IDENTIFICATION OF A NOVEL TRANSLLOCATION DOMAIN*

Sprouty (Spry) was first identified in a genetic screen in Drosophila to be an antagonist of fibroblast growth factor and epidermal growth factor (EGF) signaling, seemingly by inhibiting the Ras/MAP kinase pathway. Data base searches lead to the identification and cloning of, to date, four mammalian sprouty genes. The primary sequences of the mammalian sprouty gene products share a well conserved cysteine-rich C-terminal domain with the Drosophila protein, The N-terminal regions, however, do not exhibit significant homology. This study aimed at determining the disposition of Spry proteins in intact cells before and after stimulation of the EGF receptor tyrosine kinase. Full-length or deletion mutants of Spry, tagged at the N termini with the FLAG-epitope, were expressed in COS-1 cells by transient transfection and analyzed by immunofluorescence microscopy before and after EGF stimulation of the cells. In unstimulated cells, the Spry proteins were distributed throughout the cytosol except for human Spry2 (hSpry2), which, although generally located in the cytosol, co-localized with microtubules. In all cases, the Spry proteins underwent rapid translocation to membrane ruffles following EGF stimulation. The optimal translocation domain was identified by deletion and immunofluorescence analysis to be a highly conserved 105-amino acid domain in the C-terminal half of the hSpry2 protein. The translocation of this conserved domain, based on hSpry2 data, was independent of the activation of phosphatidylinositol-3-kinase.

In recent years, there have been several examples of key mammalian signaling proteins being first detected in Caenorhabditis elegans, and Drosophila melanogaster or Caenorhabditis elegans, both of which are amenable to mutational studies. The delineation of the mammalian Ras/MAP kinase pathway had its roots in these systems. Receptor tyrosine kinase (RTK)-initiated signaling plays key roles in the developmental process of mammals, Drosophila, and C. elegans. In Drosophila, the mutation of the RTK sevenless causes R7 cells to fail to differentiate into photoreceptor cells, and instead they become lens-secreting cone cells in the eye ommatidia (1). In screening for an allele-specific suppressor of the sevenless phenotype, son of sevenless was isolated (2). Similarly, analysis of mutant phenotypes of Drosophila eye ommatidia resulted in the identification of the adaptor protein Drk (Sem-5 in C. elegans/Grb2 in mammals) (3). It became clear that son of sevenless is linked to RTKs by the adaptor Grb2 and acts downstream of various RTKs as a guanine nucleotide exchange factor for Ras. The discovery of Raf, a target protein for active Ras, also came about following similar genetic studies (4). The activation of the serine kinase Raf leads to the phosphorylation of mitogen-activated protein kinase kinase (MAP kinase kinase), which in turn activates MAP kinase (Erk) by phosphorylation on tyrosine and threonine. Collectively, these studies allowed the first delineation of a signaling pathway from receptor activation to gene expression and highlighted the value of screening for signal transduction mutants in systems suitable for genetic analysis and extrapolating these findings into mammalian signaling systems.

In Drosophila, the fibroblast growth factor (FGF) receptors are expressed as the products of two different gene products, breathless (Btl) (5) and heartless (Htl) (6), the former involved in the formation of the tracheal system and the latter involved in the formation of the vascular system. The branchless (bnl) gene, encoding an FGF-like protein, is expressed in discrete clusters of cells surrounding developing tracheal placodes, exactly where a new branch will form. The clusters of epithelial cells that form tracheal sacs failed to migrate and elongate to develop the respiratory network in mutants of FGF (branchless) (7), FGF receptor (breathless) (6), and Downstream of FGF receptor (8). Contrary to these observations, sprouty mutations cause excessive branching (9), suggesting that Sprouty is an inhibitor of FGF signaling. Casci et al. (10) later reported that Sprouty is also involved in the inhibition of the epidermal growth factor (EGF) receptor-triggered cell recruitment in the eye of Drosophila. It was postulated that in both the EGF receptor and FGF receptor signaling pathways, Sprouty was a negative regulator of growth factor-induced Ras/MAP kinase pathways. Two different mechanisms were proposed to account for this inhibition. The first suggested that Sprouty was secreted and competes for the receptor with growth factors (9), whereas the other proposed that Sprouty inhibits a pathway upstream of Ras and is itself associated with the inner surface of plasma membrane (10). Expression of Drosophila Sprouty (dSpry) was detected in a wide range of developmental tissues such as eye imaginal discs, embryonic chordotonal organ precursors, and the midline glia (11) and thus indicated it to be a
FIG. 1. Human Sprouty2 co-localized with microtubules in quiescent cells and is translocated to membrane ruffles upon EGF stimulation. COS-1 cells transfected with FLAG-tagged full-length hSpry2 were serum-depleted overnight and either left untreated (0) or
general inhibitor of RTK signaling (12). More recently, genetic evidence was presented that in mammalian development Sprouty also inhibited the Ras/MAP kinase pathway, but the inhibition occurred downstream of Ras at the level of Raf or MAP kinase kinase (12).

