Mammalian Sprouty-1 and -2 Are Membrane-anchored Phosphoprotein Inhibitors of Growth Factor Signaling in Endothelial Cells

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Abstract. Growth factor–induced signaling by receptor tyrosine kinases (RTKs) plays a central role in embryonic development and in pathogenesis and, hence, is tightly controlled by several regulatory proteins. Recently, Sprouty, an inhibitor of *Drosophila* development-associated RTK signaling, has been discovered. Subsequently, four mammalian Sprouty homologues (Spry-1–4) have been identified. Here, we report the functional characterization of two of them, Spry-1 and -2, in endothelial cells. Overexpressed Spry-1 and -2 inhibit fibroblast growth factor– and vascular endothelial growth factor–induced proliferation and differentiation by repressing pathways leading to p42/44 mitogen-activating protein (MAP) kinase activation. In contrast, although epidermal growth factor–induced proliferation of endothelial cells was also inhibited by Spry-1 and -2,

activation of p42/44 MAP kinase was not affected. Biochemical and immunofluorescence analysis of endogenous and overexpressed Spry-1 and -2 reveal that both Spry-1 and -2 are anchored to membranes by palmitoylation and associate with caveolin-1 in perinuclear and vesicular structures. They are phosphorylated on serine residues and, upon growth factor stimulation, a subset is recruited to the leading edge of the plasma membrane. The data indicate that mammalian Spry-1 and -2 are membrane-anchored proteins that negatively regulate angiogenesis-associated RTK signaling, possibly in a RTK-specific fashion.

Key words: angiogenesis • endothelial cells • fibroblast growth factors • signal transduction • vascular endothelial growth factor

Introduction

Many organs, including lung, kidney, some glands, and in particular the vascular system, are composed of a network of tubes. The formation of these three-dimensional structures involves the coordination of several cellular processes, such as the specification of cell fate, changes in cell shape, migration, and proliferation (Metzger and Krasnow, 1999). Many of the molecular mechanisms that underlie the regulation of these complex processes have been elucidated by studying the development of *Drosophila melanogaster*. Normal development requires the spatial and temporal regulation of receptor tyrosine kinase (RTK)[1] activities and, recently, a novel negative regulator of RTK signaling, *Drosophila* Sprouty (DSpry), has been identified (Hacohen et al., 1998). During the formation of

the tracheal system, DSpry inhibits fibroblast growth factor (FGF) receptor–mediated branching of epithelial cells (Hacohen et al., 1998; Placzek and Skaer, 1999). DSpry appeared to block mainly branching of cells lateral to the FGF signaling center, and it was proposed that DSpry acts extracellularly as a secreted protein (Hacohen et al., 1998). More recently, however, Casci and co-workers (1999) have demonstrated that DSpry is an intracellular protein lacking a signal peptide for secretion. It is localized to the inner leaflet of the plasma membrane by its cysteine-rich domain, where it acts as an inhibitor of FGF receptor– and epidermal growth factor (EGF) receptor–transduced signals in wing morphogenesis (Casci et al., 1999). Moreover, it is found to associate with two intracellular *Drosophila* RTK signal transduction components, Gap1 and Drk, and an SH2-SH3 adaptor protein that is homologous to mammalian Grb-2, but not with Sos, Dos, Csw, Ras1, Raf, or Leo (Casci et al., 1999). Also, DSpry has been found to interfere with FGF receptor– and EGF receptor–mediated signaling during *Drosophila* eye development and oogenesis (Casci et al., 1999; Kramer et al., 1999; Reich et al., 1999). In the developing eye imaginal disc, the embryonic chordotonal organ precursors, and the midline glia, EGF

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[1]*Abbreviations used in this paper:* BCE, bovine capillary endothelial cell; DSpry, *Drosophila* Sprouty; EGF, epidermal growth factor; FGF, fibroblast growth factor; hSpry, human Sprouty; HUVEC, human umbilical vein endothelial cell; LDH, lactate dehydrogenase; MAP, mitogen-activating protein; MEK, MAP kinase kinase; mSpry, mouse Sprouty; PPC, particles per cell; RTK, receptor tyrosine kinase; VEGF, vascular EGF.

receptor signaling is part of the regulatory circuit to control the correct number of neurons and glia, and the loss of DSpry function results in supernumerary neurons and glia (Kramer et al., 1999). Since DSpry is also able to interfere with signaling originating from other RTKs in *Drosophila*, such as Torso and sevenless, it is thought to be a general inhibitor of RTK signal transduction (Casci et al., 1999; Reich et al., 1999). However, DSpry's mode of action has remained elusive. For example, during eye development, DSpry appears to inhibit p42/44 mitogen-activating protein (MAP) kinase activation at the level of Ras (Casci et al., 1999), whereas during wing development, it may interfere downstream of Raf (Reich et al., 1999).

Since RTK signaling pathways appear to be highly conserved during evolution, it was conceivable that, in analogy to DSpry, a mammalian Spry may also regulate RTK signaling during mammalian development and pathogenesis. Recently, three human, four murine, and two avian genes have been identified that encode protein homologues of DSpry (Hacohen et al., 1998; de Maximy et al., 1999; Minowada et al., 1999; Tefft et al., 1999; Chambers and Mason, 2000). Overexpression of mSpry (mouse Sprouty)-2 and -4 resulted in the repression of FGF-mediated limb development in chicken (Minowada et al., 1999), whereas ablation of Spry-2 expression in cultured embryonic mouse lungs lead to an increase in lung branching morphogenesis, a process that is thought to be induced by FGFs (Tefft et al., 1999). Moreover, similar to results obtained in *Drosophila* development (Hacohen et al., 1998), expression of mSprys and chicken Sprys is also upregulated by the FGF signaling pathway (Minowada et al., 1999; Chambers and Mason, 2000), suggesting a feedback loop involved in the regulation of growth factor–mediated signal transduction. However, as in *Drosophila*, the mammalian Sprys' physiological role and, in particular, the mechanisms by which they inhibit RTK signaling are also not understood in any detail.

Similar to the tracheal system in *Drosophila*, during the development of the cardiovascular system, the formation of new blood vessels from preexisting ones (angiogenesis) also involves sprouting of endothelial cells out of an epithelial layer and branching of tubular structures (Flamme et al., 1997). In the adult, angiogenesis only takes place during the female reproductive cycle, wound healing, and in pathological situations, including tumor growth, diabetic retinopathy, arthritis, atherosclerosis, and psoriasis (Folkman, 1995; Flamme et al., 1997; Risau, 1997). Angiogenesis is tightly regulated by a balance between inducing and inhibitory signals (Hanahan and Folkman, 1996; Hanahan, 1997). Peptide growth factors, such as vascular EGF (VEGF), FGF, and angiopoietins, by binding to their cognate RTKs, positively regulate angiogenesis by inducing endothelial cell proliferation, migration, differentiation, and survival (Hanahan, 1997; Gale and Yancopoulos, 1999). In contrast, factors that negatively regulate angiogenesis by specifically blocking RTK signaling are less well characterized.

Motivated by the intriguing similarities in cell biological processes and gene function between *Drosophila* trachea development and mammalian angiogenesis, we have investigated the functional role of mammalian Sproutys in endothelial cells. Our results demonstrate that Spry-1 and -2 inhibit FGF- and VEGF-induced endothelial cell proliferation and differentiation, at least in part, by repressing pathways leading to p42/44 MAP kinase activation. Our data also demonstrate that Spry-1 and -2 are anchored to

membranes by palmitoylation, posttranslationally modified by phosphorylation, and tightly associated with caveolin-1 in perinuclear and vesicular structures and in the plasma membrane. Moreover, their expression levels and their subcellular localization are modulated by growth factor stimulation. The results indicate that mammalian Sprys are membrane-anchored proteins that modulate RTK-mediated signal transduction in endothelial cells.

Materials and Methods

Cloning

Fragments of the cysteine-rich domains of mSpry-1–4 were amplified by PCR using primers derived from EST sequences homologous to human Sprouty (hSpry) and DSpry. The amplification products were used to screen an e14.5 mouse embryo cDNA library in λGT11 (CLONTECH Laboratories, Inc.) following the manufacturer's recommendations. For all mSprys, the nucleotide sequence of both strands of the coding region was determined.

