcium-dependent manner (28). To assess whether membrane targeting of hSpry2 is abolished when PtdIns(4,5)P_2 is depleted, we treated MDCK cells with the calcium ionophore ionomycin to induce a calcium influx. We observed a relocation of hSpry2 from the plasma membrane to the cytosol (Fig. 5Ae). It should be noted that although a high proportion of the previously membrane-located probes was now in the cytosol, the transition was not complete and some weak membrane staining is apparent. The reverse effect was observed when hSpry2-transfected MDCK cells were sequentially treated by ionomycin and the cell-permeant, calcium-chelating reagent BAPTA (Fig. 5Ac and Af). Following the BAPTA [1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid] treatment, both probes show a more definitive membrane staining than was apparent in unstimulated cells.

An alternative method was employed to specifically reduce the level of PtdIns(4,5)P_2 in cell peripheral membranes. We utilized a constitutively active, membrane-targeting, GFP-tagged yeast 5P which has previously been shown to greatly decrease levels of PtdIns(4,5)P_2 in target cells (18). We cotransfected this construct with hSpry2 into MDCK cells. When the 5-phosphatase was overexpressed in cells (Fig. 5Ba), the plasma membrane distribution of hSpry2 was almost completely abolished (Fig. 5Bb). Moreover, the microtubule staining was enhanced and was as prominent as that observed with unstimulated COS-1 cells (11).

In short, the membrane-targeting behavior of hSpry2 was demonstrated to be most likely due to the specific interaction

FIG. 4. hSpry2/TD comigrates with a well-characterized PtdIns(4,5)P_2-binding probe in vivo. (A) COS-1 cells were transfected either with FLAG-hSpry2/TD (a and b) or with GFP-PH (PLCδ) (c and d). At 24 h posttransfection, cells were serum deprived overnight and then either left untreated (a and c) or stimulated with EGF (50 ng/ml) for 10 min at 37°C (b and d). (B) Swiss 3T3 cells were microinjected with FLAG-hSpry2, HA-Rac1 V12, and either GFP-PH (PLCδ) or GFP-PH (PLCδ) D40, and the respective locations of hSpry2 were visualized either by antibody-conjugate staining (a) or by endogenous GFP-PH (PLCδ) immunofluorescence (b), as indicated. The colocalization of hSpry2 and the PH domain is apparent as a yellow signal in the merged image (c). A similar experiment was performed except for the inclusion of a non-PtdIns(4,5)P_2-binding construct, GFP-PH (PLCδ) D40, in place of the wild-type sequence (d through f). Bar, 20 μm. The label "hSpry2/TD" indicates that similar results were obtained with full-length hSpry2 and hSpry2TD.


A
a
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d
e
f
FLAG-hSpry2
α-FLAG
Untreated
Ionomycin
Ionomycin
BAPTA
B
GFP
α–FLAG
GFP-5-phosphatase
FLAG-hSpry2
a
b