In the Expressed Sequence Tag data base, three mammalian homologs of DsSpry were found. The murine Sprouty2 (mSpry2) negatively modulates respiratory organogenesis (13) suggesting some similarity to the Drosophila tracheal development system. Moreover, Sprouty overexpression caused a reduction in FGF-induced limb bud outgrowth (14). The alignment of the mammalian Sprouty isoatypes with the Drosophila protein reveals that the only conserved part is the C-terminal cysteine-rich region. It was the C-terminal half of Drosophila Sprouty that apparently directed the protein to a membrane location, whereas the N-terminal part of the protein interacted with Gap1 and Drk (10).

We have been characterizing novel proteins that act proximal to the FGF receptor. Such proteins include FRS2, a necessary component of the MAP kinase signaling system. Because Sprouty was postulated to inhibit the MAP kinase pathway in the vicinity of Ras, we were interested to know what the functions of the mammalian homologues of Sprouty are. The emphasis in this initial phase of research was to investigate the intracellular location of human Sprouty2 (hSpry2) and to observe the effect of stimulation of cells with either FGF or EGF on the protein disposition.

**MATERIALS AND METHODS**

**Cell Culture**—COS-1 monkey kidney cells or 293T human kidney epithelial cells were grown and maintained in Dulbecco’s modified Eagle’s medium (1 g/ml glucose) or RPMI medium, respectively. Culture medium was supplemented with 10% fetal bovine serum (Hyclone Lab- oratories), 2 mM L-glutamine, 10 mM HEPES (PH 7.4), 100 units/ml penicillin, and 100 units/ml streptomycin. Cells were seeded in six-well plates containing glass coverslips for immunofluorescence studies or in 60-mm culture dishes for Western analyses. Cells at 80% confluency were transfected with various expression plasmids using LipofectAMINETM 2000 reagent (Life Technologies, Inc.) 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. Before being fixed or lysed, cells were either left untreated or stimulated with 50 ng/ml EGF (Sigma) for different time points. Various chemicals were added to media at the following concentrations: nocodazole, 10 μM; cytochalasin A, 1 μM; phorbol 12-myristate 13-acetate (PMA), 1 μM; and wortmannin, 50 or 100 nM. All of the above chemicals were from Sigma. In the EGF washout experiment, cells were washed with serum-free medium three times following 10 min of EGF stimulation. The cells were then reincubated in fresh, serum-free medium at 37 °C for an additional 30 or 60 min before being processed for immunofluorescence.

**DNA Expression Plasmides**—Full-length cDNA of hSpry2 was cloned from an adult brain library (CLONTECH) using the Expand Long Template polymerase chain reaction system (Roche Molecular Biochemicals) with primers designed against the published sequence (GenBank™ accession number AF039843). The hSpry2 cDNA was subcloned into the pXJ40FLAG mammalian expression vector (obtained from Dr. E. Manser, Glaxo Group Institute of Molecular and Cell Biology, Singapore) utilizing BamHI/XhoI restriction sites. Arbitrary deletion mutants Δ53–122, Δ123–177, Δ178–194, Δ195–221, and Δ265–282, and DNA fragments of the N terminus (1–177) and C terminus (178–315, 178–282, 178–264, 178–250, 178–237, 178–229, and 178–220) of hSpry2 were generated by standard polymerase chain reaction procedures. Mouse Sprouty1, mouse Sprouty4, and Drosophila Sprouty (provided by Dr. M. Krasnow, Stanford University School of Medicine, and Dr. G. Martin, University of California, San Francisco) were subcloned into pXJ40FLAG. The cDNAs of Rac1 (provided by Dr. A. Hall, University College, London, United Kingdom) and Akt (a kind gift from Dr. P. Cohen, University of Dundee, Dundee, United Kingdom) were inserted into vector pXJ40HA (Dr. E. Manser, Glaxo-Institute of Molecular and Cell Biology, Singapore). Dominant negative mutant Rac1 (N7) was produced by site-directed mutagenesis using the Quik-Change mutagenesis kit (Stratagene). PKCβ II in pcDNA3 was obtained from Dr. X-M. Cao (Institute of Molecular and Cell Biology, Singapore). The integrity of all constructs was confirmed by DNA sequencing or restriction digestion analyses.