Adenovirus Vectors

The cDNAs encoding mSpry-1 and -2 were subcloned and integrated into recombinant E1/E3 defective adenoviruses using homologous recombination in *Escherichia coli* as described (Chartier et al., 1996). All genes of interest were under control of the cytomegalovirus immediate early promoter, followed by a rabbit β-globin intron/polyadenylation signal. Virus cultures were initiated by transfecting the linearized genomes into 293 cells using polyethylenimine (Baker et al., 1997). After amplification of the culture, virus was purified by banding twice on CsCl gradients, transferred into HBS/40% glycerol by passage over a gel filtration column, and stored at −80°C as previously described (Michou et al., 1999). Viral quantitation was based on protein content using the conversion of 1 mg viral protein/3.4×10^{12} virus particles.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) and mouse microvascular endothelial cells (1G11) (Dong et al., 1997) were cultured in DME medium supplemented with 20% FCS (GIBCO BRL), 2 mM glutamine, 40 μg/ml bovine brain extract, 80 U/ml heparin, and antibiotics. The medium for bovine capillary endothelial cells (BCEs) was supplemented with 10% FCS and FGF2 (2.5 ng/ml). For viral infections, the culture medium was replaced with starvation medium (5% FCS for HUVECs, 2% FCS for BCEs, 0% for 1G11) containing 5,000 particles per cell (PPC). After 4 h, the medium was replaced with fresh growth medium.

DNA Synthesis Assay

Endothelial cells were plated in triplicate in 24-well dishes (30,000/well) and infected with adenoviruses (5,000 PPC) in reduced serum medium for 2–3 h. Cells were starved for 24 h in serum-free 1G11, 2% FCS medium (BCE), or 5% FCS medium (HUVEC), and then stimulated overnight with recombinant FGF2 (Promega) or VEGF (R&D Systems). After stimulation, 1 μCi of [³H]thymidine (Amersham Pharmacia Biotech) was added per well, and incorporation of thymidine was determined by scintillation counting as described (Shing et al., 1993).

Differentiation Assay

HUVECs were infected with 5,000 PPC of adenoviruses; after 4 d, they were resuspended in 5% FCS medium and plated in 24-well plates (40,000 cells/well) in the presence of growth factors on a thin layer of Matrigel (Becton Dickson) diluted 1:1 with 5% FCS medium.

Subcellular Fractionation

HUVECs (10^6) were infected with AdmSpry-1, or AdeGFP; and, after 48 h, the cells were extracted with 90 mM KAc, 2.5 mM $MgCl_2$, 1 mM EDTA, 0.2 mM $CaCl_2$, 12 mM glucose, 0.003% digitonin, 0.5 mM $NaVO_4$, 10 mM NaF, 10 mM Pefabloc, 1 mM aprotinin, 1 mM leupeptin. After 7 min on ice, the supernatant (cytoplasmic extract) was collected, and the remaining cells were reextracted with Na_2CO_3 (pH 11). After 10 min on ice, the supernatant (peripheral membrane protein 1) was collected. The remaining cells were homogenized in PBS and centrifuged at 1,000 *g* to

sediment nuclei and unbroken cells. Membranes in the postnuclear supernatant were pelleted at 100,000 g, and the supernatant was collected (peripheral membrane protein 2). The membrane pellet was extracted twice with Na_2CO_3 for 10 min on ice. Proteins in the aqueous phases (peripheral membrane proteins 3) were pooled with peripheral membrane protein fractions 1 and 2, precipitated as described (Oliferenko et al., 1999), and analyzed by SDS-PAGE and immunoblotting together with the proteins in the membrane pellet (integral membrane proteins).

Flotation Assay

Actively proliferating HUVECs infected with AdmSpry-1 or AdeGFP were washed twice with ice-cold PBS, removed from the plates by scraping in PBS, and centrifuged at 1,000 g at 4°C. Cells were lysed in 200 µl TN (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM DTT, protease inhibitors as above, 10% sucrose, and 1% Triton X-100) on ice and incubated for 30 min on ice. Samples were mixed with 400 µl ice-cold Optiprep™, transferred into SW60 centrifuge tubes, and overlaid with each 600 µl of 35, 30, 25, 20, and 0% Optiprep™ in TN. The gradients were centrifuged at 35,000 rpm in a SW60 rotor for 12 h at 4°C. Fractions were collected from top to bottom of the centrifuge tubes, and proteins were precipitated and analyzed by immunoblotting.

Immunoblotting

Total cell lysates, protein fractions, or immunoprecipitates was analyzed by SDS-PAGE and immunoblotting as described (Yu and Sato, 1999). Rabbit sera against mSpry-1 and -2 were generated with peptides corresponding to the NH_2-terminal residues AVEGRQRLDYDRDTQ of mSpry-1 and HERLHGLPEHRQPPRLQPSQVHSSRAP of mSpry-2 conjugated to keyhole limpet hemocyanin. Antibodies from rabbit sera were affinity purified on antigenic peptides coupled to Sepharose. The specificity of the antibodies against mSpry-1 and -2 was further confirmed by peptide competition experiments, as well as by excluding cross-reactivity with mSpry-4. The following antibodies against other proteins were used: p42/44 MAP kinase and total p42/44 MAP kinase (Sigma-Aldrich), activated MAP kinase kinase (MEK) and total MEK (New England Biolabs, Inc.), β-catenin and caveolin-1 (Signal Transduction Laboratories), lactate dehydrogenase (LDH) (Chemicon International Ltd.), annexin II (Santa Cruz Biotechnology, Inc.), transferrin receptor (a gift from L. Huber, Research Institute of Molecular Pathology, Vienna, Austria).

Immunofluorescence

HUVECs were plated on gelatine-coated glass coverslips and grown to 70% confluency. For the stimulation experiments, the cells were starved for 24 h and then stimulated with 10 ng/ml FGF2 for various times. The cells were rinsed twice with PBS, fixed with 3% paraformaldehyde in PBS for 20 min at room temperature, permeabilized with 0.3% Triton X-100 for 3 min on ice, and washed with PBS. After blocking first with 3% BSA/PBS and then with 1% goat serum/0.1% BSA/PBS, the cells were incubated with the primary antibody for 30 min at room temperature and were washed with 0.1% BSA/PBS, followed by 30 min incubation with a secondary antibody conjugated to a fluorochrome. After washing with 0.05% Tween-20 in 0.1% BSA/PBS, the coverslips were mounted onto slides in mounting medium. Mouse monoclonal antibodies against caveolin-1, syntaxin-4, ergic-53, giantin, protein disulfide isomerase, transferrin receptor, and VE–cadherin were from Signal Transduction Laboratories; secondary antibodies anti–rabbit or anti–mouse IgG (Cy2 or Cy3 conjugated) were from Jackson ImmunoResearch Laboratories.

Immunoprecipitations

HUVECs infected with AdmSpry-1, AdmSpry-2, or AdeGFP were lysed in different extraction buffers (150 mM NaCl, 10 mM Tris, 10 mM NaF, 2 mM Na_3VO_4, 10 mM Pefabloc, 1 mM aprotinin, 1 mM leupeptin, 1 mM DTT, 1% Triton X-100 or 1% digitonin). After 30 min incubation (at room temperature for the samples in 1% digitonin and on ice for the samples in Triton X-100), lysates were cleared by centrifugation (14,000 g for 30 min), and equal amounts of lysates were immunoprecipitated with anti-Spry antibodies and protein G agarose at 4°C. The immunoprecipitates were washed three times at room temperature with the corresponding lysis buffers; immunoprecipitated proteins were resolved by 12% SDS-PAGE.

Radiolabeling

HUVECs infected with AdmSpry-1 or -2 were metabolically labeled with [35S]methionine/[35S]cysteine (Amersham Life Science) in methionine and
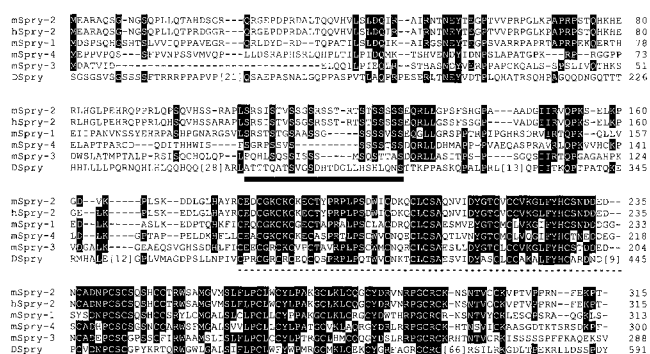

Figure 1. Amino acid alignment of the four mSpry proteins (mSpry-1–4) with hSpry-2 and DSpry. Dashes indicate spaces introduced to maximize alignment. Shaded residues indicate residues that are identical in at least four of the aligned sequences. A putative Sprouty domain and a putative serine-rich domain are underlined by a dotted line and a gray bar, respectively.

cysteine serum-free medium for the time indicated, or with [3H]palmitate (30–60 Ci/mmol; DuPont) in DME containing 10% FCS for 2 h. For orthophosphate labeling, serum-starved HUVECs overexpressing mSpry-1 or mSpry-2 were labeled for 4 h in phosphate-free medium containing 5% FCS and 1 mCi/ml [32P]orthophosphate (Amersham Life Science) and then stimulated with FGF2 (10 ng/ml). Cells were then lysed, and mSpry-1 and -2 were immunoprecipitated with affinity-purified antibodies against mSpry-1 and -2, resolved by SDS-PAGE, and visualized by autoradiography and fluorography, respectively. For phosphoamino acid analysis, the proteins were transferred to polyvinyldifluoride membranes and hydrolyzed in 6 M HCl for 60 min at 110°C. The hydrolytic products were separated in the presence of phosphoserine, phosphothreonine, and phosphotyrosine markers in two dimensions on TLC plates (pH 1.9 and 3.5).

