The C Terminus of Sprouty Is Important for Modulation of Cellular Migration and Proliferation*

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The Drosophila Sprouty (SPRY) protein has been shown to inhibit the actions of epidermal growth factor and fibroblast growth factor. However, the role of mammalian SPRY proteins has not been clearly elucidated. We postulated that human Sprouty 2 (hSPRY2) is an inhibitor of cellular migration and proliferation. Indeed, using stably transfected HeLa cells, which expressed hemagglutinin (HA)-tagged hSPRY2 or hSPRY2 tagged at the C terminus with red fluorescent protein, we demonstrated that hSPRY2 inhibits the migration of cells in response to serum, epidermal growth factor, fibroblast growth factor, and platelet-derived growth factor. Additionally, hSPRY2 also inhibited the growth of HeLa cells in response to serum. Previously, two C-terminal domains on hSPRY2, which are necessary for its colocalization with microtubules (residues 123–177) or translocation to membrane ruffles (residues 178–194), have been identified (Lim, J., Wong, E. S., Ong, S. H., Yusoff, P., Low, B. C., and Guy, G. R. (2000) J. Biol. Chem. 275, 32837–32845). Therefore, using TAT-tagged hSPRY2 and its mutants, we determined the role of these two C-terminal domains in the inhibition of cell migration and proliferation. Our data show that the deletion of either of these two regions in hSPRY2 abrogates its ability to modulate cell migration in response to different growth factors and proliferation in response to serum. Therefore, we conclude that hSPRY2 inhibits the actions of a number of growth factors, and its C terminus, which is homologous among various SPRY isoforms, is important in mediating its biological activity.

In Drosophila, Sprouty has been demonstrated to be important in regulating fibroblast growth factor (FGF) and epidermal growth factor (EGF)-mediated cellular actions. Thus, in Drosophila Sprouty mutants, the fibroblast growth factor pathway is overactive, and excessive branching is seen in Drosophila airways (1). Kramer et al. (2) have shown antagonistic actions of Sprouty on both the FGF and EGF signaling pathways in Drosophila, where loss of Sprouty results in supernumerary neurons and glia. In addition, overexpression of Sprouty in wing veins and ovarian follicle cells, tissues where EGF signaling is required for proper patterning, results in phenotypes that resemble the loss-of-function phenotypes of EGF receptors (3, 4). Sprouty has also been shown to interfere with chick embryo development. Hence, infection of the prospective wing territory with a retrovirus containing the Sprouty gene inhibits proper limb growth and formation in the embryonic chick (5). Taken together, these results suggest that Sprouty acts as an antagonist of both FGF and EGF receptor signaling pathways.

To date, four isoforms of mammalian Sprouty protein have been described (SPRY1–SPRY4) (1, 4-6). Among these, only partial clones of SPRY1 and SPRY3 are available. The mouse as well as human SPRY2 (hSPRY2) and mouse SPRY4 have been cloned (4, 6, 7), and both these proteins share considerable sequence homology in the C terminus. However, their N termini are different. By Northern analysis the mouse SPRY2 mRNA is most abundant in brain, lung, and heart followed by kidney and skeletal muscle (7). Despite their role in Drosophila and chick development, relatively little is known about the biological actions of the mammalian Sprouty proteins. Recently, one study (8) has demonstrated that upon activation of cells by EGF, hSPRY2 is translocated from the vicinity of microtubules to membrane ruffles. Moreover, this translocation of hSPRY2 from microtubules to the membrane ruffles is dependent upon particular portions of the C terminus. However, the functional role of hSPRY2, its colocalization with tubulin in microtubules, and its translocation remain to be described. Therefore, the purpose of this study was to determine the functional role of hSPRY2 in growth factor actions and determine whether the translocation sequence on this protein alters its activity. Our data show that in cells that are either transfected or transduced to express hSPRY2, the migration in response to serum and a variety of growth factors is attenuated. Additionally, serum-induced proliferation of cells expressing hSPRY2 is also markedly inhibited. Moreover, our data show that deletion of regions in the C terminus, which has been shown to disrupt either the colocalization of hSPRY2 with tubulin or its translocation to membrane ruffles, obliterates the biological actions of the protein.