**Immunofluorescence**—For immunofluorescence studies, cells were seeded onto sterile glass coverslips contained in six-well plates. Quiescent COS-1 cells that were transfected with FLAG-tagged hSpry2 were either left untreated or treated with EGF (50 ng/ml). Subsequently, the cells were washed with cold phosphate-buffered saline supplemented with 10 mM calcium chloride and 10 mM magnesium chloride (PBSYM) and fixed with cold 3% paraformaldehyde in PBSYM for 30 min at 4 °C. The fixed cells were washed twice with PBSYM, twice with PBSYM containing 50 mM NH₄Cl, and twice again with PBSYM. For cell permeabilization, cells on the coverslips were incubated with 0.1% saponin (Sigma) in PBSYM at room temperature for 15 min. The primary antibody for single labeling (anti-Flag M2® monoclonal antibody, Sigma) was diluted to 1 μg/100 μl in FDB (0.1% (w/v) fetal bovine serum, 2% (w/v) bovine serum albumin in PBSYM), and each coverslip was incubated with 100 μl of diluted antibody for 1 h at room temperature. The coverslip was then washed three times for 2 min in 0.1% saponin-containing PBSYM before incubation with secondary antibodies. For monoclonal primary antibody Texas Red® dye-conjugated AffiniPure goat anti-mouse IgG was used (Jackson Immunoresearch). After the final wash (five times in 0.1% saponin containing PBSYM), each coverslip was prepared for microscopic examination by applying mounting medium (Crystal Mount, Biomed). Direct double staining, FLAG-hSpry2 was detected with polyclonal anti-Flag (Octa-Probe™, Santa Cruz Biotechnology) and fluorescein isothiocyanate-conjugated sheep anti-rabbit IgG (Roche Molecular Biochemicals). Microtubules and intermediate-filaments were visualized with anti-β-tubulin and anti-vimentin monoclonal antibody, respectively, whereas filamentous actin was labeled by Texas Red® Iso-thiocyanate-Conjugated-labeled phalloidin (all reagents from Sigma). Hemagglutinin-tagged Rac1 (wild type and dominant negative) and Akt were detected using hemagglutinin monoclonal antibody (Roche Molecular Biochemicals). Erzin and PKCβII were detected by probing with the respective monoclonal antibodies (Transduction Laboratories).

Confluent fluorescence microscopy of fixed and immunostained cells was performed at room temperature using a MRC-1024 laser scanner (Bio-Rad). All microscopic images were captured with a × 40 objective lens. Fluorescent images were processed with LaserSharp software (Bio-Rad) and Adobe Photoshop (Adobe System, Inc.). Digital manipulation of images was done using Microsoft PowerPoint software (Microsoft 1997 version).

**Western Blot Analyses**—Cells were lysed in 1 ml of lysis buffer (50 mM HEPES (pH 7.4), 150 mM sodium chloride, 1.5 mM magnesium chloride, 5 mM EGTA, 10% (v/v) glycerol, 1% Triton X-100, a mixture of protease inhibitors (Roche Molecular Biochemicals), and 0.2 mM orthovanadate). The lysates were analyzed by SDS-PAGE followed by Western blotting with the previously described antibodies, anti-phospho-Akt (New England Biolabs), or anti-Akt (New England BioLabs). The Western blots were developed by ECL (Amersham Pharmacia Biotech).

**Alignment of Amino Acid Sequences**—The C-terminal sequences of Sprouty isoatypes, hSpry2 (AF039843), mSpry1 (AF176903.1), hSpry2, mSpry4 (AF176906.1), and Drosophila Syp (AF039842.1) were aligned using the Clustal method by DNASTAR.