Results

Cloning of mSpry cDNAs

ESTs that partially encoded mouse homologues of Sprouty were identified by their homology to hSpry sequences (Hacohen et al., 1998). DNA fragments were amplified by PCR and used to screen a cDNA library generated from gestation day 14.5 mouse embryos. Four different cDNAs were recovered that were highly homologous to DSpry and hSpry and that encoded mSpry-1–4 with calculated molecular masses of 34.4, 34.6, 31.6, and 32.6 kD. All four mSpry proteins contain in their COOH-terminal region a cysteine-rich region with at least 23 cysteines; in the alignment of mSpry, hSpry, and DSpry, this region shows the highest homology (Fig. 1). However, this highly conserved Sprouty domain does not exhibit a significant homology to known functional domains of other proteins. NH_2-terminal of the Sprouty domain, all Spry sequences analyzed contain a highly conserved serine-rich region (Fig. 1). No significant homologies between DSpry and mammalian Sprys are apparent at the extreme NH_2 and COOH termini. None of the mammalian Sprys contains predicted signal peptides for secretion or putative transmembrane domains.

mSpry-1 and -2 Inhibit Endothelial Cell Proliferation and Differentiation

Motivated by the similarity between the cellular processes involved in *Drosophila* trachea development and angiogenesis (see Introduction), we investigated the functional role of Sprys in endothelial cells. Northern hybridization
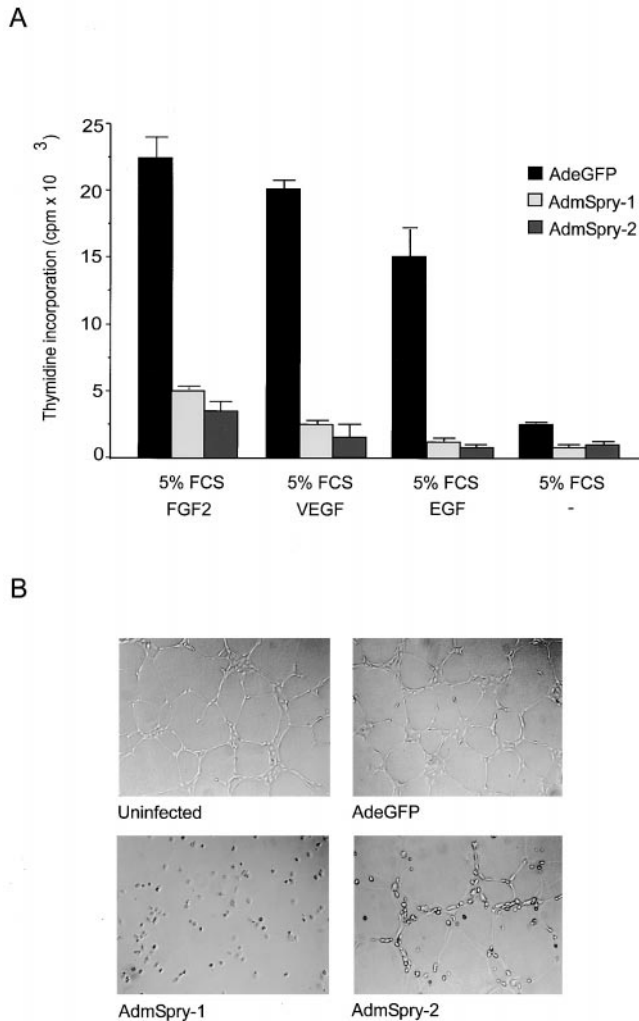
A

B

Figure 2. mSpry-1 and -2 inhibit endothelial cell proliferation and differentiation. (A) Endothelial cell proliferation. HUVECs were infected with 5,000 PPC of AdmSpry-1, AdmSpry-2, or AdeGFP as indicated. 4 h after infection, the cells were starved and then stimulated with 10 ng/ml FGF2, 10 ng/ml VEGF, or 20 ng/ml EGF. The rate of DNA synthesis was determined by the incorporation of [^{3}H]thymidine 48 h after infection. Error bars indicate the standard deviation on the average of three experiments. (B) Endothelial cell differentiation. HUVECs were infected with recombinant adenovirus as described in A and then plated on Matrigel. Representative microphotographs were taken 24 h after plating.

and reverse transcription–PCR analysis revealed that Spry-1, -2, and -4 were expressed in primary HUVECs and in mouse microvascular endothelial cells (1G11) (data not shown). To assess whether the expression of Sprys in endothelial cells could directly affect angiogenesis, we examined the influence of Spry expression on endothelial cell proliferation and differentiation, two processes that are central to angiogenesis. For these experiments, we used cultured primary endothelial cells that have not been immortalized, such as HUVECs and BCEs. To obtain high levels of Spry expression in endothelial cells, we generated recombinant replication-defective adenoviruses encoding for mSpry-1 and -2 (AdmSpry-1 and -2, respectively). After infection with adenovirus, HUVECs were stimulated
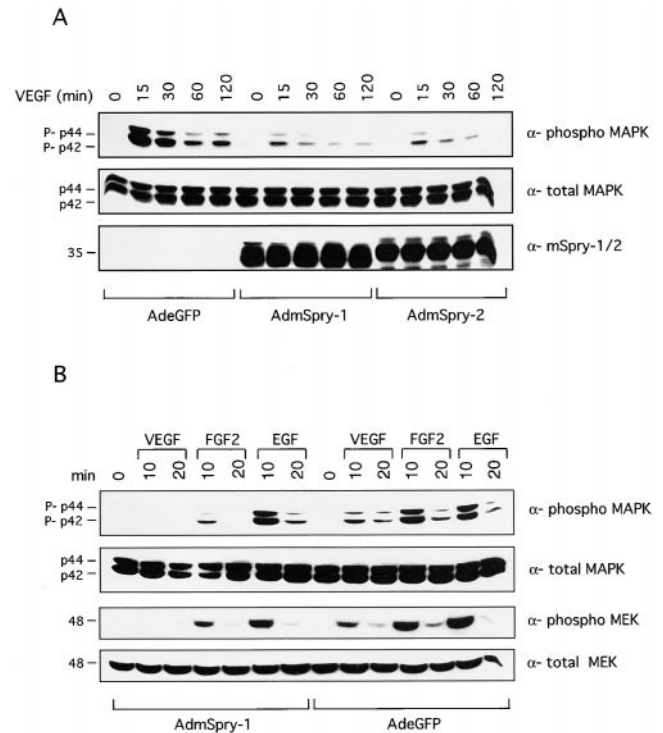

A

B

Figure 3. Inhibition of p42/44 MAP kinase activation. HUVECs infected with AdmSpry-1, AdmSpry-2, or AdeGFP were serum starved 24 h before stimulation with 50 ng/ml VEGF for various time periods as indicated (A) or with 50 ng/ml VEGF, 50 ng/ml FGF2, or 20 ng/ml EGF for 10 or 20 min (B). Cells were lysed, and levels of activated p42/44 MAP kinase, total p42/44 MAP kinase, activated MEK, and total MEK were determined by immunoblotting with specific antibodies as indicated. Expression levels of mSpry-1 and -2 were determined by stripping and reprobing the blots with antibodies specific for mSpry-1 or -2 (A).

with FGF2, VEGF, or EGF for various time periods. Subsequently, incorporation of [^{3}H]thymidine was used to determine the rate of DNA synthesis (Fig. 2 A), and cell numbers were determined to calculate proliferation rates (data not shown). DNA synthesis and proliferation rates induced by FGF2, VEGF, and EGF were dramatically reduced in HUVECs overexpressing mSpry-1 or -2, compared with cells that have been infected with a control virus expressing enhanced green fluorescent protein (Ade-GFP; Fig. 2 A). Similar results were obtained with BCEs (data not shown).