MATERIALS AND METHODS

Plasmid Constructs—Full-length hSPRY2 was cloned from two expressed sequence tag clones (accession numbers AA305092 and AA431912) using the unique AflII site in their overlapping sequence. The full-length clone was polymerase chain reaction (PCR)-amplified with a forward primer containing KpnI and BamHI sites (5-TATATATA GGT ACC GGA TCC CAC GGC AGA CAG GCT AGT GGC-3) and a reverse primer containing a stop codon as well as XhoI and EcoRI sites (5-ATATAT GAAATC TTGACG TTA GTA TGT TGG TTT TCC AAA GGT CCT-3). This PCR product was digested with KpnI and EcoRI and
ligated to the KpnI/EcoRI site of pRSET B. To generate a construct to express hemagglutinin (HA)-hSPRY2, the full-length hSPRY2 cDNA from pRSET B was digested with KpnI and subcloned into the KpnI/EcoRI sites of HA-tag-containing mammalian expression vector pHM6 (Roche Molecular Biochemicals).

To construct a TAT-hSPRY2 expression plasmid, the hSPRY2 cDNA in pRSET B was digested with KpnI and subcloned into the KpnI/EcoRI site of pTAT-HA bacterial expression vector (gift from Dr. Steven Dowdy, Washington University School of Medicine, St. Louis, MO). Similarly, the cDNAs encoding hSPRY2 deletion mutants (Δ123-177 and Δ178-194), which were generated by PCR, were subcloned into the pTAT-HA expression vector using KpnI and EcoRI restriction sites. To express the C-terminal-fragment tagged hSPRY2, the full-length clone of hSPRY2 was amplified by PCR with a forward primer containing a Xhol site (5'-TATATACTCGAGATGCAGGCCAGAAGCT-3) and a KpnI site containing reverse primer (5'-ATATAGGTACCCTTTTCAAAAGT-3) devoid of a stop codon. The PCR product was digested with Xhol and KpnI and inserted into the Xhol/KpnI sites of the RFP expression mammalian vector pDsRed1-N1 (CLONTECH). All hSPRY2 constructs were sequenced fully for tag and sequence verification.

Selection of Stable Clones—HeLa cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum without antibiotics for 2 days. The cells were transfected with plasmid constructs encoding HA-hSPRY2, hSPRY2-RFP, RFP alone, or vectors alone using the FuGENE transfection reagent (Roche Molecular Biochemicals). Twenty-four hours later, cells were grown in media supplemented with the antibiotic Geneticin (G418, BioWhittaker) at 200-800 μg/ml, and selection of clonal cell lines was initiated. Individual G418-resistant clones were expanded and maintained in 800 μg/ml G418. Overexpression of HA-hSPRY2 was confirmed by Western blotting and immunofluorescence. Similarly, overexpression of hSPRY2-RFP was confirmed by fluorescence.

Expression and Purification of TAT-GFP and TAT-HA-hSPRY2—The TAT-HA tag encodes for an N-terminal hexahistidyl leader followed by the 11-amino acid TAT protein transduction domain (YGRKKRRQRRR) and an HA tag (YPYDVPDYA) (9, 10). The TAT-HA-hSPRY2 protein was expressed in BL21(DE3)pLys strain of E. coli by induction with IPTG (0.4 mM) and overnight growth at 37 °C. TAT fusion proteins were purified after lysis of E. coli with 6 M guanidinium hydrochloride. Cellular lysates were resolved by centrifugation, loaded on a nickel-nitrilotriacetic acid column, washed successively with buffer (20 mM Tris HCl, pH 7.5) containing 8 M urea, 4 M urea, and buffer alone. Proteins were eluted with an imidazole gradient (40-500 mM) in the same buffer containing 1 M NaCl. Fractions containing TAT-HA-hSPRY2 were collected and pooled. This pool of TAT-HA-hSPRY2 was then desalted on a PD-10 column (Amersham Pharmacia Biotech) into phosphate-buffered saline (PBS). After determining protein concentration, TAT-HA-hSPRY2 was stored in 10% glycerol at −80 °C. In order to minimize antibody recognition, cells were treated with serum-free medium containing 0.1% bovine serum albumin and mounted.