stimulated with EGF (50 ng/ml) for 10 min. A. lysates were analyzed by 10% SDS-PAGE and probed with PY20 antibodies to detect tyrosine-phosphorylated EGF receptors (top panel) or anti-FLAG to determine the expression levels of FLAG-tagged-hSpry2. B. immunofluorescence studies were performed using monoclonal anti-FLAG antibodies to probe for transfected hSpry2. C–E, immunofluorescence studies were performed using polyclonal anti-FLAG antibodies to probe for transfected hSpry2 (middle panels in C–E). Cells were co-stained with tubulin antibodies (C, top panels), Texas Red® Isothiocyanate-Conjugated-phalloidin (D, top panels), or ezrin antibodies (E, top panels). Images were also overlaid (C–E, bottom panels) for comparison of staining; yellow indicates co-localization of the two proteins.
RESULTS

hSpry2 Colocalized with Microtubules in Unstimulated Cells and Translocated Rapidly to Membrane Ruffles upon EGF Stimulation—FLAG-tagged hSpry2 was overexpressed in COS-1 cells, and the distribution of the expressed protein was assessed by immunofluorescence with FLAG monoclonal antibody and confocal microscopy. Cells were serum-deprived for 18 h prior to various times of EGF stimulation. Prior to immunofluorescence studies, experiments were conducted to check that hSpry2 was present at similar levels in the different lysates and that the protein did not exhibit unexpected molecular weight changes (e.g. proteolysis) following EGF stimulation. To this end, whole cells lysates were separated by SDS-PAGE and probed with PY20 to locate tyrosine-phosphorylated EGF receptors to demonstrate EGF induced activation (Fig. 1A, top panel) or with α-FLAG to locate and determine the integrity of FLAG-tagged hSpry2 (bottom panel). The hSpry2 protein did not change from its expected molecular weight upon EGF treatment. It was apparent in this and later experiments, though, that full-length or various protein fragments of hSpry2 often appear as double bands on Western blots, whether the cells were stimulated or not.

The results from immunofluorescence experiments showed that in the unstimulated cells, hSpry2 was distributed along elements of the cytoskeleton (Fig. 1B, left panel). Upon EGF stimulation, a high proportion of the overexpressed hSpry2 rapidly (within 2 min) translocated to the cell periphery, more specifically to a wavy structure that resembled membrane ruffles (Fig. 1B, right panel), which shows the 10-min time point. Control experiments, using overexpressed FLAG-tagged Annexin II, showed that EGF-induced translocation of hSpry2 was not a spurious effect of overexpression as Annexin II did not translocate upon stimulation of cells with EGF (data not shown).

To confirm the subcellular distribution of hSpry2, co-staining of hSpry2 with each of the major cytoskeletal structural proteins tubulin, vimentin, and actin was performed. In unstimulated cells, the disposition of hSpry2 most closely resembled that of tubulin, as can be seen by a comparison of tubulin staining (Fig. 1C, top panels) with hSpry2 staining (Fig. 1C, middle panels) and a subsequently overlay of the two images (Fig. 1C, bottom panels). In support of the evidence that hSpry2 co-localized with tubulin, we noted that pretreating the cells with nocodazole (10 μg/ml for 1 h at 37 °C), a well characterized chaotropic agent for microtubules, disrupted both the microtubule and the hSpry2 protein disposition (data not shown). In contrast, hSpry2 colocalized only partially with vimentin (one of the components of intermediate filaments) at the periphery of nuclei (data not shown), whereas it showed a staining pattern completely different from that of actin (microfilaments) in unstimulated cells (see Fig. 1D).

Actin and ezrin are two proteins shown to be present in membrane ruffles (reviewed in Ref. 15). To verify that hSpry2 translocated to membrane ruffles when cells were stimulated with EGF, co-staining of hSpry2 with actin or ezrin was performed on EGF-treated cells. It is apparent from the immunofluorescence images of actin staining shown in Fig. 1D (top panels) that actin was in the same cell peripheral location as hSpry2 in EGF stimulated cells (Fig. 1D, middle panel), and this is further indicated by overlaying these images (Fig. 1D, bottom panel). Likewise, ezrin (Fig. 1E, top panel) is located in similar membrane peripheral structures as hSpry2 (Fig. 1E, middle panel), as is also seen by overlaying the two images (Fig. 1E, bottom panel).

The formation of membrane ruffles is dependent on the activation of the small G-protein Rac1 (16). To further confirm that hSpry2 is localized to membrane ruffles in EGF-stimulated cells, COS-1 cells were cotransfected with FLAG-tagged hSpry2 and hemagglutinin-tagged dominant negative Rac1 (N17). No formation of ruffles and no translocation of hSpry2 was observed in the cells when both constructs were co-expressed, indicating that hSpry2 translocated into newly formed Rac-dependent ruffles (data not shown).