Endothelial cells differentiate into net-like structures when plated on Matrigel in the presence of growth factors, such as FGF1, FGF2, or VEGF (Baatout, 1997; Riccioni et al., 1998). This differentiation process can be observed within a few hours after stimulation and does not require endothelial cell proliferation. To determine whether overexpression of mSpry might affect endothelial cell differentiation, HUVECs were infected with AdmSpry-1, AdmSpry-2, or AdeGFP and subsequently plated on Matrigel in the presence of FGF2. Under these conditions, noninfected HUVECs or HUVECs infected with AdeGFP formed stable net-like structures (Fig. 2 B). In contrast, cells infected with AdmSpry-1 and -2 were significantly impaired in their ability to elongate and to form net-like structures (Fig. 2 B). VEGF-stimulated endothelial cell
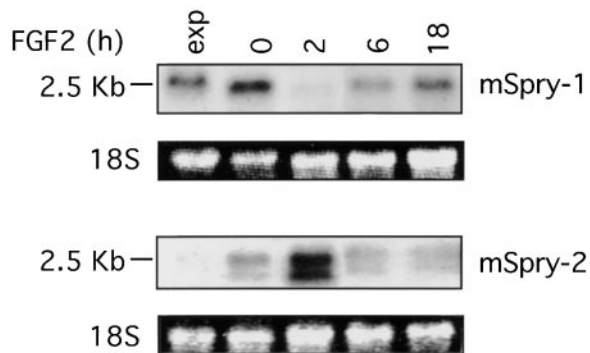
Figure 4. mSpry-1 and -2 expression is regulated by FGF2 in endothelial cells. Uninfected 1G11 cells were cultured under exponential growth conditions (exp) or serum starved for 48 h and then stimulated with 10 ng/ml FGF2 for the times indicated. Northern blot analysis was performed on total RNA isolated from each time point, using full-length cDNAs for mSpry-1 and mSpry–2 as probes. Equal loading of RNAs was confirmed by staining of the gels with ethidium bromide. The lengths of Spry mRNAs (Kb) and 18S ribosomal RNA are indicated.

differentiation was inhibited in a similar manner (data not shown). Together, the data demonstrate that overexpression of mSpry-1 or -2 inhibits growth factor–induced endothelial cell proliferation and differentiation. In these experimental settings, mSpry-2 proved to be a less potent inhibitor than mSpry-1.

mSpry-1 and -2 Inhibit MAP Kinase Activation

Previously, it has been reported that DSpry inhibits tyrosine kinase receptor signal transduction by inhibiting pathways that lead to the activation of p42/44 MAP kinase

(Reich et al., 1999). The mitogenic function of the angiogenic factors FGF and VEGF also requires the activation of p42/44 MAP kinase (Marshall, 1995; Yu and Sato, 1999). Thus, we investigated whether overexpression of mSpry is able to affect p42/44 MAP kinase activity. HUVECs were infected with AdmSpry-1, AdmSpry-2, or AdeGFP, and, after serum starvation, the cells were stimulated with VEGF (50 ng/ml) for different times. Activation of MAP kinase was determined by immunoblotting with an antibody that specifically recognized the phosphorylated (activated) form of p42/44 MAP kinase (Fig. 3 A). Adenoviral expression of either mSpry-1 or -2 but not of eGFP resulted in a significant reduction in the levels of activated p42/44 MAP kinase, whereas the overall levels of p42/44 MAP kinase were unaffected, as determined by probing the immunoblot with an antibody that recognizes all forms of p42/44 MAP kinase (Fig. 3 A). Adenoviral expression of mSpry-1 and -2 was confirmed by probing the immunoblot with affinity-purified antibodies generated against mSpry-1 and -2 peptides (Fig. 3 A).

An inhibition of p42/44 MAP kinase activation by mSpry-1 and -2 was also observed when HUVECs were stimulated with FGF2 (Fig. 3 B). Due to the stronger p42/44 MAP kinase activation by FGF2 compared with VEGF, Spry-mediated inhibition of FGF2-induced p42/44 MAP kinase activation appeared less efficient than repression of VEGF-induced p42/44 MAP kinase activity (Fig. 3 B). Surprisingly, although EGF-induced proliferation of HUVECs was inhibited by mSpry-1 and -2, EGF-mediated activation of p42/44 MAP kinase was not at all affected by Spry overexpression (Fig. 3 B). Probing of the immunoblots with antibodies against the phosphorylated (activated) form of MEK and against all forms of MEK revealed that mSpry-1 and -2 also repressed FGF2- and VEGF-induced
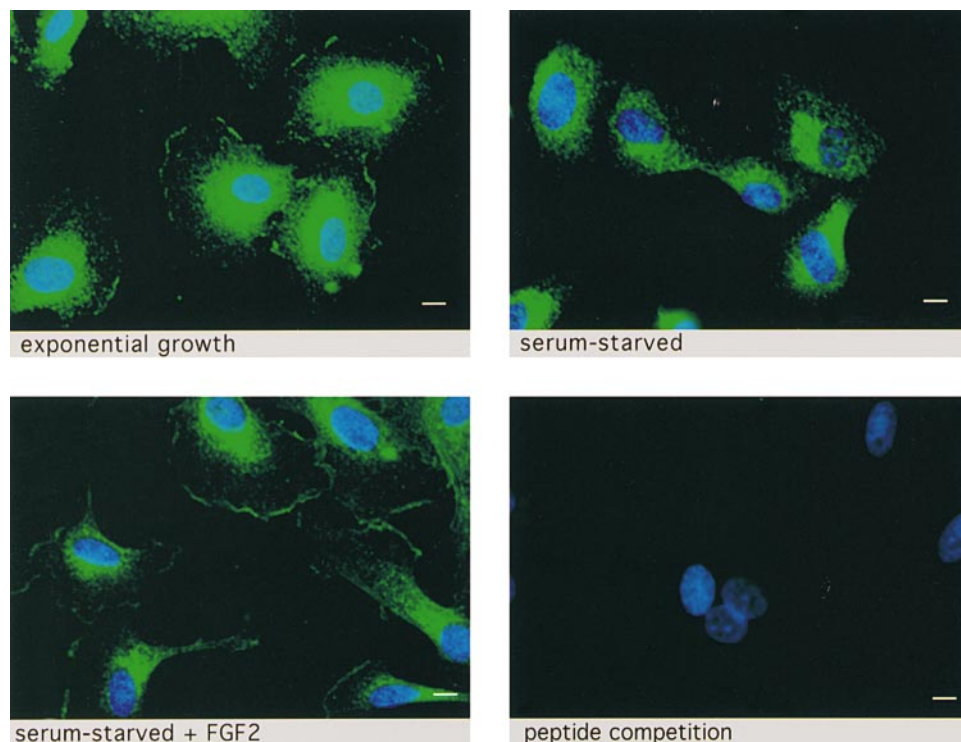