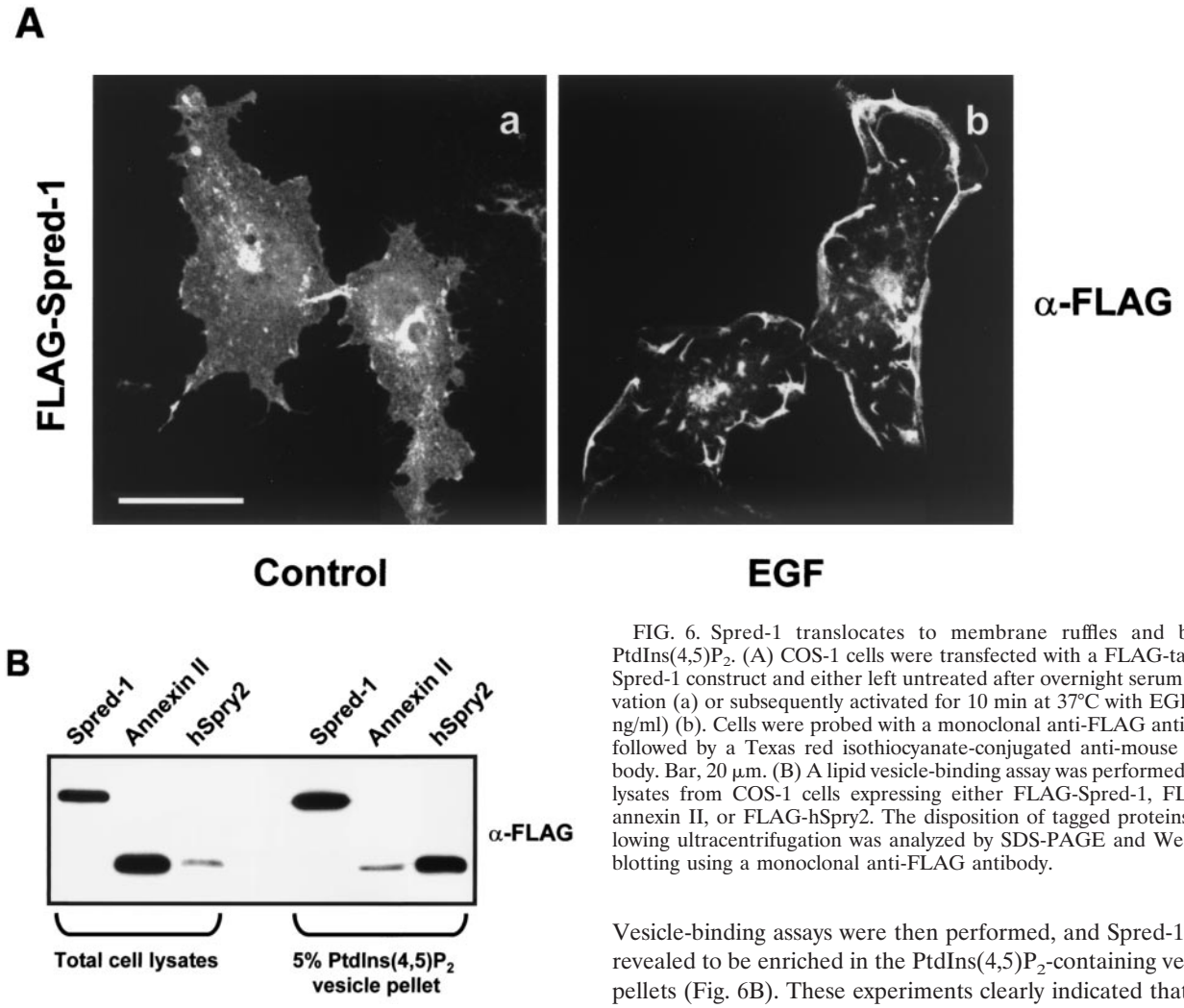


FIG. 6. Spred-1 translocates to membrane ruffles and binds PtdIns(4,5)P_2. (A) COS-1 cells were transfected with a FLAG-tagged Spred-1 construct and either left untreated after overnight serum starvation (a) or subsequently activated for 10 min at 37°C with EGF (50 ng/ml) (b). Cells were probed with a monoclonal anti-FLAG antibody followed by a Texas red isothiocyanate-conjugated anti-mouse antibody. Bar, 20 μm. (B) A lipid vesicle-binding assay was performed with lysates from COS-1 cells expressing either FLAG-Spred-1, FLAG-annexin II, or FLAG-hSpry2. The disposition of tagged proteins following ultracentrifugation was analyzed by SDS-PAGE and Western blotting using a monoclonal anti-FLAG antibody.

with PtdIns(4,5)P_2, since reduction of PtdIns(4,5)P_2 levels in the membrane by various means resulted in the relocalization of hSpry2/TD to the cytosol.