From the collective evidence, we concluded that hSpry2 is associated with microtubules in quiescent cells, and it rapidly translocated to membrane ruffles when cells were stimulated with EGF. We also observed a similar but less profound translocation of hSpry2 with FGF stimulation in COS-1 cells. hSpry2 translocation was not cell-specific, as we also showed that the protein translocated to membrane ruffles in 293T cells when these cells were similarly transfected and stimulated with EGF or FGF (data not shown).

The Translocation of hSpry2 Is Independent of PI-3 Kinase Stimulation—The rapid translocation of the hSpry2 protein upon stimulation of cells with EGF is reminiscent of the rapid membrane translocation of various signaling proteins containing pleckstrin homology (PH) domains. PH domains target to membrane lipids containing products of PI-3 kinase, such as phosphatidylinositol 3,4,5-trisphosphate (17). Similar stimulation-directed translocation has also been observed with proteins containing the FYVE domain, which also targets products of PI-3 kinase-activated catalysis (18). We asked whether full-length or C-terminal hSpry2 was targeted to similar lipid products in EGF stimulated cells. To investigate this possibility, COS-1 cells were transfected either with full-length hSpry2 or with Akt, which contains a PH domain, to compare their respective translocations as a function of time. Fig. 2A (top panels) shows the disposition of Akt for various times following EGF addition, compared with the location of hSpry2 as shown in Fig. 2A (bottom panels). Akt was detectable in a peripheral membrane location at the optimal time of 10 min after EGF addition, compared with a higher proportion in the cytosol at both earlier and later times. This is in accordance with the observations from other studies on PH domain translocation (19). In contrast, hSpry2 rapidly translocated to ruffles but essentially remained associated with those structures for at least 60 min.

In a parallel experiment, to assess the reversibility of any translocation, the cells were stimulated for 10 min with EGF, following which they were extensively washed before serum-free medium was added again to the cells. Even when EGF was removed after 10 min of stimulation, hSpry2 remained associated with ruffles for at least another 60 min (Fig. 2A, bottom right panel), whereas Akt relocated into the cytosol soon after EGF was removed and was predominantly present in the cytosol at 60 min (Fig. 2A, top right panel).

Although the kinetics of hSpry2 translocation does not appear to be similar to a prototypical PH domain-containing protein, we asked whether the translocation could be abrogated by the PI-3 kinase inhibitor wortmannin. COS-1 overexpressing hSpry2 or Akt were treated with either 50 or 100 nM wortmannin for 10 min before cells were either left unstimulated or treated with EGF. Fig. 2B shows the disposition of Akt (left panel) compared with the location of hSpry2 (right panel). In both cases, the cells had been treated with 100 nM wortmannin prior to EGF stimulation. Although Akt appeared to be predominantly located in the cytosol, hSpry2 still showed a profound ruffles location (compare the respective 10 min time points in Fig. 2A that show the disposition of the proteins following EGF stimulation without the inhibitor).

Parallel experiments were conducted on cells to show that the doses of wortmannin employed effectively inhibited PI-3
kinase activity in these experiments. It has been established that the phosphorylation and subsequent activation of Akt kinase occurs downstream of PI-3 kinase (20). We assessed the activation status of Akt by probing for phosphorylation of the kinase in whole cell lysates using anti-phospho-Akt antibodies. In Fig. 2C, phosphorylation of Akt is not apparent in lane 1 (unstimulated) but is apparent in lane 2 (EGF stimulated). The phosphorylation of Akt upon EGF stimulation was slightly decreased with 50 nM wortmannin (lane 3) but was almost completely abrogated with 100 nM wortmannin treatment (lane 4). The blot was then stripped and reprobed with Akt antibody to demonstrate equal loading of the protein in each lane (data not shown). It can therefore be concluded that the translocation of hSpry2 was independent of PI-3 kinase activation.

Based on the kinetics of translocation, the disposition of hSpry2 was more reminiscent of the translocation seen with various proteins containing a C2 domain. This domain, found in the conventional PKCs (PKCβII, for example) and a variety of other proteins, targets the proteins to diacylglycerol, another lipid-derived product of RTK stimulation (21, 22). Various phorbol esters mimic the presence of diacylglycerol in membranes and application of these tumor promoters to cells can result in the translocation of certain members of the PKC family. We therefore investigated whether the prototypical tumor promoting phorbol ester, PMA, could induce the translocation of hSpry2 to membrane structures. In a parallel study, COS-1 cells were transfected with either hSpry2 or with PKCβII. It can be seen in Fig. 2D (top panels) that PKCβII translocated to a peripheral membrane location following PMA treatment for 10 min. It is noteworthy that only about 10% of the transfected cells showed the degree of translocation illustrated and that the translocation destination was not characteristic of ruffles. Interestingly, hSpry2 also showed a weak peripheral membrane location following 10 min of PMA stimulation to an extent similar to that of PKCβII but significantly less than that for EGF stimulation (Fig. 2D, bottom panels). Although hSpry2 assumed a weak membrane peripheral location following PMA stimulation, it did not have the typical wavy ruffles appearance that can be seen following EGF stimulation. In essence, it seemed that diacylglycerol, or its mimic PMA, was unlikely to be the target for the translocation of hSpry2 upon EGF stimulation. Likewise, there was no trans-
location of hSpry2 when the calcium ionophore ionomycin, 2.5 μM was used as stimulating agent, and when ionomycin, 2.5 μM was added in conjunction with PMA there was no enhancement of effect (data not shown).