Figure 5. Growth factor–induced recruitment to the plasma membrane. The subcellular localization of endogenous hSpry-1 was determined in HUVECs by immunofluorescence microscopy using an affinity-purified antibody that specifically recognized mSpry-1 and hSpry-1 (green) and Hoechst staining of nuclei (blue). In actively proliferating endothelial cells (exponential growth), Spry-1 is localized to the perinuclear region, vesicular structures, and lamellipodia in the leading edge of the plasma membrane. In serum-starved cells, Spry-1 is not found in the plasma membrane (serum-starved), but a subset of it is recruited to the plasma membrane 30 min after stimulation with 10 ng/ml FGF2 (serum-starved + FGF2). Preabsorption of the affinity-purified antibodies with corresponding antigenic peptide (1 µg/ml) resulted in a complete abrogation of the immunofluorescence signal (peptide competition). Bars, 10 µm.
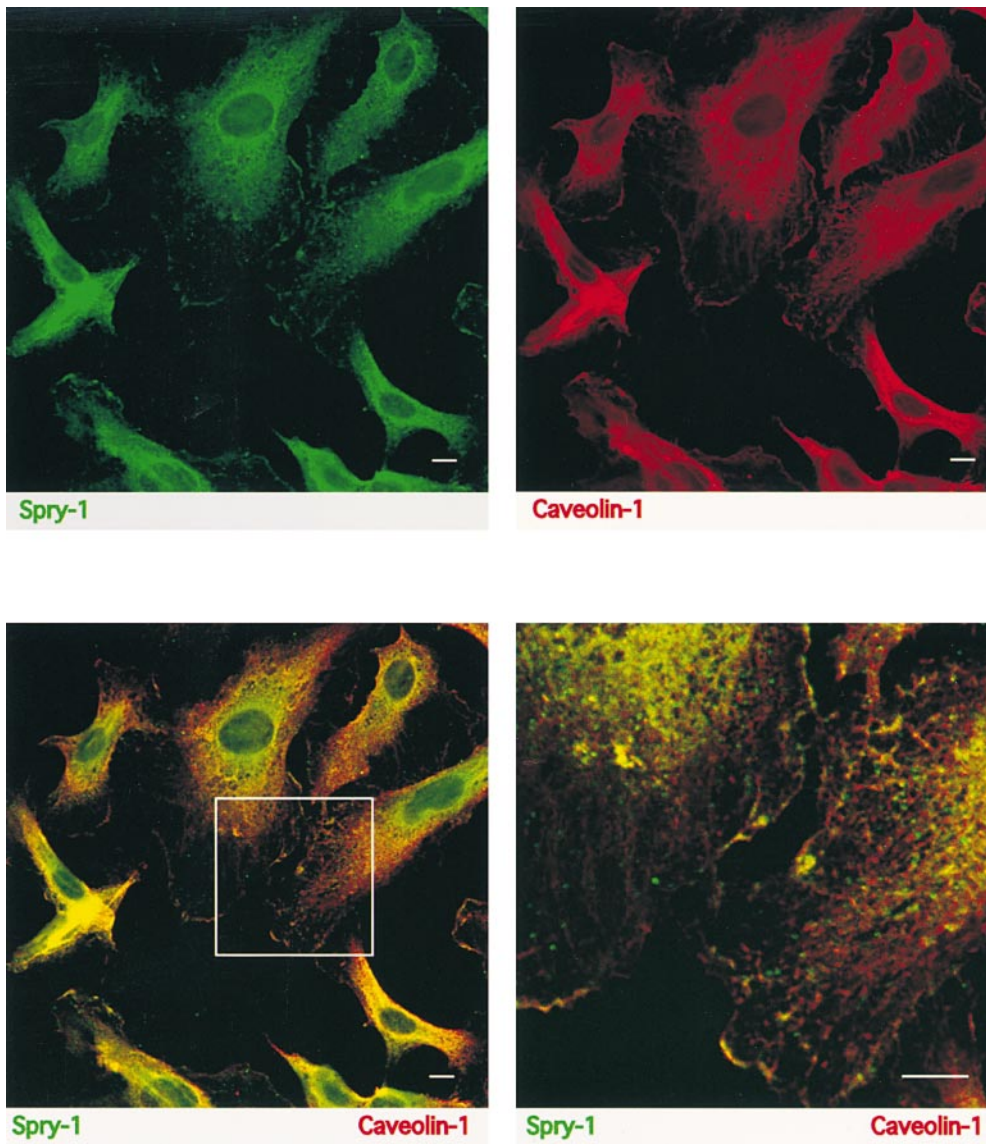
Figure 6. Colocalization with caveolin-1. The localization of endogenous hSpry-1 was determined in HUVECs by confocal immunofluorescent doublestaining with antibodies specific for hSpry-1 (green) and caveolin-1 (red) as indicated. Colocalization was determined by overlaying the green and the red staining. Note the colocalization of Spry-1 with caveolin-1 adjacent to the perinuclear region, in a subset of vesicular structures, and in the plasma membrane (visualized by yellow staining in the two bottom panels). A higher magnification of vesicular structures and plasma membrane (box, bottom left) is shown in the bottom-right panel. Bars, 10 μm.

but not EGF-induced MEK activation (Fig. 3 B). Adenoviral overexpression of mSpry-4 also repressed growth factor–induced MEK and p42/44 MAP kinase activation (data not shown).

Regulation of mSpry Expression by Angiogenic Factors

In *Drosophila*, DSpry not only interferes with growth factor–mediated signal transduction, but the expression of DSpry is also induced by the factors it subsequently inhibits. For example, DSpry expression is induced by FGF during trachea development (Hacohen et al., 1998) and EGF during oogenesis and eye development (Casci et al., 1999; Reich et al., 1999); in other regions, DSpry expression is induced with active EGF receptor signaling (Kramer et al., 1999). During chicken and mouse embryogenesis, Spry expression was found specifically in centers of FGF signaling or was upregulated in areas where recombinant FGF was applied to cultured embryos (Minowada et al., 1999; Chambers and Mason, 2000). To assess whether Sprys are regulated in a similar way in endothelial cells upon stimulation with angiogenic factors, HUVECs (data not shown) and 1G11 cells (Fig. 4) were serum starved and then stimu-

lated with FGF2 for different times. Levels of mRNAs for endogenous Spry-1 and -2 were determined by Northern blot hybridization (Fig. 4). Although Spry-1 mRNA was found at high levels in starved cells, it was transiently downregulated 2 h after treatment with FGF2 and regained high levels of expression 6 h after growth factor stimulation. Inhibition of protein synthesis by cycloheximide during growth factor stimulation prevented the transient degradation of Spry-1 mRNA (data not shown). In contrast, expression of Spry-2 (Fig. 4) and -4 (data not shown) was low in starved cells; it was induced 2 h after treatment with FGF2 and subsequently downregulated at later time points. The results indicate that the expression of Sprys is directly modulated by growth factor stimulation and that the various members of the Spry family may be affected in different ways.

The Subcellular Localization of mSpry Is Modulated by Growth Factors

To determine the subcellular localization of Spry-1 and -2 in mammalian endothelial cells, we performed immunofluorescence experiments using affinity-purified polyclonal
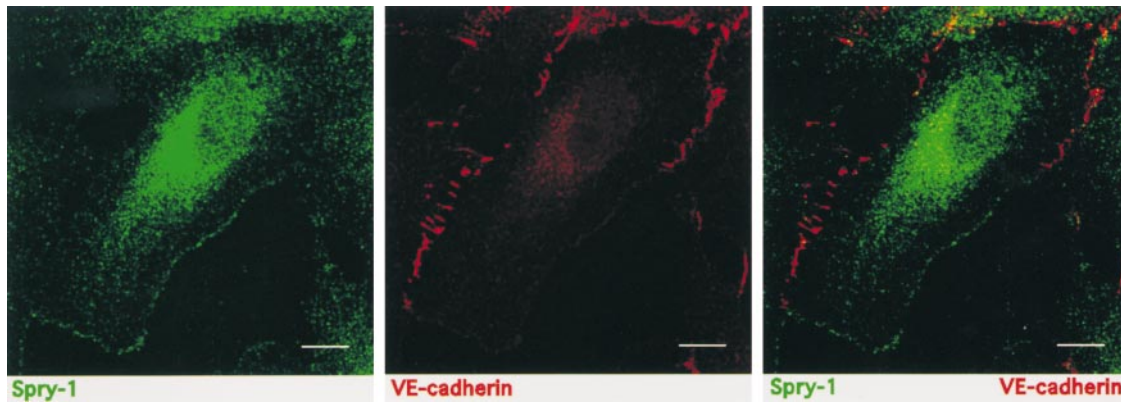
Figure 7. Colocalization with VE–cadherin. Confocal immunofluorescent doublestaining of HUVECs with antibodies specific for hSpry-1 (green) and VE–cadherin (red) as indicated. Colocalization was determined by overlaying the green and the red staining. Note the mutual exclusive localization of Spry-1 and VE–cadherin in the plasma membrane. Bars, 10 μm.

antibodies that specifically recognized mSpry-1 and -2 hSpry-1 and -2. In actively proliferating HUVECs, endogenous (human) hSpry-1 was widely distributed throughout the cells; it was found predominantly in perinuclear regions, in vesicular structures, and in the plasma membrane at the leading edge of the cells (Fig. 5, exponential growth). Preabsorption of the affinity-purified antibodies with the corresponding Spry-specific antigenic peptide completely abrogated the immunofluorescence signal, indicating that the antibodies specifically visualized hSpry-1 (Fig. 5, peptide competition). Upon serum-starvation of HUVECs, hSpry-1 was found to be absent from the plasma membrane (Fig. 5, serum-starved). In contrast, upon stimulation with FGF2 (Fig. 5, serum-starved + FGF2) or VEGF (data not shown), a small subset of hSpry-1 was recruited within 30 min to the plasma membrane, mainly to the lamellipodia of the leading edge of the cells. Identical results were obtained for endogenous mSpry-1 in 1G11 cells (data not shown). Expression levels of Spry-2 in endothelial cells were too low to be detected by immunofluorescence; however, a subcellular localization similar to endogenous hSpry-1 was observed when either mSpry-1 or mSpry-2 were overexpressed in the cells by transient transfection or adenoviral gene transfer (data not shown). Thus, not only Spry gene expression but also the subcellular localization of Spry proteins is directly modulated by growth factors.