The SpryTD in Spred proteins also mediates translocation and PtdIns(4,5)P_2 binding. In our initial studies with the SpryTD, we queried why this highly conserved domain was not found in other proteins outside the Spry family. Recent identification of the Spred proteins revealed that the cysteine-rich translocation domain is also present in these proteins (29). We therefore asked if Spred proteins behave in a manner analogous to that of Spry proteins when cells are stimulated with growth factors. The cellular disposition of Spred-1 was assessed by immunofluorescent staining combined with confocal microscopy. Not surprisingly, Spred-1 translocated to the membrane ruffles in EGF-stimulated COS-1 cells (Fig. 6A). Vesicle-binding assays were then performed, and Spred-1 was revealed to be enriched in the PtdIns(4,5)P_2-containing vesicle pellets (Fig. 6B). These experiments clearly indicated that the SpryTD in Spred-1 also functions to direct the protein toward enhanced concentrations of free PtdIns(4,5)P_2 in the plasma membrane.

The hSpry2 D252 mutant fails to translocate upon EGF stimulation of cells. We next set about engineering a mutant of Spry2 that fails to bind PtdIns(4,5)P_2 but retains tertiary integrity. Previous research on the PH and FERM domains indicated that the key residues responsible for direct contact with PtdIns(4,5)P_2 are the basic amino acids arginine and lysine (14). Alignment of the amino acid sequences of Spry and Spred family members using the DNASTAR Clustal method identified three conserved candidate residues: K183, K185, and R252 of hSpry2 (Fig. 7A). Each of the candidates was replaced by an acidic residue to produce respective point mutants, and these were subjected to immunofluorescent staining to assay their translocation behavior. The E183 and E185 point mu-

FIG. 5. Membrane targeting of hSpry2 is abolished following depletion of PtdIns(4,5)P_2. (A) MDCK cells were transfected with GFP-PH (PLCδ) (a, b, and c) or FLAG-hSpry2 (d, e, and f). The location of hSpry2 was revealed by anti-FLAG, and that of the PH domain was revealed by the native fluorescence of the GFP signal. Cells were either left untreated (a and d), treated with ionomycin (1 μM) in the presence of 1 mM $CaCl_2$ in the medium for 10 min (b and e), or treated with ionomycin for 10 min plus BAPTA (50 μM) for an additional 10 min (c and f). (B) FLAG-hSpry2 and GFP-5P were cotransfected into MDCK cells. The location of the 5P was revealed by the native fluorescence of the GFP signal (a), and that of hSpry2 was revealed by anti-FLAG (b). The images depicted are typical of more than 80% of the transfected cells. Bar, 20 μm.

