A FUNCTIONAL INTERACTION BETWEEN SPROUTY PROTEINS AND CAVEOLIN-1*

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Growth factor-mediated signal transduction cascades can be regulated spatio-temporally by signaling modulators, such as Sprouty proteins. The four mammalian Sprouty family members are palmitoylated phosphoproteins that can attenuate or potentiate numerous growth factor-induced signaling pathways. Previously, we have shown that Sprouty-1 and Sprouty-2 associate with Caveolin-1, the major structural protein of caveolae. Like Sprouty, Caveolin-1 inhibits growth factor-induced mitogen-activated protein kinase activation. Here, we demonstrate that all four mammalian Sprouty family members physically interact with Caveolin-1. The C-terminus of Caveolin-1 is the major Sprouty-binding site, while Sprouty binds Caveolin-1 via its conserved C-terminal domain. A single point mutation in this domain results in loss of Caveolin-1 interaction. Moreover, we demonstrate that the various Sprouty isoforms differ dramatically in their cooperation with Caveolin-1-mediated inhibition of mitogen-activated protein kinase activation and that such cooperation is also highly dependent on the type of growth factor investigated and on cell density. Together, the data suggest that the Sprouty/Caveolin-1 interaction modulates signaling in a growth factor- and Sprouty isoform-specific manner.

Receptor tyrosine kinases (RTKs) are single membrane-spanning receptors that transduce signals from extracellular proteins, such as growth factors and hormones, thereby controlling numerous cellular processes, including migration, proliferation, differentiation, apoptosis, and survival (1,2). Once ligated by their ligands, RTKs assemble on their intracellular domains a multi-protein complex (signalosome) consisting of a variety of signaling adaptor and effector molecules, which themselves are substrates of the RTKs’ kinase activities and/or exert subsequent scaffolding or enzymatic signal transducing activities. Each RTK signalosome possesses common elements as well as those that are unique to particular RTKs (3). For instance, the Ras-Raf-mitogen-activated protein kinase (MAPK), also known as p42/44 extracellular-regulated kinase (p42/44 ERK), pathway is a common cascade that is activated upon ligand binding to many RTKs (2). The molecules controlling RTK signaling are numerous, including negative and positive regulators of signal transduction (reviewed in (4)). Some of these signaling modulators are shared to varying degrees by different RTK-induced signaling pathways. The recently identified Sprouty (Spry) proteins are one such family of common regulatory proteins (5-9). Initially discovered in Drosophila as an inhibitor of fibroblast growth factor (FGF) signaling during tracheal development, dSpry has been also found to inhibit epidermal growth factor (EGF)-mediated development of the eye, wing and ovary among other organs (10-13).

Four mammalian Spry isoforms (Spry1-4) have been identified and found to be palmitoylated phosphoproteins that translocate to the plasma membrane upon growth factor stimulation (14-22).
Subsequent studies in mice, chicken, and zebrafish have shown that FGF-induced p42/44 ERK activation is inhibited by Spry and that FGF in turn induces Spry expression (15,16,18,22-25). Furthermore, overexpression studies have revealed that cells expressing Spry proteins exhibit reduced migration, proliferation, invasion and differentiation (15,19,26-31). Recent reports, including murine knockout studies of mSpry1 and mSpry2, have demonstrated that Spry proteins not only attenuate FGF- and EGF-mediated p42/44 ERK activation, but also inhibit signaling induced by glial-cell line derived neurotrophic growth factor, c-kit, insulin, platelet-derived growth factor (PDGF), nerve growth factor, hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) (19,29,32-37).

All Spry proteins have a unique, highly conserved cysteine-rich C-terminal domain, which is required for their inhibitory function as well as for their translocation to the plasma membrane upon growth factor stimulation (14,30,38-40). The N-terminal domains of the Spry proteins are variable, but possess a conserved tyrosine residue that, when mutated, leads to the creation of dominant-negative Sprys which can not attenuate p42/44 ERK activation and even repress wildtype Spry function (20,22,37).

Identification of various Spry-binding partners has provided some insights into the functional differences among the Spry isoforms. For example, when cells are stimulated with EGF, Spry2 associates with the E3 ubiquitin ligase c-Cbl and CIN85, leading to an inhibition of EGF receptor internalization and degradation and thus to a potentiation of p42/44 ERK activity (40-45). However, during FGF stimulation, Spry2 also binds to c-Cbl, but in this case FGF receptors are downregulated and FGF-stimulated p42/44 ERK activity is repressed (42). Furthermore, Spry2 binds to Grb2, a signaling adaptor protein, thereby uncoupling the signal transduction cascade and leading to a failure to activate p42/44 ERK (20,21,27,46). Other components of the FGF receptor signalosome, including Frs2, Raf, and Gap, have also been shown to interact with Spry2 (21).

Previously, we have reported the co-localization and co-immunoprecipitation of endogenous Caveolin-1 (Cav-1) with mouse (m) Spry1 and mSpry2 in human endothelial cells (15). Cav-1 binds cholesterol, plays a role in cholesterol transport to the plasma membrane, and is the main structural component of caveolae, flask-shaped invaginations on the cell membrane (47). It acts as a scaffolding protein for signal transduction events, binds to many RTKs, such as the EGF-, FGF-, and VEGF receptors, and directly inhibits p42/44 ERK activation (48,49). Disruption of caveolae by either cholesterol depletion or antisense Cav-1 results in p42/44 ERK hyperactivation (50,51). Recent knockout studies revealed that mice lacking Cav-1 exhibit impaired cardiac and vascular function and have a shorter lifespan (52-54). At first thought to be a tumour suppressor, Cav-1 exerts a cell type-dependent role in mouse models of tumour progression that is mirrored in human cancers: acting as a tumor suppressor in some cases (e.g. breast) and as a tumor promoter in others (e.g. prostate) (55-57). These data suggest different roles for Cav-1 that vary among cell types.

Here, we have set out to characterize the biochemical interaction between Spry and Cav-1, explore its functional significance in the context of Spry activity (i.e. inhibition of p42/44 ERK activation), and systematically compare the four murine Spry proteins in the same cellular context. We demonstrate that all Spry isoforms physically interact with Cav-1 in a complex, multidomain-dependent manner. Yet, despite their binding to Cav-1, the various Spry family members exhibit differential cooperativity with Cav-1 in repressing p42/44 ERK activation. Moreover, dependent on the type of growth factor stimulation, Spry family members exert varying inhibitory activities on signal transduction. Thus, the interaction of the four Spry proteins with Cav-1 appears to play a complex, growth factor- and Spry isoform-dependent role in modulating signal transduction.