Immunofluorescence—Cells were grown on glass coverslips to ~75% confluence. The cells were then washed with PBS and fixed by exposure to 4% paraformaldehyde in PBS for 10 min at room temperature followed by 5% Triton X-100 in PBS for 15 min at room temperature. After three washes with PBS, the coverslips were bleached overnight with 1% bovine serum albumin in PBS. The coverslips were exposed to a 1:1000 dilution of anti-HA antibody for 1 h at 37 °C followed by incubation with a 1:500 dilution of fluorescein isothiocyanate-labeled goat anti-mouse secondary antibody (Jackson Laboratories) for 1 h at 37 °C. Cells were then washed with PBS containing 1% bovine serum albumin and mounted.

RESULTS AND DISCUSSION

Initially, we transfected HeLa cells with plasmid encoding either HA-hSPRY2 or hSPRY2-RFP and selected several clones. Several G418-resistant clones were isolated. Fig. 1A shows a Western blot of several stable cell lines which either expressed HA-hSPRY2 (clones 3 and 9) or were G418-resistant.
but did not express HA-hSPRY2 (clones 5 and 8). Fluorescence microscopy was also used to confirm the presence of HA-hSPRY2 by immunocytochemistry (Fig. 1B). Similarly, as assessed by fluorescence microscopy, several clones expressing hSPRY2-RFP were also isolated. An example is shown in Fig. 1B with hSPRY2-RFP. Compared with control cells expressing RFP alone, hSPRY2-RFP was exclusively located in the cytoplasm (Fig. 1B); RFP was distributed in both the nuclei and the cytoplasm. This would suggest that the hSPRY2 is responsible for the differential localization of RFP and hSPRY2-RFP (Fig. 1B). This contention is supported by the similar localization of HA-hSPRY2 (Fig. 1B). Lim et al. (8) have demonstrated that in COS-1 cells, hSPRY2 is localized in proximity to the microtubules, resulting in similar staining. Thus, the localization of the tagged hSPRY2 proteins in our cells is similar to that reported previously in Lim et al. (8). Moreover, our data (Fig. 1B) demonstrate that neither the N terminus (HA) tag nor the C terminus (RFP) tag interferes with the location of hSPRY2 in cells.

Because the Drosophila SPRY has been shown to be important for tracheal formation and branching by opposing the actions of EGF and FGF (2), we postulated that hSPRY2 would inhibit growth factor actions and disrupt the migration of HeLa cells. For this purpose, we utilized the scratch wound assay. As shown in Fig. 2A, control cells, which were G418-resistant but did not express hSPRY2 (e.g. HA-control, Fig. 1A), did not migrate in serum-free medium but demonstrated increased migration when exposed to either 10% serum or 100 nM EGF. Similar results (not shown) were obtained with a second clone, which does not express HA-hSPRY2 (Fig. 1A). In contrast to these findings, however, when cells expressing HA-hSPRY2 were exposed to either serum or EGF, no migration was observed (Fig. 2A). Results similar to those shown for HA-hSPRY2 (Fig. 2A) were also observed with another clone, which also expresses HA-hSPRY2 (not shown). In additional migration assays, the ability of hSPRY2 to modulate the migration of HeLa cells in response to a variety of growth factors was tested. As shown in Fig. 2B, in control G418-resistant cells not expressing HA-hSPRY2, serum, EGF, PDGF, and FGF markedly enhanced migration of HeLa cells. However, in HA-hSPRY2-expressing cells, migration in response to serum, EGF, PDGF, and FGF was inhibited (Fig. 2B). Likewise, in hSPRY2-RFP-expressing cells, but not in control cells expressing RFP alone, migration in response to serum, EGF, PDGF, and FGF was inhibited (Fig. 2C). These data demonstrate that hSPRY2 opposes the migration-enhancing actions of a number of growth factors in HeLa cells. Moreover, the data in Fig. 2 show that the N- and C-terminal tags in SPRY do not interfere with its ability to inhibit migration in response to a variety of stimuli.