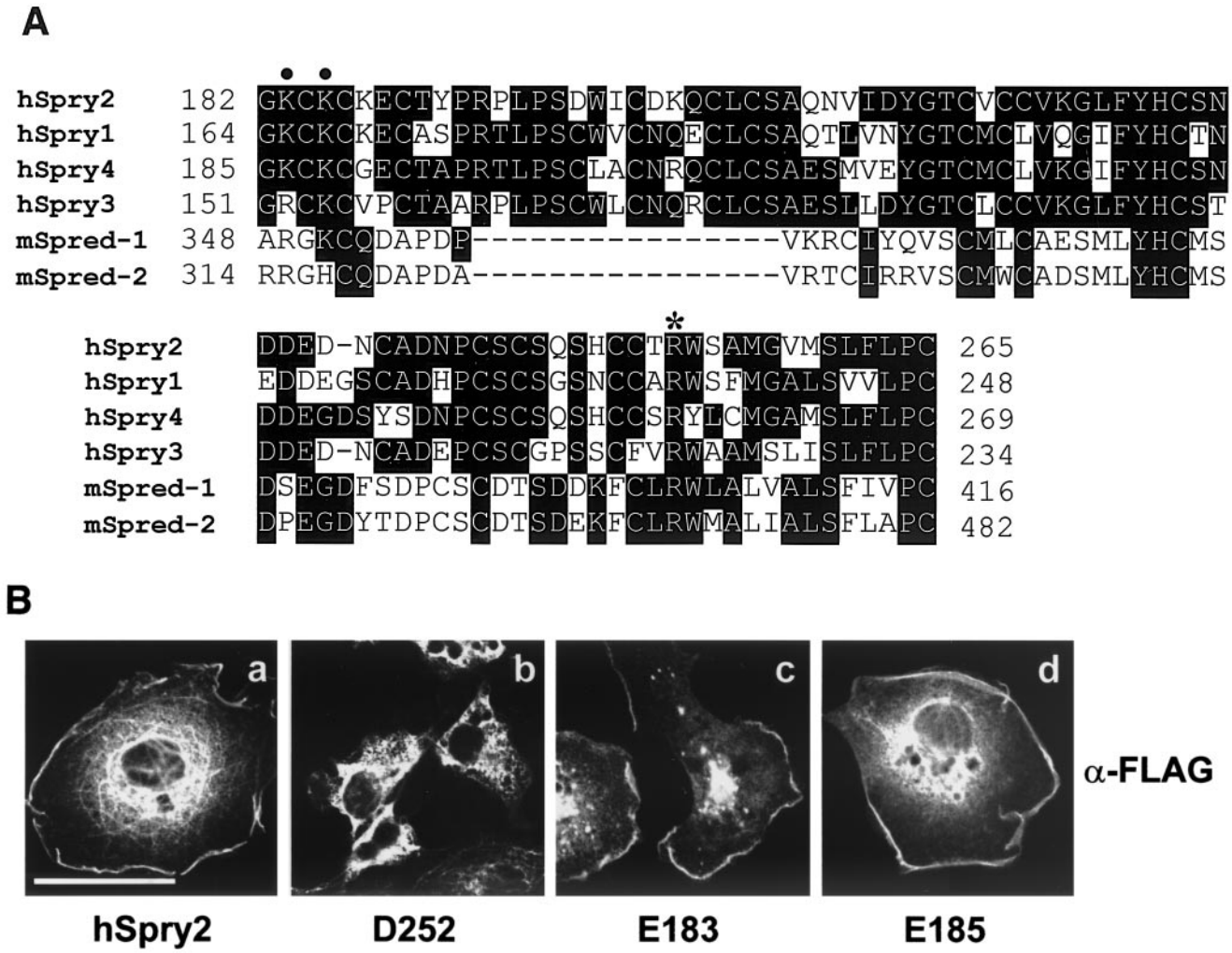
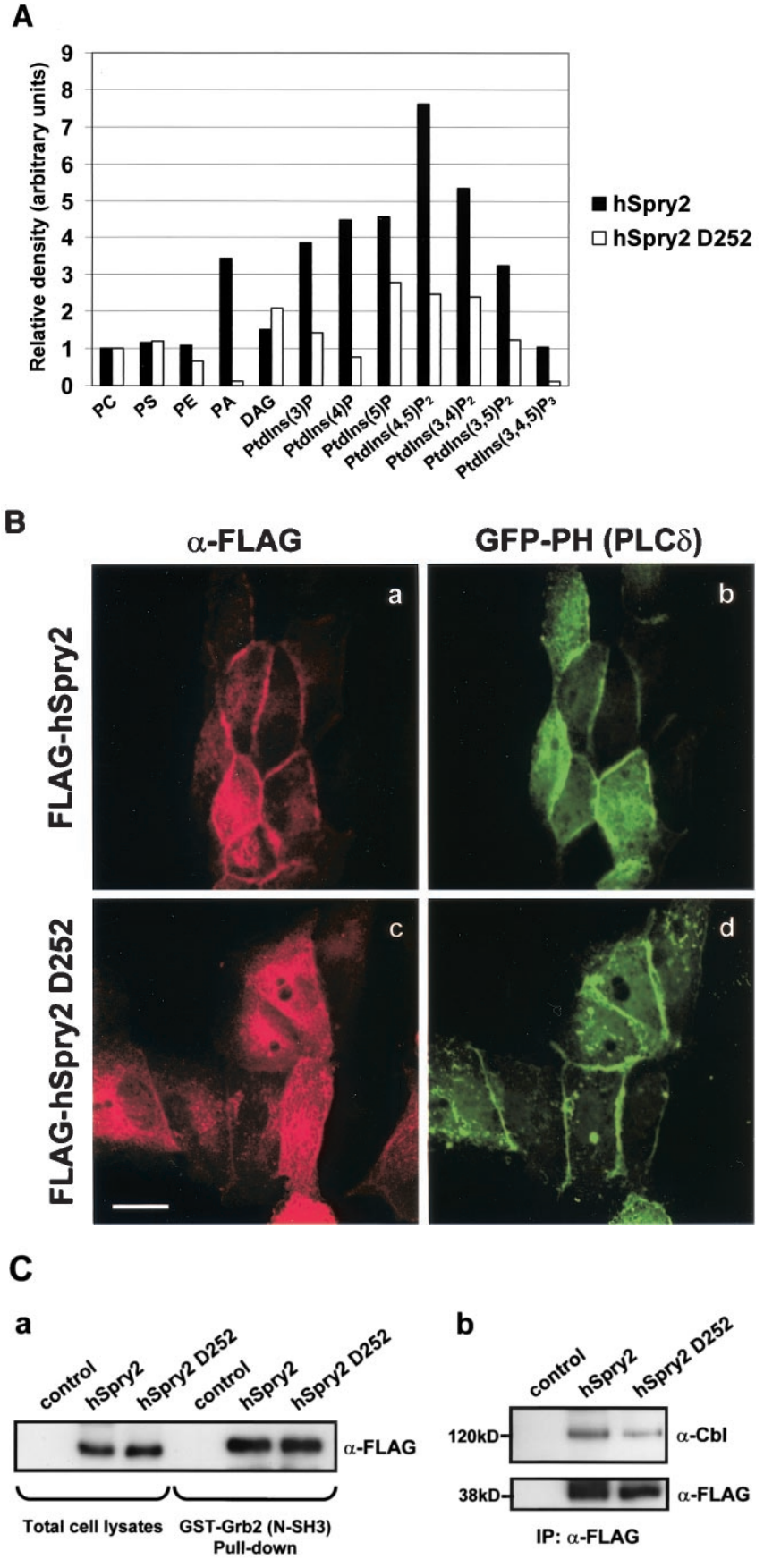