At Least Two Regions on hSpry2 Are Required for the Association with Microtubules, and a Highly Conserved C-terminal Domain Is Required for Ligand-stimulated Translocation—To investigate the domains responsible for directing hSpry2 to microtubules and ruffles, a series of deletion mutants was constructed, as shown in Fig. 3. These mutants, which included two N-terminal deletions, Δ53–122 and Δ123–177, as well as three C-terminal deletions, Δ178–194, Δ195–221, and Δ265–282, were transfected into COS-1 cells. Cells were either left untreated or stimulated with EGF (50 ng/ml) for 10 min. Immunofluorescence studies were performed using monoclonal anti-FLAG to detect transfected hSpry2.

Fig. 3. Effect of deletions on the localization of hSpry2. COS-1 cells were transfected with FLAG-tagged hSpry2 deletion mutants Δ53–122, Δ123–177, Δ178–194, Δ195–221, or Δ265–282. A, lysates were analyzed by 10% SDS-PAGE and probed with PY20 antibodies to detect tyrosine-phosphorylated EGF receptors (top panel) or anti-FLAG to determine the expression levels of FLAG-tagged-hSpry2 (bottom panel). B, the filled bar represents the conserved, cysteine-rich domain, and the open bar denotes the nonconserved N-terminal sequence. Serum-depleted cells were either left untreated (0) or stimulated with EGF (50 ng/ml) for 10 min. Immunofluorescence studies were performed using monoclonal anti-FLAG to probe for transfected hSpry2.

FIG. 4. Minimum amino acid sequence required for membrane ruffle translocation. COS-1 cells were transfected with FLAG-tagged hSpry2 N-terminal or C-terminal fragments or with a series of C-terminal deletion mutants: amino acids 178–282, 178–237, or 178–229, as indicated. A, lysates were analyzed by 15% SDS-PAGE and probed with PY20 antibodies to detect tyrosine-phosphorylated EGF receptors (top panel) or anti-FLAG to determine the expression levels of FLAG-tagged-hSpry2 (bottom panels). B, serum-depleted cells were either left untreated (0) or stimulated with EGF (50 ng/ml). Immunofluorescence studies were performed using monoclonal anti-FLAG as a probe to detect transfected hSpry2 fragments.
locate tyrosine-phosphorylated EGF receptors (Fig. 3A, top panel). The expression of the various mutants was shown to be approximately equivalent and intact following EGF stimulation as assessed by Western blotting with FLAG monoclonal antibodies (Fig. 3A, bottom panel). It is noteworthy that the Δ53–122 hSpry2 protein ran anomalously when subjected to SDS-PAGE in that it consistently migrated at the same rate as the full-length hSpry2 protein.

From the immunofluorescence analysis (Fig. 3B), the disposition of Δ53–122 (top panels) was similar to that of full-length hSpry2 in both stimulated and unstimulated cells, indicating that this region of the protein was not involved in determining the subcellular localization. In contrast, both Δ123–177 and Δ195–221 (second and fourth rows of panels) showed no colocalization with the microtubules. This suggested that the association of hSpry2 with tubulin was via at least two distinct regions, one from each half of the protein.

We were particularly interested in defining the region on hSpry2 that was responsible for the translocation of the protein to a membrane ruffles location. From Fig. 3B, it can be seen that both Δ178–194 and Δ195–221 (third and fourth rows of panels) failed to translocate to the membrane ruffles upon EGF stimulation. Δ265–282 (bottom panels) translocated to ruffles upon stimulation but was less effective than full-length hSpry2 (see Fig. 1B). It thus appeared that the region of C-terminal hSpry2 from 178–221 contained a novel membrane-targeting domain.