Because hSPRY2 inhibited migration in response to a number of mitogenic growth factors and serum, we assessed the effects of hSPRY2 on proliferation of HeLa cells in response to serum. In HA-hSPRY2- (Fig. 3A) and hSPRY2-RFP-expressing cells (Fig. 3B), the ability of serum to induce growth was markedly attenuated. Therefore, as observed with the cell migration assay, the N- and C-terminal tags do not alter the ability of hSPRY2 to inhibit cell proliferation.

Lim et al. (8) have shown that in COS-1 cells growth factors such as EGF translocate hSPRY2 from the proximity of microtubules to membrane ruffles. Moreover, these authors demonstrated that the deletion of amino acids 178-194 in hSPRY2 abolishes this translocation without changing the colocalization of hSPRY2 in the proximity of microtubules (8). On the other hand, deletion of amino acids 123-177 selectively disrupts the colocalization of hSPRY2 with tubulin without altering its translocation in response to growth factor (8). Therefore, using the deletion mutants described by Lim et al. (8) we investigated whether the regions of hSPRY2 that are important for colocalization with tubulin and translocation are important for the functional effects of hSPRY2 on cell migration and proliferation. To facilitate these studies, we employed the approach of transducing proteins into cells. It has been demonstrated that the addition of an 11-amino acid sequence from the TAT pro-
tein of human immunodeficiency virus in the N terminus of proteins permits their transport across cell membranes, and cells readily take up the protein of interest (9, 10, 14, 15). By this method, more than 50 proteins ranging in size from 15kDa to 115kDa have been shown to be successfully transduced into cells (14, 15). There are several advantages to this approach. First, transduction is achieved in nearly 100% of the cells (15) and data not shown). Therefore, one does not have to select clonal lines and assay these. Second, the proteins enter the cells very rapidly (9, 10, 14, 15). Thus, 10 min after the cells were exposed to TAT-HA-hSPRY2, the protein was detected inside the cells by Western analysis (Fig. 4A). Similarly, as monitored by fluorescence, fluorescein isothiocyanate-labeled TAT-HA-hSPRY2 and its C-terminal deletion mutants were observed inside the cells after exposing the cells to the protein for 30 min and up to 24 h (Fig. 4B). It should be noted that for the experiments in Fig. 4, A and B, the cells were extensively washed to remove the TAT-HA-hSPRY2 or its deletion mutants that may have adhered to the outside of the cells. Moreover, the proteins were visualized by confocal microscopy, and several “z” plane images demonstrated that the fluorescent tagged proteins were located in the cytoplasm (data not shown).

Next we investigated whether the TAT-HA-hSPRY2, like its FLAG-tagged counterpart (8), colocalized with microtubules and was translocated to membrane ruffles in the presence of growth factors such as EGF. We could not detect membrane ruffling in HeLa cells under any experimental condition, and therefore, these experiments were performed in COS-7 cells. COS-7 cells are identical to the COS-1 cells that Lim et al. (8) had used to characterize the cellular localization and translocation of hSPRY2. As shown in Fig. 5A, TAT-HA-hSPRY2 was colocalized with tubulin, a microtubule component. On the other hand, the deletion mutant TAT-HA-hSPRY2Δ123-177 was not colocalized with tubulin to the same extent as was the full-length TAT-HA-hSPRY2 (Fig. 5A). These results demonstrate that the TAT-tagged proteins localized like their FLAG-tagged counterparts (8). Moreover, after treatment with EGF for 10 min, the TAT-HA-hSPRY2, but not its deletion mutant TAT-HA-hSPRY2Δ178-194, translocated to membrane ruffles (Fig. 5B) as described for the FLAG-tagged hSPRY2 (8). In these latter experiments, translocation of filamentous actin to the membrane ruffles in response to EGF was used as a positive control (8). This control also permitted the colocalization of filamentous actin with TAT-HA-hSPRY2 in membrane ruffles to be discerned.