MATERIAL AND METHODS

Chemicals and Reagents
All chemicals used were obtained from Sigma/Fluka (Basel, Switzerland) or Merck (Darmstadt, Germany) unless otherwise indicated. Restriction enzymes were obtained from New England Biolabs (Frankfurt, Germany), Invitrogen (Basel, Switzerland), or Roche Diagnostics (Rotkreuz, Switzerland).

Cloning and Plasmids
The four mSpry cDNAs were isolated as previously described (15) and were cloned into pSG9M (58,59), which encodes a myc epitope on the N-terminus. Dominant-negative versions of all four myc-tagged mSpry proteins (mSpry1-Y53A, mSpry2-Y55A, mSpry3-Y27A, mSpry4-Y53A) and the RD mutants (mSpry1-R249D, mSpry3-R221D, mSpry4-R235D) were produced using the QuickChange Site-Directed Mutagenesis kit (Stratagene; Amsterdam, Netherlands) according to the manufacturer’s instructions and sequenced for confirmation. Full-length mSpry2 was cloned in frame into the pcDNA3.1/V5-His-TOPO (Invitrogen) using KpnI/XbaI by employing Pwo polymerase (Roche Diagnostics) and the following primers, 5’-CGCGGTACCGCCACCACATGGAGGCCAGAGCTCAAGAG-3’ and 5’-TGCTCTAGAAATGTCGCTTTTCAAAAGTTCTG-3’, in a PCR amplification reaction. The N-terminal portion of mSpry2 (encoding aa 1-176) was cloned in frame into pcDNA3.1/V5-His-TOPO using KpnI/XbaI by employing the following primers, 5’-CGCGGTACCGCCACCACATGGAGGCCAGAGCTCAAGAG-3’ and 5’-TGCTCTAGAAATGTCGCTTTTCAAAAGTTCTG-3’. The C-terminal portion of mSpry2 (encoding aa 177-315) was cloned in frame into pcDNA3.1/V5-His-TOPO using BamHI/XhoI by employing the following primers, 5’-CGCGGATCCGCCACCATGGAGGTGTGAGGCTGCGGC-3’ and 5’-TGCTCTAGAAATGTCGCTTTTCAAAAGTTCTG-3’. The murine Cav-1 cDNA was isolated from NIH3T3 cells by RT-PCR. Briefly, RNA was isolated from NIH3T3 cells using TRIzol (Invitrogen), and random hexamers and Superscript II (Invitrogen) were used to generate single-stranded DNA. Pwo polymerase and the following primers, 5’-CGGGATCCGCCACCATGGAGGAGCTGTAGGCTGCTGAGGAGACCTG-3’ and 5’-TGCTCTAGAAATGTCGCTTTTCAAAAGTTCTG-3’, were used for subsequent PCR amplification. The Cav-1 cDNA was cloned into pSG9M and into pcDNA3.1 (Invitrogen) using BamHI/NotI and cloned into the respective sites of the pcDNA3.1 plasmid to generate the pcDNA3.1/E GF construct. The human (h) Spry2 cDNA was amplified by PCR from human genomic DNA isolated (60) from HEK293T cells using the primers, 5’-CGGGATCCGCCACCATGGAGGAGCTGTAGGCTGCTGAGGAGACCTG-3’ and 5’-TGCTCTAGAAATGTCGCTTTTCAAAAGTTCTG-3’. All cDNAs were cloned into pcDNA3.1 and sequenced. The pcDNA3.1/EGFP transfectants (T47D-EGFP), cells cultured in 0.5 mg/ml G418/Geneticin were selected, pooled, and employed in the experiments described below.

Cell Culture

All reagents used for cell culture were obtained from Sigma/Fluka. Human embryonic kidney cells (HEK293T), human breast carcinoma cells (T47D), African green monkey kidney cells (Cos7) and human cervical carcinoma cells (HeLa) were obtained from the American Tissue Culture Collection (ATCC; Manassas, VA). All cells were maintained at 37°C with 5% CO2. HEK293T, HeLa, and Cos7 cells were cultured in high glucose Dulbecco’s Modified Eagle medium, containing 10% fetal bovine serum and 2 mM glutamine, while T47D cells were cultured in RPMI-1640 with the same supplements as above. Cells were passaged using trypsin-EDTA and counted manually or using a Coulter Counter (Beckman Coulter; Nyon, Switzerland).

Stable Transfections

The pcDNA3.1/Cav-1 and pcDNA3.1/EGFP plasmids were linearized with PvuII and transfected into T47D cells in a 6-well plate using PerFectin (Gene Therapy Systems, Inc; San Diego, CA) according to the manufacturer’s instructions. 48 hours post-transfection cells were trypsinized and plated onto 10 cm dishes. Stably-transfected clones for pcDNA3.1/mCav-1 (T47D-Cav-1) were selected for by addition of 0.5 mg/ml G418/Geneticin (Invitrogen). A few clones were selected and characterized by immunoblotting as described below and one clone was used for the experiments described herein. For the pcDNA3.1/EGFP transfectants (T47D-EGFP), cells cultured in 0.5 mg/ml G418/Geneticin were selected, pooled, and employed in the experiments described below.
**Transient Transfections**

HEK293T cells were transiently transfected using PerFectin or Metafectene (Biontex, Munich, Germany). Cos7 cells were transiently transfected using Metafectene. For co-transfections, the ratio of two plasmids was 1:1 and was normalized using empty vectors. HeLa cells were transiently transfected using Fugene 6 (Roche Diagnostics). Transfection with pcDNA3.1/EGFP was used to monitor the transfection efficiency.

**Adenoviral Vectors and Stimulation Experiments**

Adenoviral constructs encoding the mSpry cDNAs (AdmSpry1-4) and the firefly luciferase cDNA (AdLite) were generated as described previously (15,62). Amplification of the virus was carried out in HEK293 cells and virus particles were purified from cell lysates using cesium chloride gradients and gel filtration (62). Viral quantities were based on protein content using the conversion of 1 mg viral protein/3.4 x 10^{12} virus particles. For viral infection of T47D cells, virus particles were added to the cells and allowed to adsorb for 2 h at 4°C. After adsorption, the medium was replaced with fresh growth medium. After viral infection, cells were allowed to recover for approximately 5 h and then starved over night (at least 14 h) in starvation medium. Next, cells were stimulated for the times indicated by adding 50 ng/ml of either recombinant human basic FGF (FGF2) or EGF (both from either Catalys AG/Promega, Wallisellen, Switzerland or Sigma/Fluka).