FIG. 7. Identification of hSpry2 D252 as a translocation-defective mutant. (A) Amino acid sequence alignment of partial translocation domains of hSpry2, hSpry1, hSpry4, hSpry3, mSpred-1, and mSpred-2 (accession numbers, NP_005833, XP_036349, XP_096029, O43610, BAB62848, and BAB62849, respectively). The positions of the conserved basic residues K183 and K185 are indicated by solid circles, while that of R252 is indicated by an asterisk. (B) COS-1 cells were transfected with either the wild-type FLAG-tagged hSpry2 construct (a) or its D252 (b), E183 (c), or E185 (d) mutant and were activated for 10 min at 37°C with EGF (50 ng/ml). Cells were stained sequentially with monoclonal anti-FLAG and Texas red isothiocyanate-conjugated antibodies. The images are representative of more than 85% of the transfected cells. Bar, 20 μM. (C) A lipid vesicle-binding assay was performed with lysates from COS-1 cells expressing either wild-type FLAG-hSpry2 or its D252, E183, or E185 mutant. Following ultracentrifugation, pellets (P) of lipid vesicles containing PtdIns(4,5)P_2 and supernatants (S) were analyzed by SDS-PAGE and Western blotting using a monoclonal anti-FLAG antibody to detect bound, tagged proteins.

tants translocated to the membrane ruffles in EGF-stimulated cells (Fig. 7Bc and Bd). The D252 mutant (Fig. 7Bb), however, failed to translocate under conditions where wild-type hSpry2 was seen to migrate to the plasma membrane (Fig. 7Ba).