Having shown that the TAT-tagged hSPRY2 and its deletion mutants were behaving like their FLAG-tagged counterparts, we investigated whether these proteins alter migration and proliferation of HeLa cells. As demonstrated in Fig. 6A, in cells exposed to TAT-HA-hSPRY2, the migration in response to serum, EGF, PDGF, and FGF was markedly diminished compared with control cells, which were exposed to TAT-tagged GFP. These data demonstrate that the TAT-HA-hSPRY2 was inhibiting migration in the same manner as that observed with transfected HA-hSPRY2 (c.f. Figs. 2B and 6A). Additionally, because the TAT-GFP-transduced cells did not alter migration (c.f. controls in Figs. 2B and 6A), our data show that the effects of TAT-HA-hSPRY2 on cell migration cannot be attributed to the TAT tag. Most interestingly, our data demonstrated that

**Fig. 3.** HA-hSPRY2 and hSPRY2-RFP inhibit the proliferation of HeLa cells in response to serum. Panel A, HeLa cell lines which either expressed HA-hSPRY2 or did not express this protein were plated at a density of 10^5 cells per 60-mm dish and treated with 10% serum. Cell proliferation was monitored by counting the cells at 24, 48, and 72 h after plating the cells. The means ± S.E. from six experiments are presented. Panel B, same as A except that cells expressing hSPRY2-RFP or RFP alone (control) were used. The means ± S.E. from six experiments are presented. **,** p < 0.001; Student’s unpaired t test.

**Fig. 4.** Transduction of TAT-tagged hSPRY2 proteins in HeLa cells. Panel A, HeLa cells were transduced with TAT-HA-hSPRY2 for the times indicated. Cells were then washed extensively, and cell lysates were subjected to Western analysis with anti-HA antibody for the presence of TAT-HA-hSPRY2 protein. Panel B, HeLa cells were exposed to fluorescein isothiocyanate-labeled TAT-HA-hSPRY2 or its deletion mutants (1 μg of protein/ml) at 37 °C for the different times indicated. Cells were washed extensively at the end of the incubation and fixed as described under “Materials and Methods.” The fluorescein isothiocyanate-labeled hSPRY2 was detected by confocal fluorescence microscopy.
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Fig. 5. Intracellular localization of TAT-HA-hSPRY2 and its deletion mutants. COS-7 cells (10⁴ cells/cm²) were exposed to TAT-HA-hSPRY2 or its deletion mutants (10 μg of protein/ml) for 1 h in serum-free medium. Thereafter, the cells were washed with PBS and incubated in the presence or absence of EGF (100 nm) for 10 min. The cells were then fixed as described under “Materials and Methods” for immunocytochemistry. Panel A, TAT-HA-hSPRY2 and TAT-HA-hSPRY2Δ123–177 transduced COS-7 cells were exposed to anti-tubulin (1:100 dilution) and anti-HA antibodies to visualize tubulin and TAT-HA-hSPRY2 (and its mutant), respectively. The localization of tubulin (red) and TAT-tagged hSPRY2 and its Δ123–177 mutant (green) are shown separately and together. Panel B, COS-7 cells transduced with TAT-HA-hSPRY2 or its deletion mutant Δ178-194 were incubated in the presence (lower two rows) and absence (top row) of EGF (100 nm). After fixing (red) the cells were exposed to Texas red isothiocyanate-labeled phalloidin, (1:100 dilution), (red) to visualize filamentous actin and anti-HA antibody to visualize TAT-HA-tagged hSPRY2 and its deletion mutant (Δ178-194) (green). Note: in the presence of EGF, TAT-HA-hSPRY2Δ178-194 does not translocate to membrane ruffles.

when cells were transduced with either hSPRY2Δ123-177 or hSPRY2Δ178-194 the inhibitory effect of the protein on cell migration in response to growth factors and serum was obliterated. Because residues 123-177 and 178-194 in hSPRY2 are necessary for colocalization with microtubules and translocation to membrane ruffles, respectively (8), it would follow that the disruption of either of these functions obliterates the activity of SPRY as an inhibitor of cell migration in response to growth factors and serum.