**Immunoprecipitations**

Cells were lysed for 30 min on ice in lysis buffer (1% Triton X-100, 160 mM NaCl, 20 mM Tris pH8.0, 2 mM Na3VO4, 10 mM NaF and 1:200 dilution of stock protease inhibitor cocktail for mammalian cells (Sigma/Fluka)) and cleared by centrifugation (14,000g, 30 min, 4°C). Protein concentration was determined using a modified Bradford protocol (Bio-Rad Protein Assay; BioRad Laboratories, Reinach, Switzerland). For immunoprecipitations, equal amounts of lysates were incubated overnight (4°C on rotator) with either preimmune sera or sera specific for one of the Spry isoforms. Protein G sepharose beads (Sigma/Fluka) (10% v/v in lysis buffer) were added to each tube and allowed to incubate with the immune complexes for at least 1 hour (4°C on rotator). Immunoprecipitate/bead complexes were washed three times in cold lysis buffer, an equal volume of 2x SDS-PAGE loading buffer (20% glycerol, 4% SDS, 0.13 M Tris, bromophenol blue 1mg/100ml, 2% beta-mercaptoethanol) was added to the washed beads followed by boiling of the samples.

**Immunoblotting**

Total cell lysates and immunoprecipitates were resolved by 12% SDS-PAGE (63). SDS-PAGE gels were transferred to PVDF (Millipore, Volketswil, Switzerland) by semi-dry transfer in Towbin’s buffer (20% methanol, 25 mM Tris, 192 mM glycine), blocked with either 4% bovine serum albumin or 5% skim milk powder in Tris-buffered saline with 0.05% Tween 20 (TBST). All primary and secondary antibodies were diluted in 5% skim milk powder in TBST. Detected proteins were visualized using enhanced chemiluminescence (GE Health Sciences/Amersham Biosciences, Otelfingen, Switzerland or Interchim, Montluçon, France). Membranes were stripped by incubation in 200 mM glycine, pH 2.5 (30 min, room temperature) followed by washing with TBST. Rabbit sera against mSpry1 and mSpry2 were generated as previously described (15). Rabbit sera against mSpry3 were generated with a peptide corresponding to C-terminal residues (RKISSSSSPFPKAQEKSV) conjugated to keyhole limpet hemocyanin. Rabbit sera against mSpry4 were generated with peptides corresponding to either N-(PLLDSRAPHSRLQHP) or C-terminal (AASGDTKTSRSDKPF) residues conjugated to keyhole limpet hemocyanin. Antibody specificity was confirmed by peptide competition experiments and by excluding cross-reactivity with other Spry proteins. The following primary antibodies were also used: goat polyclonal anti-6-His (Bethyl Laboratories, Montgomery, Texas), mouse monoclonal anti-V5 (Invitrogen), mouse monoclonal anti-diphosphorylated p42/44 ERK (Sigma/Fluka), rabbit polyclonal anti-p42/44 ERK (Sigma/Fluka), mouse monoclonal and rabbit polyclonal anti-Cav-1 (Becton Dickinson Pharmingen/Transduction Laboratories, Zymed/Invitrogen), mouse monoclonal anti-myc (clone 9E10 supernatant), mouse monoclonal anti-GFP (Roche Diagnostics), and goat polyclonal anti-actin (Santa Cruz Biotechnology, Heidelberg, Germany). The following secondary antibodies were used: horseradish peroxidase-conjugated goat polyclonal anti-rabbit IgG (1:10,000 dilution) or donkey polyclonal anti-goat IgG (1:10,000 dilution).
conjugated to horseradish peroxidase were used: goat anti-mouse IgG (Sigma/Fluka or Jackson Immunoresearch, Cambridgeshire, UK), goat anti-mouse IgM (Sigma/Fluka), rabbit anti-goat IgG (Sigma/Fluka), and donkey anti-rabbit IgG (Amersham or Jackson Immunoresearch). Immunoblots were scanned and analysed using densitometry using Adobe Photoshop 7 (San Jose, CA). Immunoblots were also visualized, analyzed and quantitated using the Odyssey Imager (Li-Cor Biotechnology, Bad Homburg, Germany). In these cases, the following secondary antibody conjugates were used: goat anti-mouse Alexa 680 (Invitrogen) and goat anti-rabbit IRDye 800 (Rockland Immunochemicals, Gilbertsville, PA).

**GST Pulldown Experiments**

HA-tagged versions of the four murine Spry isoforms were generated using PCR and cloned in frame into pGEX4T1 (GE Health Sciences/Amersham Biosciences). Also, the following fragments of murine Cav-1 were generated by PCR and cloned in frame into pGEX4T1: aa 1-61, aa 61-101, aa 1-101, aa 135-178. All resulting constructs were confirmed by sequencing. The constructs were transformed into Top10 Escherichia coli (Invitrogen). GST protein production was induced by treatment of cultures with 0.1 mM IPTG at either 30°C or 37°C for 2-4 h. Bacteria were harvested and pellets were processed as described previously (64). GST fusion proteins were purified using GST sepharose (GE Health Sciences/Amersham Biosciences) or GST agarose (Sigma/Fluka). Levels of purified fusion proteins bound to beads were determined by SDS-PAGE followed by Coomassie staining. Bound purified fusion proteins were incubated with an equal volume of HEK293T cell lysates (4°C for 2 h), washed three times in PBS. Then 2x SDS-PAGE loading buffer was added to the washed beads, the samples were boiled and resolved by SDS-PAGE.

**Overlay of Cav-1 peptides with Spry proteins**

Custom-made murine Cav-1 peptides were synthesized on cellulose sheets using SPOT peptide synthesis (Sigma-Genosys) (65). This panel of peptides was previously used to investigate protein-protein interactions between Cav-1 and dynamin-2 and consisted of eighteen consecutive peptides, each 12-13 amino acid residues long, with each peptide overlapping the previous by three residues (66). The Cav-1 peptide arrays were incubated with blocking buffer (90 ml of TBS-T plus 10 ml of Sigma-Genosys 10x blocking buffer plus 5 g of sucrose) overnight at room temperature on a shaker. The following day, membranes were washed with TBS-T. Purified recombinant mSpry proteins (either GST-HA-mSpry1, GST-HA-mSpry2, GST-HA-mSpry3 or GST-HA-mSpry4; each 13 µg in 10 ml of blocking buffer) were incubated at room temperature for three hours on a shaker, then washed with TBS-T three times. Next, membranes were incubated with mSpry-specific antibodies (1:5,000) in blocking buffer at room temperature for 1.5 hours followed by three washes with TBS-T and then incubated with anti-rabbit antibodies conjugated to horseradish peroxidase (1:20,000) and washed three times with TBS. Bound antibodies on the peptide array were detected using enhanced chemiluminescence and relative binding was determined using densitometry of scanned blots (ImageJ, [http://rsb.info.nih.gov/ij/](http://rsb.info.nih.gov/ij/)) followed by statistical analysis (Prism (GraphPad Software, San Diego, CA)). Six peptide-containing membranes were used for this study, with each recombinant Spry isoform being tested three times on a different membrane. All membranes were stripped according to the manufacturer's instructions and reprobed as described above with a different recombinant Spry isoform.