FIG. 8. The hSpry2 D252 mutant loses specific PtdIns(4,5)P_2 binding and colocalization with the PH domain of PLCδ. (A) Cell lysates from COS-1 cells expressing either wild-type FLAG-hSpry2 or its D252 mutant were subjected to a lipid vesicle-binding assay. The resultant Western blot was analyzed by densitometry, and the relative binding of the two Sprys was plotted as a bar chart. Bars represent the relative amount of hSpry2 or its D252 mutant in the pellet. (B) MDCK cells were transfected with either wild-type FLAG-hSpry2 (a and b) or its D252 mutant (c and d) in combination with GFP-PH (PLCδ). The location of hSpry2 was revealed by anti-FLAG (a and c), and that of the PH domain was revealed by the native fluorescence of the GFP signal (b and d). (C) Wild-type hSpry2 and its D252 mutant were tested for both Grb2 and c-Cbl binding. (a) For Grb2 binding, a GST pulldown experiment employed the N-terminal SH3 domain of Grb2. (b) For Cbl binding, the presence of c-Cbl was assessed in FLAG-hSpry2 immunoprecipitates (IP). In each case the associated proteins were analyzed by SDS-PAGE and Western blotting with the indicated monoclonal antibodies.


A
Relative density (arbitrary units)
0
1
2
3
4
5
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7
8
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PC
PS
PE
PA
DAG
PtdIns(3)P
PtdIns(4)P
PtdIns(5)P
PtdIns(4,5)P2
PtdIns(3,4)P2
PtdIns(3,5)P2
PtdIns(3,4,5)P3
hSpry2
hSpry2 D252
B
α-FLAG
GFP-PH (PLCδ)
FLAG-hSpry2
FLAG-hSpry2 D252
a
b
c
d
C
a
control
hSpry2
hSpry2 D252
control
hSpry2
hSpry2 D252
α-FLAG
Total cell lysates
GST-Grb2 (N-SH3) Pull-down
b
control
hSpry2
hSpry2 D252
120kD
α-Cbl
38kD
α-FLAG
IP: α-FLAG

Wild-type hSpry2 and the three point mutants were next assessed for their abilities to bind to phospholipid vesicles containing 5% PtdIns(4,5)P_2. The assay was performed as described in Materials and Methods, and Fig. 7C shows the resultant Western blot demonstrating the dispositions of the hSpry2 derivatives. Wild-type hSpry2 preferentially binds to vesicles, as seen from the differential binding to the pellet and the supernatant. Both the E183 and E185 point mutants show a similar binding preference to the vesicles (pellet). The D252 mutant, however, shows the reverse preference, with less binding to the pellet than to the supernatant.

Taken together, these data demonstrate that with the D252 point mutation in SpryTD, there is substantially reduced binding or no binding to PtdIns(4,5)P_2 and consequently no translocation to membrane ruffles in appropriately stimulated cells.

The hSpry2 D252 mutant has reduced binding to PtdIns(4,5)P_2 compared to that of wild-type hSpry2 but binds similarly to associated proteins. Next we analyzed more closely the characteristics of the hSpry2 D252 mutant in terms of phospholipid binding, normality of folding, and its disposition in unstimulated MDCK cells. We subjected the hSpry2 D252 mutant to the vesicle-binding assay with the range of phospholipids previously used to characterize the binding of wild-type hSpry2/TD. From Fig. 8A it can be seen that the binding of the hSpry2 D252 mutant to the various inositol phospholipids is severely reduced from that of the wild-type protein in all cases.

We have previously argued that there is free PtdIns(4,5)P_2 in the plasma membranes of resting MDCK cells. Given the reduced binding of the D252 mutant to PtdIns(4,5)P_2, the hSpry2 D252 mutant should not be found in the plasma membranes of these cells. An experiment, which used the PH domain of PLCδ as a control, was performed to investigate this possibility. From the immunofluorescent images shown in Fig. 8B, it is apparent that while the PH domain (PLCδ) (Fig. 8Bb and Bd) and wild-type hSpry2 (Fig. 8Ba) locate to the plasma membrane, the D252 mutant is located in the cytosol (Fig. 8Bc) with little or no membrane binding.

To ensure that the D252 mutant protein was being expressed in a native conformation, we assessed whether it could still associate with c-Cbl (30) and Grb2 (our unpublished data), previously characterized binding partners of the wild-type protein. We have shown that the N-terminal SH3 domain of Grb2 interacts directly with a canonical proline-rich sequence (PXXPXR) on the extreme C terminus of hSpry2 (unpublished data). COS-1 cells were transiently transfected with wild-type hSpry2 or the D252 mutant, the cells were lysed, and the lysates were subjected to a pulldown experiment with GST-Grb2 (N-terminal SH3 domain). Bound proteins were analyzed by Western blotting with anti-FLAG (Fig. 8Ca). Both full-length hSpry2 and the point mutant bound equally well to the N-terminal SH3 domain of Grb2.