The Translocation of hSpry2 Is Determined by a Highly Conserved C-terminal Amino Acid Sequence—To define the region on hSpry2 that was responsible for the growth factor-stimulated translocation to membrane ruffles, we first investigated the ability of either the N- or C-terminal half of the protein to translocate to ruffles. Constructs were made as shown in Fig. 4 and transfected into COS-1 cells. Control experiments to demonstrate EGF activation and approximate equality and integrity of the expression levels of the various hSpry2 constructs were performed as described above and are shown in Fig. 4A. The illustrated experiment demonstrated that the protein product from each construct migrated at the expected molecular weight and retained these physical characteristics following EGF stimulation. The immunofluorescence experiments resulting from the expression of the various constructs is shown in Fig. 4B. The N-terminal half of hSpry2 (N-hSpry2) in unstimulated and EGF stimulated cells is distributed randomly throughout the cytosol. In contrast, the C-terminal fragment (C-hSpry2), although it exhibits a random cytosolic distribution in unstimulated cells, translocated to membrane ruffles when cells were activated by EGF. It is interesting to note that neither N-terminal nor C-terminal fragments of hSpry2 associated with microtubules in unstimulated cells. This observation is in line with the results described above that demonstrated that the binding of hSpry2 to microtubules is determined by sites on both the N- and C-terminal fragments (see Fig. 3B).

From the disposition of the N- and C-terminal fragments of hSpry2, it was concluded that the translocation domain of hSpry2 resides in the C-terminal region from amino acids 178–282. In order to investigate the minimal translocating sequence a series of truncated C-terminal hSpry2 mutants were made from amino acid 282 back toward 178 and tested for their ability to translocate to membrane ruffles (Fig. 4B, bottom three panels). The shortest sequence required for optimal translocation was determined to be a 105 aa-fragment, 178–282 (third panel from top). Diminished translocation was observed with smaller fragments (residues 178–266, 178–252 (images not shown) and 178–237 (Fig. 4B, fourth panel from top)). Fragments containing smaller sequences, however, failed to translocate (residues 178–229, Fig. 4B, bottom panel).

The Optimal Translocation Domain in hSpry2 Is Highly Conserved in Other Sprouty Proteins—We next investigated whether the minimal "translocation sequence" was conserved in the other Sprouty sequences. When compared with the other Sprouty sequences, the optimal and minimal translocation sequences from hSpry2 are very highly conserved from Drosophila through the mammalian Sprouty homologues (Fig. 5). The Sprouty translocation domain (SpryTD) (i.e. the 105-amino acid fragment from residues 178–282) sequence is currently not found in other protein sequences in the data bases and may therefore represent a novel translocation sequence.

Ectopically Expressed Marine Sprouty1 and Sprouty4 and Drosophila Sprouty Also Translocate to Ruffles upon RTK Activation—We next investigated the disposition of other Sprouty homologues to see whether they also showed similar translocation profiles to hSpry2. Constructs of FLAG-tagged hSpry2, mSpry1, mSpry4, or dSpry were transfected into COS-1 cells that were stimulated with EGF or left unstimulated. An initial experiment, as described above, was performed to demonstrate expression levels of the various constructs as well as the level of EGF-induced tyrosine phosphorylation (Fig. 6A). The protein levels of the various Sprouty proteins were approximately equivalent, and the predicted molecular weights did not vary after EGF stimulation. From the immunofluorescence data obtained, the most significant feature, shown in Fig. 6B, is that all Spry proteins translocated to membrane ruffles upon EGF.
stimulation. It is interesting to note that neither mSpry1, mSpry4, nor dSpry interacted with microtubules or any obvious cytoskeletal structure. This is not surprising because the binding of hSpry2 to microtubules was manifested to a significant degree by the nonconserved N-terminal end of the protein, as seen in Fig. 3B.

These data indicate that like hSpry2, both mouse proteins and Drosophila Sprouty are likely to translocate to membrane locations (ruffles) following stimulation of RTKs in the respective organisms.

DISCUSSION

We have demonstrated that a highly conserved region in the C-terminal half of human Sprouty2 protein is responsible for translocating it from the cytosol to a membrane peripheral ruffles structure when receptor tyrosine kinases are activated. This cysteine-rich domain has currently been found in only four protein isotypes, and each of these shows similar translocation from the cytosol to membrane ruffles when the proteins are expressed in COS-1 cells followed by stimulation with EGF. These proteins, although all designated as Sprouty proteins, may have different functions from each other, although evidence suggests that dSpry and mSpry2 have parallel functions in developing trachea in Drosophila and mice, respectively (13).