**Immunofluorescence Experiments**

Cos7 cells were plated on glass coverslips and transiently transfected one day later as described above. After 24-48h, cells were washed in PBS and fixed using 4% paraformaldehyde (37°C for 15 min). Following three washes with PBS, cells were permeabilized with cold 0.1% Triton X-100 (v/v) in PBS (4°C for 10 min). Afterwards, cells were blocked with 4% normal goat serum or 4% bovine serum albumin in PBS (room temperature for 1h or 4°C overnight). All primary and secondary antibody stainings were performed at room temperature with three PBS washes in between. Prior to mounting, nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (diluted 1:10,000 in PBS from a 1 mg/ml stock) and washed twice with PBS. The following secondary antibodies were used: goat anti-rabbit conjugated to Alexa 488 and goat anti-mouse conjugated to Alexa 546 (Molecular Probes/Invitrogen). Coverslips were mounted with fluorescent mounting medium (Dako, Baar,
RESULTS

All Spry Isoforms Interact with Cav-1 in Vivo

Previously, we had demonstrated that mSpry1 co-localized with endogenous Cav-1 and that Cav-1 co-immunoprecipitated with mSpry1 and mSpry2 in endothelial cells (15). To determine if Cav-1 interacts with all four members of the mammalian Spry family, we first performed co-immunoprecipitation experiments. HEK293T cells were transiently co-transfected with plasmids encoding all four myc-tagged mSpry and myc-tagged Cav-1, and immunoprecipitated using specific anti-Spry sera (Fig. 1). All four mSpry proteins co-immunoprecipitated Cav-1 in a specific fashion, suggesting that this is a general characteristic of the Spry family. Notably, dominant-negative versions of all four Spry family members, in which the highly conserved N-terminal tyrosine residue is mutated (Y53A in mSpry1, Y55A in mSpry2, Y27A in mSpry3, Y53A in mSpry4) (22,41), all co-immunoprecipitated Cav-1 indicating that the conserved N-terminal tyrosine did not play a role in binding of Cav-1 (data not shown).

Next, reverse immunoprecipitation experiments were performed. Lysates from HEK293T cells (transfected as described above) were incubated with anti-Cav-1 antibodies (Fig. 2). Each overexpressed mSpry isoform was co-immunoprecipitated Cav-1 in a specific fashion, suggesting that this is a general characteristic of the Spry family. Notably, dominant-negative versions of all four Spry family members, in which the highly conserved N-terminal tyrosine residue is mutated (Y53A in mSpry1, Y55A in mSpry2, Y27A in mSpry3, Y53A in mSpry4) (22,41), all co-immunoprecipitated Cav-1 indicating that the conserved N-terminal tyrosine did not play a role in binding of Cav-1 (data not shown).

Two Distinct Cav-1 Domains Interact with Spry

To confirm a direct physical interaction between Cav-1 and Spry, we performed GST pull down in vitro binding experiments. Since all Spry proteins behaved in a similar manner in the experiments described above, mSpry1 was used as a representative Spry in the following experiments. GST-Cav-1 fragments (aa 1-61, aa 61-101, aa 1-101, aa 135-178) as well as GST-HA-mSpry2 were used in GST pulldown experiments. Lysates from HEK293T cells transfected with cDNA encoding myc-mSpry1 were incubated with the recombinant purified GST fusion proteins described above. As shown in Fig. 3, myc-mSpry1 interacted specifically with two fragments of Cav-1: aa 61-101 (weakly binding) and aa 135-178 (strongly binding). Unexpectedly, myc-mSpry1 failed to bind the GST-Cav-1 aa 1-101 fusion protein, despite the fact that it was produced and purified to similar levels as the other recombinant proteins (Fig. 3). This observation raises the possibility that the Cav-1 N-terminal 61 aa interfere with the Spry-binding domain (aa 61-101). Furthermore, recombinant purified GST-HA-mSpry2 bound to myc-mSpry1 indicating that mSpry proteins heterodimerize, confirming previously reported results from co-immunoprecipitation and functional experiments (37,67). Altogether, these observations suggest that mSpry1 interacts specifically with the Cav-1 oligomerization/scaffolding domain and the C-terminus of Cav-1 (Fig. 3).

To verify the in vitro mSpry/Cav-1 association and to eliminate the possibility that an unknown additional cellular component was required to mediate mSpry/Cav-1 binding, we performed direct protein-protein binding experiments. Peptide arrays have been used to pinpoint more accurately the sites of protein-protein interactions (65,66). Eighteen overlapping peptides, 12-13 aa in length, that span the Cav-1 sequence, were synthesized and immobilized on nitrocellulose (Fig. 4). The four recombinant purified GST-HA-mSpry proteins were incubated three times with these peptide arrays, and bound proteins were detected with Spry-specific antibodies (Fig. 4). The results from this experiment showed that all four mSpry isoforms bound to Cav-1 at more than one site. All mSpry isoforms interacted to varying degrees with the extreme C-terminal peptide (peptide 18) that corresponds to aa 167-178 of Cav-1. The recombinant proteins also bound, although to a lesser extent, to two other sequences in the C-terminal region: peptide 14 (corresponding to aa 131-142) in the case of all Spry isoforms and peptide 16 (corresponding to aa 149-160) for Spry1, Spry2, and Spry4. In the so-called scaffolding domain (peptides 7-9, which span aa 61-101) of Cav-1, the recombinant Spry isoforms...
bound peptides to varying specificities and degrees: Spry1 (peptides 5, 7 and 8), Spry2 (peptide 9), Spry3 (peptide 7), and Spry4 (peptide 7). These results confirm the previous GST pulldown experiments demonstrating that Spry1 could bind Cav-1 via two different regions: the C-terminal end and the N-terminal region containing the oligomerization and scaffolding domains, with the latter appearing to be more complex. Taken together, the in vitro binding experiments demonstrate that the mSpry/Cav-1 association represents a direct protein-protein interaction with all four Spry family members binding to the N- and C-termini of Cav-1.