In an attempt to identify the subcellular structures of Sprys' localization, we performed immunohistochemical double-labeling studies using confocal laser scanning microscopy. Although Spry-1 was found within the subcellular compartments identified by some of the markers, it did not significantly colocalize with protein disulfide isomerase (PDI; a marker for the ER), ergic-53 (a marker for the ER/Golgi region), giantin (a marker for the Golgi network), transferrin receptor (a marker for early endosomes), and syntaxin (a marker for secretory vesicles) (data not shown). In contrast, costaining of Spry-1 with caveolin-1, a cholesterol-binding protein in cholesterol- and sphingolipid-rich surface domains (lipid rafts) and the major structural protein of caveolae (Anderson, 1998), revealed significant colocalization predominantly in the perinuclear region of the Golgi region and in the plasma membrane (Fig. 6). Spry-1 and caveolin-1 were also found

to colocalize in vesicular structures; however, a major fraction of the two proteins appeared also to be part of distinct vesicles. Costaining with VE–cadherin, the predominant cell–cell adhesion molecule involved in the formation of adherens junctions in endothelial cells, revealed a mutually exclusive localization: whereas VE–cadherin was found exclusively in the portions of the cell membrane that were involved in cell–cell adhesion, Spry-1 was found in the parts of the plasma membrane that were not in the vicinity of any neighboring cells (Fig. 7). These results are consistent with the notion that caveolins are also not localized to the regions of adherens junctions, further supporting an association of Sprys with caveolin-containing membrane domains (caveolae).

Sprys Are Tightly Associated with Membranes and with Caveolin-1

Next, we performed coimmunoprecipitation experiments to assess whether Sprys could directly associate with caveolin-1. HUVECs infected with AdmSpry-1 or AdeGFP were lysed in buffers that differed in their strength of membrane disaggregation: 1% Triton X-100 for solubilization of conventional membranes, and 1% digitonin for the quantitative depletion of cholesterol from cholesterol-rich membrane domains (rafts). Under these different buffer conditons, mSpry-1 was immunoprecipitated with antibodies specific for Spry-1, and precipitated mSpry-1 and coprecipitated caveolin-1, respectively, were analyzed by immunoblotting (Fig. 8 A). These experiments revealed that overexpressed mSpry-1 was tightly associated with caveolin-1, even under conditions known to disrupt lipid rafts (Fig. 8 A). A similar caveolin-1 association was also observed for overexpressed mSpry-2 (data not shown). Control immunoprecipitations using unrelated rabbit immunoglobulins did not precipitate caveolin-1 (Fig. 8 A, right). Moreover, annexin II, a peripheral membrane protein, did not coprecipitate with mSpry-1, confirming a specific association between overexpressed mSpry-1 and caveolin-1. In AdeGFP-infected HUVECs, precipitation of endogenous hSpry-1 did not recover significant amounts of hSpry-1 and caveolin-1, most likely due to the low levels of endogenous hSpry-1 in HUVECs (Fig. 8 A). Notably, immunoprecipitations of caveolin-1 did not recover detectable
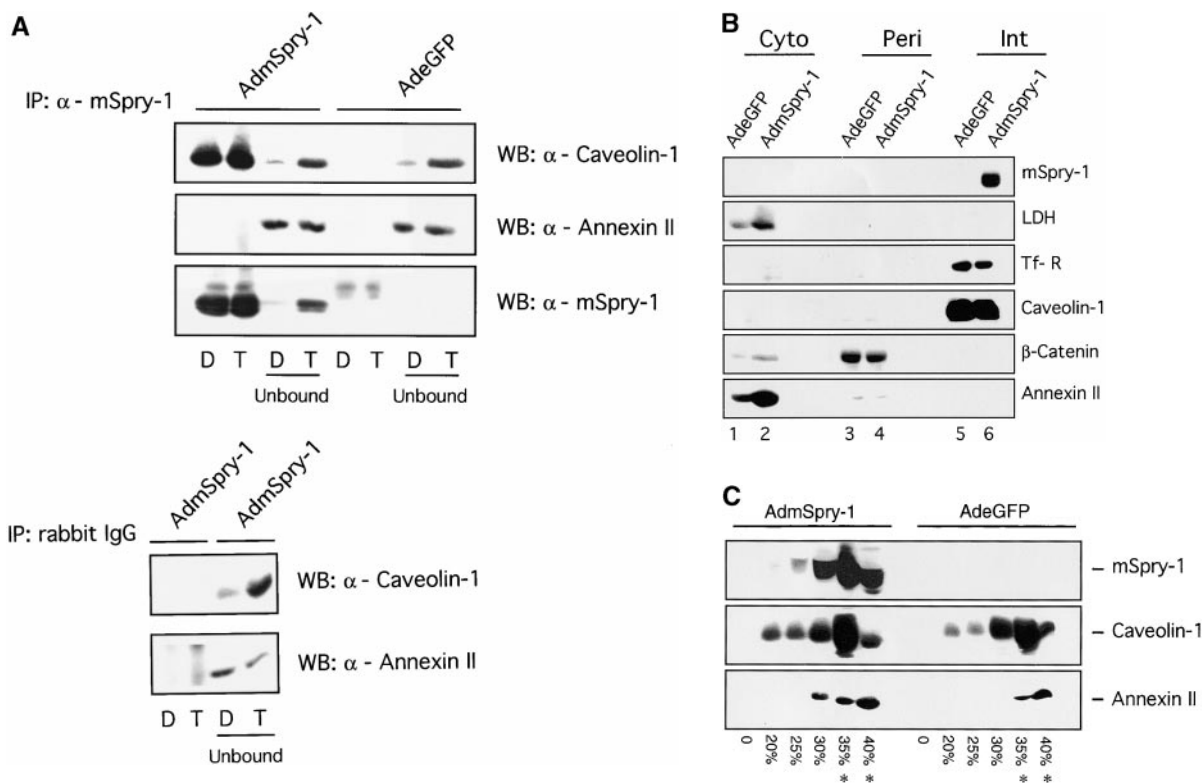
Figure 8. Sprys are tightly associated with caveolin-1 and membranes. (A) mSpry-1 associates with caveolin-1. HUVECs infected with AdmSpry-1 or AdeGFP were lysed in digitonin (D) or Triton X-100 (T) buffers, as described in Materials and Methods and indicated below the lanes. Aliquots of the cell lysates were subjected to immunoprecipitations with antibodies specific for mSpry-1 (top) or unrelated rabbit immunoglobulins, as negative control (bottom), in the respective buffers. Precipitated proteins and nonprecipitated proteins (Unbound) were resolved by SDS-PAGE, and caveolin-1, mSpry-1, and annexin II were detected by immunoblotting as indicated. (B) mSpry-1 is tightly associated with membranes. HUVECs infected with AdmSpry-1 or AdeGFP were subjected to a subcellular fractionation protocol, as described in Materials and Methods. Lanes 1 and 2 (Cyto) show cytoplasmatic extract obtained by permeabilizing the cells with digitonin (0.003%). Lanes 3 and 4 (Peri) show peripheral membrane proteins washed from partially purified membranes with sodium carbonate (Na_2HCO_3). Lanes 5 and 6 (Int) show integral membrane proteins that were not removed from the membrane fraction by sodium carbonate treatment. Protein levels of mSpry-1, LDH, transferrin receptor (Tf-R), caveolin-1, and annexin II in the various fractions were determined by immunoblotting as indicated. (C) The majority of mSpry-1 and caveolin-1 are not in detergent-resistant lipid rafts. HUVECs infected with AdmSpry-1 or AdeGFP were lysed in a Triton X-100 buffer and sedimented on Optiprep gradients as described in Materials and Methods. mSpry-1, caveolin-1, and annexin II in the gradient fractions were visualized by immunoblotting as indicated. The Optiprep concentrations of the fractions are given below the lanes. *Indicates that half of the fractions has been loaded in these lanes.

amounts of overexpressed mSpry-1, raising the possibility that only a subset of caveolin-1 is associated with mSpry-1 (data not shown).

To characterize the membrane association of Sprys, we performed biochemical fractionation experiments. Actively proliferating HUVECs infected with either AdmSpry-1 or AdeGFP were permeabilized with 0.003% digitonin to extract the cytoplasm. The remaining membrane-associated proteins were further separated into peripheral membrane proteins and integral membrane proteins by washing the membranes with sodium carbonate (Fig. 8 B) or using a Triton X-114 extraction protocol (data not shown). Under both extraction conditions, overexpressed mSpry-1 was not released from the membranes; it cofractionated exclusively with transferrin receptor and caveolin-1, both prototype integral membrane proteins (Fig. 8 B, lanes 5 and 6). In contrast, the peripheral membrane protein β-catenin was quantitatively released from the membranes under these conditions (Fig. 8 B, lanes 3

and 4). LDH was used as a marker for cytoplasmic proteins (Fig. 8 B, lanes 1 and 2). Under the Ca^{2+} concentration used, the peripheral membrane protein annexin II dissociated from membranes and was predominantly found in the cytoplasmic fraction, whereas only a subset remained in the peripheral membrane fraction (Fig. 8 B, lanes 1–4). Similar results were obtained for overexpressed mSpry-2 in HUVECs (data not shown).