To test the effects of the deletion mutants of hSPRY2 on cell proliferation, the experiment depicted in Fig. 6B was performed. Similar to our findings with the migration assay, in cells exposed to TAT-HA-hSPRY2 proliferation in response to serum was attenuated. This is similar to the findings with cells overexpressing HA-hSPRY2 and hSPRY2-RFP (Fig. 3). However, in cells transduced with hSPRY2Δ123-177 and hSPRY2Δ178-194, serum-induced cell growth was not affected. Therefore, as discussed above for cell migration, these latter data also demonstrate that the C-terminal regions encompassed by amino acids 123-177 and 178-194 in hSPRY2 are necessary to observe inhibition of cell growth.

Although SPRY has been shown to be important in modulating the actions of growth factors in the Drosophila and chicken, the role of SPRY2 in mammalian systems has remained unknown. More elusive has been the determination of the mechanism(s) and signaling pathways involved in the actions of SPRY. Therefore, the identification of hSPRY2 as a modulator of migration and proliferation is a starting point in the elucidation of the mechanisms underlying the actions of this protein. It has been shown previously that Drosophila SPRY associates with the Grb2 homologues Drk and Gab1 and that the Ras pathway below the activation of Ras may be the locus of SPRY action (3, 4). However, whether the mitogen-activated protein kinase pathway in mammalian cells is the target of hSPRY2 is a question that remains to be resolved. Interestingly, among the various isoforms of SPRY, the N-terminal domains are different, but the C terminus is similar. Because the C-terminal domains that are conserved are important in the actions of hSPRY2 as an inhibitor of cell migration and proliferation, it would appear that these functions may be
displayed by all isoforms of SPRY in different species. Indeed, lack of this inhibitory action in SPRY mutants of Drosophila would explain the excessive branching of trachea (1).

We used HeLa cells in our studies because we were interested in determining whether hSPRY2 inhibits the migration and/or proliferation of transformed carcinoma cells. Because the migration of HeLa cells may be an index of their metastatic capacity, it would be tempting to speculate that hSPRY2 may decrease the metastatic potential of cancer cells. Likewise, one would predict that in cells that proliferate and migrate very rapidly, the amount of hSPRY2 protein may be decreased. Presently, there are no antibodies available that can detect the endogenously expressed hSPRY2 in HeLa or other cell types. However, using reverse transcription-PCR methodology, we did detect endogenous hSPRY2 mRNA in HeLa cells (data not shown). Whether the amount of this transcript and its protein are regulated by specific growth factors or other stimuli remains to be determined.

In conclusion, our data demonstrate that hSPRY2 is an inhibitor of cell migration in response to a number of growth factors and also exerts anti-proliferative actions. These actions of hSPRY2 require the domains in the molecule that have been shown previously to be necessary for colocalization with tubulin in microtubules or translocation of hSPRY2 to the ruffles. The deletion of either of these latter domains of hSPRY2 obliterates its ability to inhibit migration and cell proliferation. Because hSPRY2 inhibits cell migration in response to several growth factors it appears to be a general inhibitor of receptor tyrosine kinases. Moreover, because the conserved C-terminal domains are important for SPRY function and because these regions are conserved in the different isoforms, our data suggest that all SPRY isoforms may inhibit migration and proliferation of cells.

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Note Added in Proof—After acceptance of this article for publication, two other groups have reported that Spry4 (Lee, S. H., Schloss, D. J., Jarvis, L., Krasnow, M. A., and Swain, J. L. (2001) J. Biol. Chem. 276, 4128–4133) and SPRY-1 and -2 (Impagnatiello, M. A., Weitzer, S., Gannon, G., Compagni, A., Cotten, M., and Christofori, G. (2001) J. Cell Biol. 152, 1087–1096) inhibit endothelial cell proliferation, migration, and differentiation. Thus the inhibition of cellular migration and proliferation by SPRY proteins may be a general phenomenon.

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