The Spry C-terminal End is Necessary and Sufficient for its Interaction with Cav-1

Next, we wanted to determine which region(s) of the Spry proteins were necessary for their binding to Cav-1. As all four Spry isoforms seemed to interact with Cav-1 in a similar manner, we used mSpry2 to address this question. We generated a number of V5- and 6-His-epitope-tagged versions of mSpry2: full-length, the N-terminus domain (mSpry2N, encoding aa 1-176) and the C-terminal domain (mSpry2C, encoding aa 177-315). Each construct was transiently co-transfected along with Cav-1 into HEK293T cells and immunoprecipitations were performed (Fig. 5A). The full-length mSpry2 co-precipitated myc-Cav-1 as has been described above. However, of the two truncated versions of mSpry2, only the C-terminal domain co-precipitated Cav-1. These observations suggest that Spry proteins interact with Cav-1 via their highly conserved cysteine-rich C-termini.

Spry Binding to Cav-1 Requires a Conserved Arginine

To follow up on the observation that the conserved C-terminal Spry domain was necessary and sufficient for interacting with Cav-1, we examined a point mutant of hSpry2, hSpry2-R252D. This mutant has been characterized by its inability to: i) bind to phosphatidylinositol 4,5-bisphosphate (PIP2), ii) translocate to the membrane upon EGF stimulation, and iii) inhibit FGF-mediated p42/44 ERK activation (61). The mutated arginine residue is highly conserved among all Spry family members as well as among the related Spred protein family. hSpry2 and the mutant, hSpry2-R252D, were transiently cotransfected along with Cav-1 into HEK293T cells and immunoprecipitations were performed and analyzed as described above. In contrast to wildtype hSpry2, the mutant hSpry2-R252D did not co-immunoprecipitate myc-Cav-1 (Fig. 5B). Despite lower expression levels for the mutant hSpry2-R252D, the immunoprecipitations were equally efficient for both the wildtype and mutant versions of hSpry2. Next, we determined the localization of the arginine-mutated mSpry1, hSpry2, mSpry3, mSpry4 and Cav-1 under growth factor-stimulated conditions using immunofluorescence microscopy. Cos7 cells were transiently transfected with wildtype or mutant Spry cDNAs, serum-starved, and stimulated with EGF (Fig. 6A, supplemental Fig. S1, and data not shown). mSpry3 and mSpry3-R221D can both be observed on the plasma membrane of EGF-stimulated cells (Fig. 6A). Plasma membrane staining was also apparent not only with wildtype hSpry2, but also in cells expressing the mutant hSpry2-R252D (supplemental Fig. S1). Endogenous Cav-1 was also observed on the plasma membrane of EGF-stimulated Cos7 cells as has been described (68) and significantly co-localized with the wildtype and mutant Spry signals at the cell membrane. Also, no differences in localization were seen between the mutant and wildtype forms of mSpry1 and mSpry4 under these conditions (data not shown). Lastly, to determine the effect of the arginine to aspartic acid mutation on Spry function, HeLa cells were transfected with mSpry3 or mSpry3-R221D, serum-starved and stimulated with EGF for the times indicated and activation of p42/44 ERK was analyzed by immunoblotting (Fig. 6B). While wildtype mSpry3 was able to ablate ERK activation, the mutant was no longer functional. These results suggest that association of Spry proteins with Cav-1 is not required for targeting of Spry to the membrane and that mutant Spry proteins at the membrane are non-functional.

Functional Consequences of the Spry/Cav-1 Interaction

Given that all members of the mSpry family bind to Cav-1, we surmised that this interaction must have functional consequences on Spry activity. To address this possibility, we employed the human breast cancer cell line T47D, lacking Cav-1 expression due to methylation of the Cav-1 promoter (69). T47D cell clones expressing high levels of Cav-1 (T47D-Cav-1) or
EGFP (T47D-EGFP) were generated by stable transfection. These cell lines provided an experimental system where both EGF and FGF2 signaling could be examined in the absence or presence of Cav-1. T47D-Cav-1 grew more slowly than control T47D-EGFP cells (data not shown), as has been previously reported (70). Cav-1 localization depended on cell confluency, since either exogenous or endogenous Cav-1 redistributes to cell-cell contacts as cells become more confluent (51,71). Thus, the inhibitory action of Cav-1 on p42/44 ERK activity was expected to vary according to cell confluency. Indeed, when T47D-Cav-1 and T47D-EGFP cells were plated at high and low cell densities, Cav-1 only inhibited FGF2-mediated p42/44 ERK activation at higher cell densities and not in sparsely seeded cells (Fig. 7). Notably, at low cell densities, Cav-1 inhibited the activity of mSpry2. All mSpry isoforms attenuated FGF2-mediated p42/44 ERK activation in the absence of Cav-1 independently of cell confluency (Fig. 7 and data not shown).

At high cell density, inhibition of p42/44 ERK activation was rather modest with mSpry1 (Fig. 8A) and highly efficient with mSpry2, mSpry3, or mSpry4 (Fig. 8B, 8C, and 8D). In cells where both an mSpry isoform and Cav-1 were present, two patterns could be observed. When either mSpry1 or mSpry3 (Fig. 8A and 8C) were expressed in the presence of Cav-1, FGF2-induced p42/44 ERK activation was synergistically attenuated. However, when either mSpry2 or mSpry4 (Fig. 8B and 8D) were present along with Cav-1, p42/44 ERK activation increased slightly compared to when Cav-1 was present by itself, suggesting that the inhibitory activity of Cav-1 was decreased. These results suggest that FGF2-induced p42/44 ERK activation can be modulated to varying extents by the various Spry proteins in the presence of Cav-1.

To examine the effects on EGF signaling, T47D-Cav-1 and T47D-EGFP cells were infected as described above, serum-starved, stimulated with EGF for the times indicated, and p42/44 ERK activation was analyzed by immunoblotting (Fig. 9). Again, Cav-1 did not affect EGF-mediated p42/44 ERK activation in cells plated at a lower cell density (data not shown). When mSpry proteins were expressed in the absence of Cav-1, only two Spry isoforms, mSpry2 and mSpry3, attenuated EGF-stimulated p42/44 ERK activity independently of cell density (Fig. 9A and 9B, respectively). mSpry1 and mSpry4 had little to no effect on EGF-induced p42/44 ERK activity (data not shown), as has been described previously (15,22). As in the experiment above, the presence of Cav-1 led to two distinct patterns of p42/44 ERK activation. When mSpry2 (Fig. 9A) was expressed in the presence of Cav-1, the levels of phosphorylated p42/44 ERK were slightly elevated when compared to Cav-1 alone, suggesting that the inhibitory activity of Cav-1 was decreased. In marked contrast, when mSpry3 (Fig. 9B) was present along with Cav-1, a cooperative effect was observed in the attenuation of EGF-induced p42/44 ERK activation. These results suggest that in this cell type EGF-induced p42/44 ERK activation is differentially modulated by only two Spry isoforms in the presence or absence of Cav-1.