Caveolin-1 is a principal component of cell surface invaginatons called caveolae (Kurzchalia et al., 1992; Rothberg et al., 1992). Caveolae represent a subset of lipid rafts, cholesterol- and sphingolipid-rich membrane microdomains that are functionally implicated in cellular transport processes and in signal transduction–related events (Simons and Toomre, 2000). The association of mSpry-1 with caveolin-1, thus, raised the question whether mSpry-1 was localized in lipid rafts. To test for this possibility, HUVECs were infected with AdmSpry-1 and AdeGFP, respectively, and cells were extracted with Triton X-100,
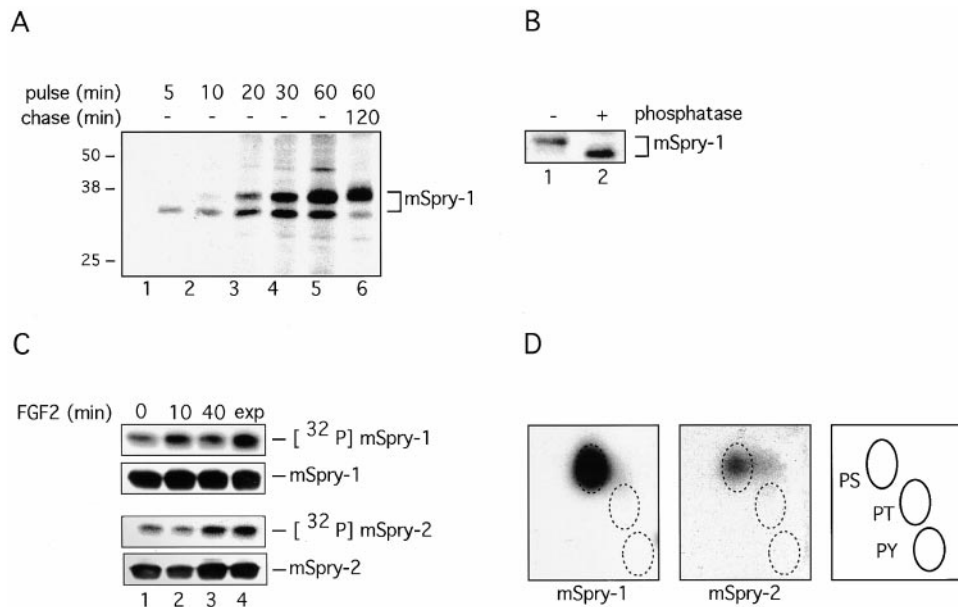
Figure 9. Spry-1 and -2 are post-translationally modified by phosphorylation on serine residues. (A) Kinetics of posttranslational modification. HUVECs infected with AdmSpry-1 were metabolically labeled with [^{35}S]methionine/cysteine for the times indicated in minutes (pulse). Cell lysates were either collected immediately, (lanes 1–5) or after the addition of normal medium for 2 h (chase; lane 6). mSpry-1 was immunoprecipitated, and the state of its posttranslational modification was assessed by the electrophoretic mobility of the protein upon SDS-PAGE and immunoblotting. (B) Phosphatase treatment. Total cell lysate from actively proliferating 1G11 cells was treated with calf intestine phosphatase (lane 2) or with buffer alone (lane 1), and endogenous mSpry-1 was detected by immunoblotting. (C) Phosphate labeling. HUVECs infected with AdmSpry-1 or -2 were serum starved, stimulated with 10 ng/ml FGF2, and metabolically labeled with [^{32}P]orthophosphate for the times indicated in minutes. mSpry-1 and -2 were immunoprecipitated with affinity-purified antibodies, and the incorporation of radioactivity was analyzed by SDS-PAGE and autoradiography. Exponentially growing cells were used as control (exp). (D) mSpry-1 and -2 are phosphorylated on serine residues. Phosphoamino acid analysis was performed on the immunoprecipitated mSpry-1 and -2 proteins described in C. The positions of phosphoserine (PS), phosphothreonine (PT), and phosphotyrosine (PY) markers on the thin layer chromatogram are indicated.

a procedure that disrupts conventional membranes but does not affect detergent-resistant lipid rafts (Simons and Toomre, 2000). Triton X-100 lysates were sedimented on Optiprep gradients, and the presence of mSpry-1 in the gradient fractions was analyzed by immunoblotting (Fig. 8 C). These experiments revealed that most of mSpry-1 and caveolin-1 sedimented as soluble proteins. Only a very small amount of mSpry-1 and caveolin-1 was found to float on top of the gradient, indicating that, in cultured endothelial cells, the vast majority of both caveolin-1 and mSpry-1 are not found in detergent-resistant rafts. This result is somewhat surprising since endothelial cells are known to contain abundant numbers of caveolae; however, our results are consistent with previous reports demonstrating that, in cultured endothelial cells, the majority of caveolin-1 is soluble in Triton X-100 (Venema et al., 1997).

Hence, consistent with the subcellular localization data (Fig. 6), the immunoprecipitation and the biochemical fractionation results indicate that, in cultured endothelial cells, Sprys tightly associate with membranes and with caveolin-1, but the majority of both Sprys and caveolin-1 are not part of detergent-resistant lipid rafts.

mSpry-1 and -2 Are Phosphorylated and Palmitoylated

The membrane association of Spry-1 and -2 and the apparent lack of membrane targeting motifs in their amino acid sequence, together with the observation that Sprys were found with varying electrophoretic mobilities (data not shown), suggested that Sprys are posttranslationally modified. To examine this, we performed a pulse–chase experiment to investigate the kinetics of mSpry-1 and -2 biosynthesis and processing. HUVECs were infected with

AdmSpry-1 and metabolically labeled with [^{35}S]methionine/cysteine for different times, and mSpry-1 was immunoprecipitated from protein lysates and analyzed by SDS-PAGE. At early time points, mSpry-1 was detected as a single band with the molecular mass predicted from its amino acid sequence (34 kD; Fig. 9 A, lane 1). With increasing time of labeling, a second band with lower electrophoretic mobility appeared that became predominant at later times (lanes 2–5). When the pulse experiments were followed by a chase with normal medium, a complete shift to the slower migrating form was observed (lane 6). Identical results were obtained for mSpry-2 (data not shown). These results raise the possibility that newly synthesized mSpry-1 and -2 undergo posttranslational modifications. The presence of tunicamycin in the culture medium or endoglycosidase treatment did not affect the electrophoretic mobility of mSpry-1 and -2, excluding protein glycosylation (data not shown). In contrast, alkaline phosphatase treatment of uninfected 1G11 cell lysates induced a shift of endogenous mSpry-1 to a faster migrating form, indicating that it was modified by phosphorylation (Fig. 9 B). Consistent with this notion, mSpry-1 and -2 were found to be labeled by the incorporation of [^{32}P]orthophosphate in immunoprecipitation experiments (Fig. 9 C). However, when AdmSpry-1- or -2–infected HUVECs were starved and then stimulated with FGF2, incorporation of [^{32}P]orthophosphate into mSpry-1 and -2 was not significantly changed with time (Fig. 9 C). Varying the experimental conditions, such as timing of the [^{32}P]orthophosphate pulse and growth factor addition, also did not reveal a strong correlation between growth factor stimulation and mSpry phosphorylation, indicating that phosphorylation of mSprys was not directly affected by growth factor stimulation (data not
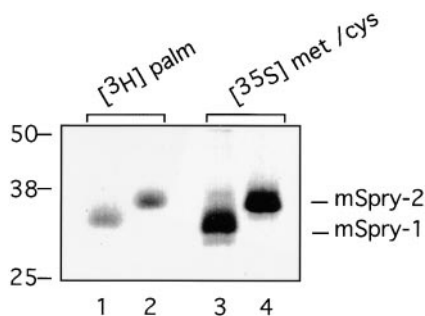
Figure 10. mSpry-1 and -2 are palmitoylated. HUVECs infected with AdmSpry-1 (lanes 1 and 3) or -2 (lanes 2 and 4) were labeled with [^{3}H]palmitate (lanes 1 and 2) or [^{35}S]methionine/cysteine (lanes 3 and 4) for 2 h. mSpry-1 and -2 were immunoprecipitated with affinity-purified antibodies, and radiolabeled mSpry-1 and -2 were resolved by SDS-PAGE and visualized by fluorography.

shown). Phosphoamino acid analysis of [^{32}P]orthophosphate-labeled and -immunoprecipitated mSpry-1 and -2 revealed that both mSpry-1 and -2 were phosphorylated exclusively on serine residues (Fig. 9 D).