The binding of hSpry2 and c-Cbl occurs distally to the C terminus, in the N-terminal region (30). Immunoprecipitation experiments were performed following transient expression of FLAG-tagged wild-type hSpry2 or its D252 mutant in COS-1 cells. Cell lysates were precipitated with anti-FLAG, and the associated proteins were analyzed by Western blotting using anti-c-Cbl (Fig. 8Cb). The blot was reprobed with anti-FLAG to demonstrate equality in expression and precipitation of the two forms of hSpry2. Taken together, these data lead to the conclusion that hSpry2 and its D252 mutant bind equally to Grb2 or c-Cbl, indicating that the D252 mutant behaves normally in terms of interaction with other proteins.

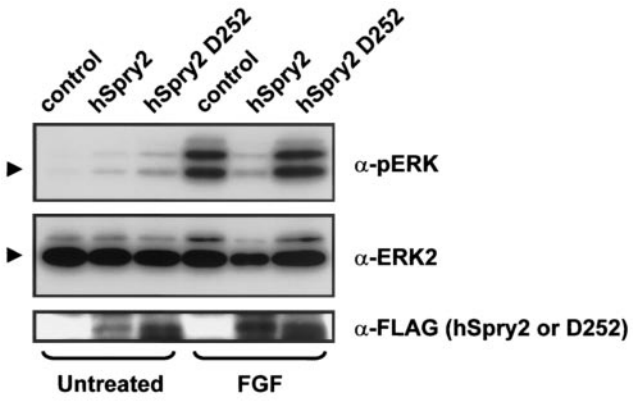

FIG. 9. The translocation-defective hSpry2 D252 mutant fails to inhibit MAPK phosphorylation. PC12 cells were transfected with either wild-type FLAG-tagged hSpry2 or its D252 mutant. Cells were harvested after 3 h of serum deprivation and stimulated with basic FGF (10 ng/ml) for 8 min at 37°C. Levels of phosphorylated ERK, ERK, and FLAG-tagged protein expression were analyzed by SDS-PAGE and Western blotting with the indicated monoclonal antibodies.

The translocation-defective hSpry2 D252 mutant loses its MAPK-inhibitory function. It has previously been shown that in a fibroblast growth factor (FGF)-signaling context, the phosphorylation of ERK was inhibited in cells overexpressing hSpry2 (33). To examine the role of the SpryTD in this inhibitory function, we transfected PC12 cells with FLAG-tagged wild-type hSpry2 or its D252 mutant and subsequently performed a phospho-ERK Western blot analysis. The ERK protein was potently phosphorylated in response to FGF stimulation, while its phosphorylation was reduced to almost basal levels in hSpry2-transfected cells. There was little or no inhibition of ERK phosphorylation in cells transfected with the D252 mutant (Fig. 9). Similar results were obtained by transfection studies in 293T cells (data not shown), indicating that the assay was not cell line specific.

Collectively, the evidence presented indicates that the SpryTD targets available PtdIns(4,5)P_2 in cells and that this targeting is necessary for the physiological effects of the various Spry and possibly Spred proteins.

DISCUSSION

Taken together, our data strongly indicate that the highly conserved cysteine-rich translocation domain from Spry and Spred targets PtdIns(4,5)P_2, which is enriched in membrane ruffles and microspikes in various cell types. Despite the lack of inherent sequence similarity to existing inositol phospholipid binding domains, the SpryTD harbors a superficial similarity to two domains that bind phospholipids: the C1 domain, which binds DAG (15), and the FYVE domain, which binds to PtdIns(3)P (2). Each of these domains consists of a sequence of strategically positioned basic residues within a matrix of cysteines. In the case of Spry and Spred proteins, the K183, K185, and R252 of hSpry2 are conserved. In accordance with

the reports that the binding of PH and ENTH domains to PtdIns(4,5)P_2 is disrupted by mutation of a key arginine or lysine residue (8, 21), the hSpry2 D252 point mutant lost the dose-responsive binding to PtdIns(4,5)P_2-containing lipid vesicles and failed to down-regulate ERK phosphorylation. It is probable that the corresponding residues in hSpry1 (R235), hSpry4 (R256), hSpry3 (R221), mSpred-1 (R403), and mSpred-2 (R469) would play a similar and crucial role in targeting the respective proteins to inositol phospholipids.