To determine the status of the mSpry2/Cav-1 interaction during growth factor stimulation, we performed co-immunoprecipitation experiments in AdmSpry2-infected T47D-Cav-1 cells before and during either EGF or FGF2 stimulation. These experiments revealed that the relative amount of Cav-1 interacting with mSpry2 after 15 or 30 min of stimulation did not change compared to unstimulated cells (supplemental Fig. S2). These data suggest that the Spry/Cav-1 interaction is stable and not changing during growth factor stimulation.

In conclusion, in T47D breast cancer cells, various Spry isoforms attenuated p42/44 ERK to varying degrees in the absence or presence of Cav-1 under different growth factor stimulations (summarized in Table 1). mSpry1, which affected only FGF2-stimulated p42/44 ERK, inhibited cooperatively with Cav-1. mSpry2 diminished both FGF2- and EGF-stimulated p42/44 ERK activation independently of Cav-1. mSpry3 was an effective inhibitor of both FGF2 and EGF signaling cascades and acted synergistically with Cav-1. Finally, mSpry4 decreased only FGF2-induced p42/44 ERK activation and did not cooperate with Cav-1. Hence, the above experiments demonstrate that the functional consequences of Spry/Cav-1 interaction are complex, since they appear to be growth factor-, cell density-, and Spry isoform-dependent.
DISCUSSION

In this report, we demonstrate, using various techniques, that all mSpry isoforms directly interact with Cav-1 via specific regions in each protein. Co-immunoprecipitation experiments (Fig. 1 and 2) suggest that all mSpry isoforms bind to Cav-1, independently of the highly conserved tyrosine residue in the N-terminus of the mSpry proteins. GST pulldown (Fig. 3) and peptide-binding (Fig. 4) experiments reveal that mSpry proteins associate with Cav-1 at more than one site. Both the scaffolding domain (aa 61-101) and, to a much greater extent, the C-terminus of Cav-1 (aa 167-178) bind to all four mSpry family members. Yet, mammalian Spry proteins do not possess the Cav-1-binding motif that has been previously identified in other signaling proteins, including Ha-Ras and many RTKs (72). Despite the lack of an easily identifiable Cav-1 binding motif, all four mSpry isoforms also interact with Cav-1’s N-terminal scaffolding domain, the interaction site of other Cav-1 binding motif-containing protein. Similar to the Spry isoforms, other proteins, such as protein kinase A and dynamin-2, have recently been shown to associate with Cav-1 at more than one site suggesting that multiple interactions of this type are not uncommon (66,73,74). The number of Cav-1 binding partners is quite large and the list continues to grow as has been previously noted (75). Thus, it will be important to determine if there are any other caveolar proteins interacting with the Spry/Cav-1 complex.

Interestingly, the GST pulldown experiments demonstrate that Spry proteins can form heterodimers (Fig. 3), as has been suggested previously (22,37). Such heterodimers differ functionally from homodimers due to the binding of different proteins to the various Spry isoforms (67). Spry isoforms are co-expressed in various cell types and can act as agonists or antagonists depending on the growth factor stimulus and cell context. Together, these observations imply that Spry hetero-oligomers may have different functions when compared to Spry homooligomers. Recently, purified recombinant Spry2 has been reported to form large spheres composed of approximately 24 subunits with iron-sulfur-binding capabilities (76). This multimeric Spry2 structure is proposed to act as a multifunctional protein complex, not only inhibiting growth factor signaling but also having a sensing function for redox state, nitric oxide or both. This hypothesis as well as the question of the functional specificity of possible Spry hetero-multimers warrants further investigation.

That the C-terminus of mSpry2 is required for Cav-1 association (Fig. 5A) is not surprising, given that all mSpry isoforms associate with Cav-1 and possess the so-called highly conserved, cysteine-rich Spry domain (aa 178-301 in mSpry2). This C-terminal domain is required for targeting hSpry2 to the membrane upon growth factor activation, as well as for inhibiting cell proliferation and migration (14,30). Further mutational analysis by Lim and co-workers, revealed that a single amino acid exchange mutant, hSpry2-R252D, was translocation defective, could not bind PIP2, and did not inhibit FGF-induced p42/44 ERK (61). We found that the hSpry2 mutant did not co-immunoprecipitate Cav-1, yet still localized to the membrane upon growth factor stimulation (Fig. 5B and supplemental Fig. S1). Furthermore, when we investigated this mutation in the context of the other Spry isoforms, we observed that they still localized to the plasma membrane upon growth factor stimulation (Fig. 6B and supplemental Fig. S1). Taken together, these data suggest that Spry translocation to the plasma membrane is independent of Cav-1 binding and that the highly conserved arginine is critical for Spry function, as previously reported (61).

The four Spry family members appear to bind various proteins, although here we demonstrate that they all have a common binding partner, Cav-1. We investigated the functional consequences of this interaction to better understand the differences in Spry isoform activity. Spry function in the presence and absence of Cav-1 was systematically analyzed using human T47D cells expressing no or high levels of Cav-1 in combination with adenoviral expression of mSpry isoforms (Fig. 7-9 and Table 1). Unlike Cav-1 inhibitory activity, Spry proteins alone function independently of cell density. However, at lower cell densities, Cav-1 inhibits Spry function (Fig. 7). All four Spry family members inhibit FGF2-mediated p42/44 ERK activity. Yet, while mSpry2- and mSpry4-mediated attenuation...
is not cooperative with that of Cav-1, mSpry1 and mSpry3 can decrease p42/44 ERK activation in a cooperative manner with Cav-1, similar to recent observations made with Spred-1 and Cav-1 (77). In the case of EGF-induced p42/44 ERK activation, only the combination of mSpry3 and Cav-1 results in a synergistic inhibition of p42/44 ERK activity, whereas mSpry2 inhibits growth-factor mediated p42/44 ERK activity independently of Cav-1. Notably, during growth factor stimulation, the levels of Cav-1 associating with mSpry2 do not change suggesting a stable interaction (supplemental Fig. S2).