Next, we analyzed how Sprys associate with membranes. Extensive protein sequence analysis did not reveal any apparent signal peptide, transmembrane domain, or conserved motifs known to be modified by prenylation or myristylation (Veit and Schmidt, 1998). In contrast, Sprys contain an unusually high number of internal cysteine residues, amino acids that, via a labile thioester bond, can be modified by palmitoylation (Veit and Schmidt, 1998). To investigate the possibility that Sprys were palmitoylated, HUVECs were infected with AdmSpry-1 and -2, respectively, and metabolically labeled with either [^{3}H]palmitate or [^{35}S]methionine/cysteine. Subsequently, mSpry-1 and -2 were immunoprecipitated, and palmitoylation of the proteins was analyzed by SDS-PAGE under nonreducing conditions and fluorography (Fig. 10). Both mSpry-1 and -2 were specifically labeled by the incorporation of [^{3}H]palmitate. Specific palmitoylation was further confirmed by soaking the SDS-PAGE gels in hydroxylamine, a treatment that is known to specifically hydrolyze thioester bonds, such as palmitoylation, but not amide bonds, such as myristylation (Linder et al., 1995). Such treatment specifically removed incorporated hydogen-3 from mSpry-1 and -2 (data not shown). These results demonstrate that both mSpry-1 and -2 are palmitoylated, most likely the molecular basis for their integral membrane localization.

Discussion

During embryonic development, DSprys, mSprys, and chicken Sprys have been shown to inhibit growth factor–mediated signal transduction, at least in part by repressing pathways that lead to the activation of p42/44 MAP kinases (Hacohen et al., 1998; Casci et al., 1999; Minowada et al., 1999; Reich et al., 1999); however, their mechanism of action is not understood. We have addressed some of the open questions about the molecular mechanisms that underlie Sprys' functions. We have cloned four mouse homologues of hSpry and DSpry and have functionally characerized hSpry-1 and -2 and mSpry-1 and -2 in endothelial cells. Our data show that mSpry-1 and -2, when overexpressed, inhibit endothelial cell proliferation and differentiation induced by FGF2 and VEGF. At least a fraction of this inhibitory effect is due to the inhibition of p42/44 MAP kinase activation, certainly upstream of the activation of MEK, since growth factor–induced MEK activity is also repressed by Spry-1 and -2. Curiously, although Sprys efficiently inhibit EGF-induced endothelial cell proliferation, EGF-mediated p42/44 MAP kinase activation in endothelial cells is not affected by Spry-1 and -2, indicating that Sprys' inhibitory functions may not exclusively act by repressing the MAP kinase pathway. The potential inhibition of other signaling pathways that are activated by FGF, VEGF, and EGF receptors remains to be determined.

We also report for the first time the subcellular localization of endogenous Spry in mammalian cells. Consistent with the previously reported localization of Dspry to membranes (Casci et al., 1999), our studies demonstrate a tight association of Sprys with membranes. First, mSpry-1 and -2 and hSpry-1 and -2 significantly colocalize with caveolin-1 to the perinuclear region, vesicular structures, and the plasma membranes of endothelial cells. Second, the modification by palmitoylation and the lack of glycosylation suggest that Spry-1 and -2 are tightly associated with membranes by a lipid anchor. Third, immunoprecipitation experiments reveal a tight association between Spry-1, Spry-2, and caveolin-1, raising the possibility that Sprys are part of lipid rafts or caveloae (see below; Simons and Ikonen, 1997; Simons and Toomre, 2000).

Upon growth factor stimulation of starved endothelial cells, a significant subset of the proteins is translocated to the plasma membrane, predominantly to the lamellipodia at the leading edge of the cells, a site where the majority of growth factor receptors is known to localize. Consistent with the observations on DSpry reported by Casci and co-workers (1999), we were not able to detect Spry-1 or -2 in the supernatant or in the extracellular matrix of cultured cells, even when overexpressing the proteins (data not shown).

The specific subcellular localization of mammalian Sprys and the relocalization induced by growth factors provides some insights into the mechanisms by which Sprys inhibit RTK signaling. Several signaling factors, including EGF receptor, platelet-derived growth factor receptor, Src kinases, Gα subunits, phospholipase C γ, protein kinaes C, Ras, Raf, and MAP kinases, have been shown to associate with lipid rafts or to cofractionate with caveolin-1 (Okamoto et al., 1998; Smart et al., 1999). Hence, lipid rafts and/or caveolae are thought to play an important role in signal transduction, possibly by assembling signaling complexes or by resetting or recycling receptors (Liu et al., 1997a,b; Furuchi and Anderson, 1998). Recently, Simons and Toomre (2000) proposed to distinguish four types of lipid rafts: native rafts that are only detectable in living cells, clustered rafts that are induced by cross-linking, detergent-resistant rafts that are characterized by, for example, their resistance to Triton X-100, and caveolae that are characterized by raft proteins, caveolins and lipids. Consistent with a previous report (Venema et al., 1997), our biochemical fractionation experiments with cultured endothelial cells have revealed that the majority of caveolin-1 and mSpry-1 is not found in detergent-resistant lipid rafts. Moreover, depletion of cholesterol from

lipid rafts by treating HUVECs with methyl-β-dextrin did not affect the inhibitory function of overexpressed mSpry-1 on VEGF-induced MEK activation (data not shown), suggesting that functional Spry may not be part of lipid rafts. On the other hand, the small subset of mSpry-1 that, together with caveolin-1, is found in lipid rafts may represent functional Spry and may not be affected by methyl-β-dextrin because it is not exposed on the outer side of the plasma membrane, for example, within caveolae. Hence, it remains to be elucidated whether their association with caveolin-1 and/or their subcellular localization brings Sprys into contact with RTK signaling components to exert their inhibitory function.

DSpry has been shown to directly bind Gap-1 and the Grb-2 homologue Drk, both adaptor molecules of RTK signaling pathways (Casci et al., 1999). However, in endothelial cells, under the conditions used to identify caveolin-1 in a tight complex with Spry-1 and -2, these signaling adaptors did not coprecipitate with Sprys (data not shown). Hence, the molecular mechanisms by which Sprys negatively modulate RTK signaling remain to be elucidated. Based on the association of Sprys with caveolin-1 and the inability of Sprys to inhibit EGF-induced MAP kinase activation, it is tempting to speculate that Sprys may not simply interfere with the activation of the MAP kinase pathway. Rather, they may modulate the assembly of RTK signaling complexes, promote the resetting of RTK activity, or play a role in the regulation of RTK recycling. The latter functions would be compatible with the observation that in previously published reports (Chambers and Mason, 2000; de Maximy et al., 1999; Minowada et al., 1999; Tefft et al., 1999) and, in our experiments, the inhibitory function of vertebrate Sprys is exclusively demonstrated by overexpression of the proteins. Hence, the function of endogenous Sprys may differ fundamentally from the effect of overexpressed Sprys. For example, Sprys, similar to or together with caveolin-1, may act as scaffolding proteins for the assembly of signaling complexes, and increasing Spry levels by overexpression may disturb the stoichiometry of complex assembly and prevent the formation of functional signaling complexes.

Consistent with previous reports on DSpry (Reich et al., 1999), mSpry, and chicken Spry (Minowada et al., 1999; Chambers and Mason, 2000) during embryogenesis, mRNA levels of Spry-1 and -2 are also tightly regulated by growth factor stimulation in endothelial cells. Notably, expression of Spry-1 and the expression of Spry-2 appear to be inversely regulated. Although the expression of Spry-1 is repressed by growth factor stimulation, the expression of Spry-2 is upregulated. Both Sprys are phosphorylated on serine residues, however, phosphorylation does not appear to be modulated by growth factor stimulation, and the functional role of this modification remains to be elucidated. For example, it is not known whether Spry-1 and -2 phosphorylation is required for their recruitment to the plasma membrane or for their inhibitory function. Thus, identification of the Spry phosphorylation sites and/or the kinase(s) that phosphorylate Sprys may shed some light on Sprys' mode of action. Moreover, the observation that Sprys are palmitoylated membrane proteins and associate with caveolin-1 sets the stage for future experiments aimed at unraveling Sprys' physiological functions.

Note added in proof. An inhibitory function in angiogenesis of murine Sprouty-4 has been reported recently by Lee and coworkers (Lee, S.H., D.J. Schloss, L. Jarvis, M.A. Krasnow, and J.L. Swain. 2001. *J. Biol. Chem.* 276:4128–4133).

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