Although the single point mutants E183 and E185 did not disrupt hSpry2 translocation, deletion of amino-acids 178 to 194 (which include K183, K185, K187, and R193 in hSpry2) resulted in failure of the protein to translocate to membrane ruffles in EGF-stimulated cells (11) and abolished the inhibitory effect of hSpry2 on cell migration in COS-7 cells (32). The various conserved basic residues within the SpryTD could contribute synergistically to inositol lipid binding. We have worked mainly with the hSpry2/TD sequence. It is possible that subtle variations in the amino acid sequences of the various translocation domains could confer different binding specificities on the Spry and Spred family members.

In the studies of Honda et al. (6) and other groups (17, 20), Arf6 activation was instrumental in the formation of membrane ruffles. During the course of these studies in which we compared the dispositions of the PLCδ PH domain and hSpryTD, we employed the constitutively active form of Arf6 (L67 mutant), either by transfection or by microinjection. We were unable to induce ruffle formation either with the Arf6 mutant or with PI5K constructs, the products of which are postulated to be downstream of active Rac and instrumental to the ruffle formation cascade. We postulate that Rac is the key molecule in signaling for membrane ruffle formation, and it is likely that other targets of Rac, besides Arf6 and PI5K, are needed synergistically to drive actin reorganization.

We suggest that Spry proteins bind to various partner proteins via their respective N termini, targeting these proteins to a peripheral membrane location via PtdIns(4,5)P_2 interaction following RTK activation, and are thus able to fulfill their respective physiological functions. The C terminus of hSpry2 is capable of translocating upon cellular stimulation and binds to PtdIns(4,5)P_2 but does not inhibit the Ras/ERK pathway (33). This indicates that hSpry2TD is not by itself able to cause inhibition of the Ras/ERK pathway; it suggests, rather, that hSpryTD is a targeting domain and that critical proteins capable of controlling the Ras/ERK pathway bind to the N-terminal sequence. So far we have characterized c-Cbl as being one of these proteins, but there is currently no evidence to suggest that c-Cbl binding is responsible for the inhibition of the Ras/ERK pathway in the context of FGF signal transduction.

There is no compelling evidence to propose that binding of the SpryTD to PtdIns(4,5)P_2 has any profound effect on the metabolism of the lipid or any downstream physiological function of transfected cells. In hSpry2-transfected cells, neither Ins(1,4,5)P_3 production nor tyrosine phosphorylation of PLCγ was affected (data not shown). PtdIns(4,5)P_2, like the other lipid messengers DAG and PA, enables the translocation of various catalytically active molecules such as PLCδ, protein kinase C, and Raf, respectively, in a diffusible, highly regulated, and rapidly reversible manner. This mode of localization-activation provides a sensitive response to rapid changes whereby subtle changes at the surface of the cell can be rapidly translated into a cytosolic signaling response.

It appears that inhibitory proteins of the Spry and Spred families utilize a novel and common PtdIns(4,5)P_2-binding domain that targets the proteins to a cellular localization that maximizes their inhibitory potential. These inhibitory proteins seem to have arisen in evolution to control complex organ patterning. They are not found in *Caenorhabditis elegans*, and only one isoform of Spry is present in *Drosophila*, compared to at least four isoforms in mammals. There is considerable variation in the N termini of the various Spry proteins, but the SpryTD is highly conserved during evolution, which indicates a close link between sequence fidelity and specificity of function.

ACKNOWLEDGMENTS

We are grateful to Cheh Peng Lim and Wing Chan for advice on microinjection. Special thanks also to Corrine Lay San Neo and Hwei Fen Leong for technical assistance.

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