Also, the activity of the Spry isoforms in the absence of Cav-1 varies dramatically between each other in T47D cells: mSpry2 clearly inhibits EGF-induced p42/44 ERK inhibition (as opposed to potentiating it), while mSpry1 and mSpry4 do not. Curiously, Spry2 (either human or mouse, no species-specific differences have been identified so far), the most studied isoform to date, has been reported to inhibit, potentiate or have no effect on EGF-mediated p42/44 ERK activation, yet it attenuates VEGF-, FGF- and HGF- mediated p42/44 ERK signaling (5-7,29). Similar contrasting observations have been made with Spry1 and Spry4 in the case of EGF signaling (15,22,43,78,79). The reasons for these seemingly contradictory observations are unclear, however, cell context, the method of Spry overexpression (transfection or viral infection), and cell types may be critical factors. Differences in tyrosine phosphorylation of three of the four Spry family members have been reported and may partially explain the bimodal function, either agonistic or antagonistic, of these proteins (37,43). For example, Spry1 is phosphorylated after FGF and PDGF stimulation, Spry2 is phosphorylated following EGF and FGF stimulation, while Spry4 is not phosphorylated following exposure to any of these growth factors. More recently, Rubin and co-workers demonstrated that different tyrosine residues found in the C-terminal domain of Spry2 were phosphorylated in distinct patterns depending on the stimulus (either EGF or FGF), thus imparting a discriminatory role on this domain (38). Spry differences in function may also vary from cell to cell depending on which components of the RTK signalosome are present and activated by different ligands (3). Thus, more work needs to be done to fully understand the differences between the four isoforms and which growth factors utilize which Spry isoform for the attenuation of p42/44 ERK activity.

Cav-1 is a major protein of caveolae, and an essential role of caveolae is to assemble and locally concentrate components of signal transduction cascades. Our results suggest that targeting of Spry to the plasma membrane upon growth factor stimulation is independent of its interaction with Cav-1 (Fig. 6 and supplemental Fig. S1). However, the fact that Spry function could be modulated by the presence of Cav-1 at high and low cell densities illustrates the importance of membrane microdomains. At present, at least three types of negative regulators of growth factor-mediated signaling are found in caveolae/lipid rafts: Cav-1, Spry, and Spred (15,77). These observations raise a number of important questions. What other proteins are the four Spry isoforms interacting with? Which ones are required for their agonistic and antagonistic activities? Cell context is important in understanding such molecular mechanisms, since Spry proteins exert differential functions in various cell types and since Cav-1 is ubiquitously, yet variably expressed (80). Cav-1 levels, which are higher in terminally differentiated cell types, such as adipocytes and endothelial cells, could influence Spry function. Moreover, different cell types have various repertoires of Spry and Spred expression. While keeping in mind the growth factor-, cell density- and Spry isoform-dependent nature of the Spry/Cav-1 association, studying these variables on a more quantitative level will be necessary to properly understand the function of each Spry isoform in the regulation of signal transduction and, hence, in many physiological and pathological processes.

REFERENCES


FOOTNOTES

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The abbreviations used are: Spry, Sprouty; Cav-1, Caveolin-1; aa, amino acids; MAPK, mitogen activated protein kinase; ERK, extracellular-regulated kinase; m, mouse; h, human; Ad, adenovirus; EGFP, enhanced green fluorescent protein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; FGF2, basic FGF; EGF, epidermal growth factor; HGF, hepatocyte growth factor; PDGF, platelet-derived growth factor; RTK, receptor tyrosine kinase; DAPI, 4',6-diamidino-2-phenylindole; pp42/44 ERK, phosphorylated p42/44 ERK.

FIGURE LEGENDS
**Fig. 1. Cav-1 co-immunoprecipitates with all mSpry isoforms.** HEK293T cells were co-transfected with cDNAs encoding for each of the designated myc-tagged Spry proteins and either myc-tagged Cav-1 or the empty vector, as indicated. After 48h, lysates were prepared and immunoprecipitations (IP) were performed using specific anti-Spry antisera (+) or preimmune sera (-). Immunoblots (IB) were probed with anti-myc antibodies. Equal amounts of protein (25 µg) were loaded in each lysate lane (Lys). The results shown are representative of four independent experiments.

**Fig. 2. All mSpry isoforms co-immunoprecipitate with Cav-1.** HEK293T cells were co-transfected with cDNAs encoding for each of the myc-tagged Spry proteins and myc-tagged Cav-1 as indicated. After 48h, lysates were prepared and immunoprecipitations (IP) were performed using anti-Cav-1 polyclonal antisera (+) or normal rabbit IgG (-). Immunoblots (IB) were sequentially probed with anti-Cav-1 monoclonal antibodies and specific anti-Spry antisera. The blots were stripped between probings. Equal amounts of protein (25 µg) were loaded in the total cell lysates blot.

**Fig. 3. mSpry1 binds Cav-1 fragments and mSpry2 in vitro.** A schematic of the Cav-1 protein illustrates where the various domains are situated in relation to the GST-Cav-1 fusion proteins (top panel). All GST fusion proteins were prepared and purified as described in Materials and Methods. Purified fusion proteins bound to GST beads were incubated overnight with lysates from HEK293T cells transfected with myc-tagged mSpry1. After washing, the resulting purified proteins were resolved by SDS-PAGE, transferred to PVDF membrane and immunoblots (IB) probed with anti-myc antibodies (left bottom panel). Levels of purified fusion proteins were visualized by Coomassie staining of SDS-PAGE gels (right bottom panel).

**Fig. 4. Peptide mapping of mSpry/Cav-1 interactions.** A schematic of the Cav-1 protein depicts where the various domains are situated in relation to the eighteen peptides. The sequence of the overlapping peptides is also shown on the left-hand side. Peptide arrays comprising of eighteen 12-13mers spanning the Cav-1 sequence were generated by spotting onto nitrocellulose (Sigma-Genosys). The membranes were incubated with recombinant Spry proteins and then with specific anti-Spry antibodies to detect bound Spry proteins as indicated. Recombinant protein binding to the peptide was quantitated by densitometry. Bar graphs represent percent above background (referenced to the dot with the lowest signal in each blot) with the error bars indicating the standard deviation between three independent experiments. Levels of purified recombinant Spry fusion proteins were visualized by Coomassie staining of SDS-PAGE gels (bottom left panel).