A number of proteins involved in various signal transduction systems have been shown to translocate to cell membrane components upon cytokine or growth factor stimulation. Various domains on these proteins that are responsible for the observed relocation have been identified. The best characterized translocation sequences are the PH, FYVE, and C2 domains. The PH (23, 24) and FYVE (reviewed in Ref. 25) domains are found on a number of diverse proteins and mainly target to derivatives of the polyphosphorylated inositol lipids that are formed when PI-3 kinase is activated. The activation of PI-3 kinase is a feature of RTK and some cytokine receptor stimulation (26, 27). The C2 domain was first discovered on members of the PKC family (28). This relatively cysteine-rich domain has been identified to target proteins to the membrane when diacylglycerol or intracellular calcium levels increase. Diacylglycerol is produced by various phospholipase enzymes, most notably by phospholipase Cγ when RTKs are activated. There appears to be a common theme that these well characterized domains target proteins to membranes via newly formed lipid products. The transient appearance of activation-derived lipid products constitutes an elegant means to rapidly and strategically target key signaling proteins to the membrane.

Although the Spry translocation domain is not dependent on products of PI-3 kinase activation, the temporal disposition of hSpry2 was also unlike that of proteins bearing PH domains, the association of which with the membrane is relatively brief. The time course of SpryTD domains associating with the membrane is more reminiscent of that of proteins containing C2 domains. The C2 domain notably also contains a relatively high proportion of cysteine residues, although there is no noticeable conservation between the spacing of the cysteines in SpryTD and C2 domains.

It is possible that translocation of proteins to the plasma membrane occurs by directed targeting to other plasma membrane proteins or cortical cytoskeletal components. Both cortactin and Eps8 are reported to translocate to ruffles from a cytosolic location when quiescent cells are stimulated with growth factors (29, 30). Neither of these proteins contains PH or C2 domains, although both have been implicated in cytoskeletal reorganization and cortactin is believed to bind directly to actin (30, 31). We tested the possibility that SpryTD targets cortical cytoskeletal components by investigating whether the addition of cytochalasin D, an inhibitor of actin polymerization,
would inhibit RTK-induced translocation. Although it inhibited
the formation of ruffles, cytochalasin D did not inhibit the translocation
of hSpry2 to the plasma membrane, which indicates an independence from actin-associated components. Furthermore, although hSpry2 associates with microtubules in unstimulated cells, neither mSpry1 or mSpry4 appears to associate with any cytoskeletal structure in unstimulated cells, yet they all translocate to ruffles upon stimulation of RTKs. There is also no tubulin apparent in ruffles after EGF stimulation, and nocodazole treatment does not inhibit the translocation of SpryTD to the plasma membrane. Taking all of the evidence together, it appears unlikely that Spry cotranslocates along with components of microtubules or microfilaments.

The function of membrane ruffles is not clear. It is one of the earliest physiological responses seen with a number of growth factors, cytokines, and chemotactants. These cell protrusions contain a meshwork of newly polymerized actin, and their formation has been shown to be dependent on small GTP-binding protein Rac (16). A number of key signal transducing proteins have also been implicated in the membrane-ruffling response, including Ras, Grb2, PI-3 kinase, phospholipase Cγ, phospholipase A2 and phorbol ester responsive proteins (Refs. 32 and 33; reviewed in Ref. 16). Changes in polyphosphoinositide metabolism and intracellular Ca2+ levels may also play a role (33–36). It will be interesting to see what the SpryTD is actually targeting to in these ruffled structures. We have shown that it is unlikely to be a lipid product of PI-3 kinase and that phorbol ester induces some translocation, although not apparently similar to that stimulated by RTKs. The translocation induced by phorbol esters could be provoked by the direct binding to these membrane-intercalating diacylglycerol mimics or could be a corollary of phorbol esters inducing Rac activation (37, 38). The target is clearly produced rapidly upon activation of RTKs. Based on precedent, lipid derivatives are the most likely, and if a protein were to be the target, a cryptic binding domain would have to be revealed consequent to RTK stimulation. Further investigations will focus on whether Spry targets lipids or proteins. It remains to be seen whether the translocation of the Spry proteins is directly involved with the inhibition of RTK signaling. A number of signaling proteins have been shown to be concentrated in membrane ruffles and it is possible that the various Sproutsys and their associated proteins are brought into close proximity with target proteins in this membrane structure following stimulation.

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