**Fig. 5. The C-terminus of mSpry2 co-immunoprecipitates Cav-1, whereas hSpry2-R252D fails to bind Cav-1.** A, HEK293T cells were co-transfected with cDNAs encoding for different versions of mSpry2-V5-His (F, full-length; N, N-terminal; C, C-terminal) or the empty vector (v) and myc-tagged Cav-1. After 48h, lysates were prepared and immunoprecipitations (IP) were performed using goat anti-6-His polyclonal antisera (+) or normal goat IgG (-). Immunoblots were probed with anti-myc and anti-V5 monoclonal antibodies. Equal amounts of protein (25 µg) were loaded in the total cell lysates blot. B, HEK293T cells were co-transfected with cDNAs encoding either hSpry2 or hSpry2-R252D and either myc-tagged Cav-1 or the empty vector. After 48h, lysates were prepared and immunoprecipitations (IP) were performed using specific anti-Spry2 antisera (+) or preimmune sera (-). Immunoblots were probed with the antibodies indicated. Equal amounts of protein (25 µg) were loaded in each lysate lane. The results shown are representative of three independent experiments.

**Fig. 6. Co-localization of wildtype and mutant mSpry3 with Cav-1 and loss of function of mSpry3-R221D.** A, Cos7 cells were transfected with cDNAs encoding for either myc-mSpry3 or myc-mSpry3-R221D. After 24h, cells were serum-starved overnight, and the following day the cells were stimulated with 50 ng/ml EGF for 10 min. Cells were fixed, permeabilized, and incubated with the primary antibodies indicated and the appropriate secondary antibodies. Images were acquired on a Carl Zeiss LSM
510 Meta confocal microscopy system. Bars = 10 µm. B, HeLa cells were transfected with cDNAs encoding for either myc-mSpry3 or myc-mSpry3-R221D. After 24h, cells were serum-starved overnight, and the following day the cells were stimulated with 50 ng/ml EGF for the times indicated. Lysates were prepared and resolved by SDS-PAGE. Equal amounts of protein were loaded in each lane. The resulting immunoblots were probed simultaneously with the indicated antibodies and visualized using an Odyssey fluorescence Imager.

**Fig. 7. Inhibitory activity of Cav-1 is dependent of cell density.** T47D cells stably transfected with either murine Cav-1 (T47D-Cav-1) or EGFP (T47D-EGFP) were plated at low and high cell densities, infected with AdmSpry2 or control virus AdLite. Cells were stimulated with 50 ng/ml FGF2 for the times indicated. Lysates were prepared and resolved by SDS-PAGE. Equal amounts of protein were loaded in each lane. The resulting immunoblots were sequentially probed with the indicated antibodies. Levels of phosphorylated p42/44 ERK (pp42/44 ERK) were quantitated by densitometry.

**Fig. 8. Modulation of Spry function in FGF2-stimulated cells by Cav-1.** T47D cells stably transfected with either murine Cav-1 (T47D-Cav-1) or EGFP (T47D-EGFP) were infected with A, AdmSpry1, B, AdmSpry2, C, AdmSpry3, D, AdmSpry4 or control virus AdLite. Cells were stimulated with 50 ng/ml FGF2 for the times indicated. Lysates were prepared and resolved by SDS-PAGE. Equal amounts of protein were loaded in each lane. The resulting immunoblots were sequentially probed with the indicated antibodies. Levels of phosphorylated p42/44 ERK (pp42/44 ERK) were quantitated by densitometry. Quantitation was also performed using the Odyssey Imager system to confirm the results obtained by densitometry (data not shown).

**Fig. 9. Modulation of Spry function in EGF-stimulated cells by Cav-1.** T47D-Cav-1 and T47D-EGFP were infected with A, AdmSpry2, B, AdmSpry3 or control virus AdLite. Cells were stimulated with 50 ng/ml EGF for the times indicated. Lysates were prepared and resolved by SDS-PAGE. Equal amounts of protein were loaded in each lane. The resulting immunoblots were sequentially probed with the indicated antibodies. Levels of pp42/44 ERK were quantitated by densitometry. Quantitation was also performed using the Odyssey Imager system to confirm the results obtained by densitometry (data not shown).
Table 1. Summary of ERK Inhibition Profiles in T47D Cells

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<tr>
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Figure 2

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**IB:**
- anti-Cav-1
- anti-Spry1
- anti-Spry2
- anti-Spry3
- anti-Spry4

**Total Cell Lysates**
Figure 3

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GST
GST Cav aa 1-61
GST Cav aa 61-101
GST Cav aa 1-101
GST Cav aa 135-178
GST HA-Spy2
Cell lysate (myc-Spy1)
Cell lysate (vector only)

IB: anti-myc
HEK293T Cell Lysates
mycSpry1
vector

Coomassie-stained gel of GST proteins

kDa
112
83
59.5
51
38
35
30
25
70
60
50
40
30
25
Figure 4

Cav-1 Peptides
1. MSGGKYDVSEGHL
2. GHLTYVPIREQGN
3. QGIYKPPNNKAMA
4. AMADEVTEKQVYD
5. VYDAHTKEIDLVN
6. LVNRDPKHLNDDV
7. DDVVKIDFEDVIA
8. VIAEPFEGTHSFDG
9. FGDGWASFTFTT
10. TFTVTKTFYRLL
11. KLLSTIFGPIMAL
12. MALIINGYFAILS
13. ILSFLHIWAVVPC
14. VPICKSFLIEIQ
15. EIQCISRKVSYSIY
16. SIYHTFCDPLF
17. PFLPEAIGKIFSN
18. FSNIRSTGKEI

GST-HA Spry
1 2 3 4
Figure 5 A

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IB: anti-myc

IB: anti-V5

Total Cell Lysates
Figure 5B

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anti-Spry2
Figure 6 A

starved

myc-mSpry3

+ EGF

starved

myc-mSpry3R221D

+ EGF

anti-myc  anti-Cav-1  DAPI  Merge


**Figure 7**

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**pp42/44 ERK levels (%)**

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**IB:**

- dense: pp42/44 ERK
- sparse: p42/44 ERK
Figure 8 A

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## Figure 8 B

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Figure 8 C

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Figure 8 D

AdVirus T47D min FGF2

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IB:
- pp42/44 ERK
- pp42/44 ERK levels (%)
- p42/44 ERK
- Spry4
- Caveolin-1
- EGFP
### Figure 9 A

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- **pp42/44 ERK**
- **pp42/44 ERK levels (%)**
- **p42/44 ERK**
- **Spry2**
- **Caveolin-1**
- **EGFP**

JPEG: 18x12 to 594x780

596x382
Figure 9 B

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- pp42/44 ERK
- pp42/44 ERK levels (%)
- p42/44 ERK
- Spry3
- Caveolin-1
- EGFP
A functional interaction between sprouty proteins and caveolin-1
Miguel A. Cabrita, Fabienne Jaggi, Sandra P. Widjaja and Gerhard Christofori

J. Biol. Chem. published online July 28, 2006

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