Efficient suppression of FGF-2-induced ERK activation by the cooperative interaction among mammalian Sprouty isoforms

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Summary

Strict regulation of the receptor tyrosine kinase (RTK)/extracellular signal-regulated kinase (ERK) pathway is essential for maintaining balanced growth in multi-cellular organisms. Several negative regulators of the pathway have been identified which include Sprouty proteins. Mammalian cells express four Sprouty isoforms (Sprouty1-4) in an ERK-dependent manner. In this study, we have examined the molecular mechanisms by which Sprouty proteins elicit their inhibitory effects on the RTK/ERK pathway, with special focus on the co-operation among Sprouty isoforms. The four mammalian Sprouty isoforms interact with each other, most probably to form hetero- as well as homo-oligomers through their C-terminal domains. Sprouty1 specifically interacts with Grb2, whereas Sprouty4 interacts with Sos1. Although any of the Sprouty isoforms by itself inhibits the fibroblast growth factor-2 (FGF-2)-induced activation of the ERK pathway significantly, hetero-oligomers show a more pronounced inhibitory activity. The hetero-oligomer formed between Sprouty1 and Sprouty4 exhibits the most potent inhibitory effect on ERK activation through its highly effective ability to suppress the association of Grb2-Sos1 complex with FRS2. The cooperative interactions observed among Sprouty isoforms could represent an advanced system that functions to regulate strictly the activation state of the RTK/ERK pathway in mammalian cells.

Supplementary material available online at
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Key words: Sprouty, ERK pathway, Receptor tyrosine kinase, Negative feedback inhibition, FGF

Introduction

Growth factor signaling mediated by receptor tyrosine kinases (RTKs) plays a central role in the regulation of a variety of responses in target cells, such as proliferation, differentiation and migration (Schlessinger, 2000; Simon, 2000). Aberrant activation of the RTK signaling pathway has been linked with cancer as well as with disorders of developmental processes (Hanahan et al., 2000; Hunter, 2000; Blume-Jensen and Hunter, 2001; Zwick et al., 2002; Voas and Rebay, 2004), implying that the intensity and duration of the activation state of this pathway must be regulated quite strictly. Negative feedback inhibition is one of the mechanisms that provide an effective way to terminate or modulate the RTK signaling. In this respect, the RTK pathway has been shown to induce the expression of its own negative regulators, which include mitogen-activated protein (MAP) kinase phosphatases (Camps et al., 1999; Farooq and Zhou, 2004) and Sprouty proteins (Minowada et al., 1999; Ozaki et al., 2001; Sasaki et al., 2001).

Sprouty was originally identified in *Drosophila* (dSprouty) as a feedback inhibitor of fibroblast growth factor (FGF) signaling during tracheal branching (Hacohen et al., 1998). Subsequent studies have shown that dSprouty also antagonizes epidermal growth factor (EGF) receptor and other RTK signaling pathways during *Drosophila* organogenesis (Casci et al., 1999; Kramer et al., 1999; Reich et al., 1999). However, its mode of action remains rather controversial; i.e. dSprouty has been shown to inhibit the activation of extracellular signal-regulated kinase (ERK) upstream of Ras during fly eye development (Casci et al., 1999), whereas it appears to inhibit ERK activation downstream of Ras during wing development (Reich et al., 1999).

Unlike *Drosophila*, mammalian cells express at least four Sprouty isoforms (Sprouty1-4) (Minowada et al., 1999; Impagnatiello et al., 2001), whose expression is dependent on the ERK signaling pathway (Ozaki et al., 2001; Sasaki et al., 2001). These proteins have highly conserved C-terminal domains and highly variable N-terminal domains. As in *Drosophila*, mammalian Sprouty proteins function as feedback inhibitors of FGF signaling during organogenesis (Minowada et al., 1999; Tefft et al., 1999). However, the molecular mechanism by which each Sprouty isoform elicits its function as well as the physiological significance of the expression of four Sprouty proteins in mammalian cells have not been well established.

Over-expression of Sprouty1, Sprouty2 or Sprouty4 inhibits FGF- and vascular endothelial growth factor-induced proliferation, migration and differentiation by repressing pathways leading to ERK activation (Impagnatiello et al., 2001; Lee et al., 2001; Sasaki et al., 2001; Yigzaw et al., 2001; Hanafusa et al., 2002; Sasaki et al., 2003). By contrast, Sprouty1/2 are unable to repress EGF-induced ERK activation, but potentiate it by attenuating Cbl-mediated endocytosis of the

EGF receptor (Egan et al., 2002; Wong et al., 2002). Sprouty1/2 can inhibit FGF-induced ERK activation by preventing the recruitment of the Grb2-Sos complex to the FGF docking adaptor protein FRS2 or SH2-containing protein-tyrosine phosphatase Shp2 (Hanafusa et al., 2002), or by acting downstream of the Grb2-Sos complex (Gross et al., 2001). It should also be mentioned that members of the mammalian Sprouty family exhibit slightly different activities and interact with different partners, which include c-Cbl, Grb2, Raf1, FRS2, caveolin-1, dual specificity kinase TESK1 and protein tyrosine phosphatase PTP1B (Christofori, 2003). It seems probable that mammalian Sprouty proteins exert their specific functions through multiple mechanisms, which probably depend on the type of growth factor stimulation, the cell type, and/or the experimental conditions.

In the present study, we have examined the molecular mechanisms by which Sprouty proteins elicit their inhibitory effects on the RTK signaling pathway, with special focus on the cooperation among Sprouty isoforms. For the analysis, we have utilized 293T cells expressing limited amounts of exogenous Sprouty isoforms as well as Swiss 3T3 cells stimulated with FGF-2. Our results demonstrate that the four mammalian Sprouty isoforms form hetero- as well as homo-oligomers through their C-terminal domains. Although Sprouty1 or Sprouty4 alone significantly inhibited the FGF-2-induced ERK activation by sequestering Grb2 or Sos1, respectively, the hetero-oligomer formed between these two Sprouty isoforms markedly suppressed the ERK activation by inhibiting the association of the Grb2-Sos1 complex with FRS2 more effectively.

Materials and Methods

Materials

Human recombinant FGF-2 and EGF were purchased from PeproTech, Inc. The monoclonal anti-Myc antibody (sc-40), the polyclonal anti Grb2 antibody (sc-225), the polyclonal anti-Sos1 antibody (sc-256), the monoclonal anti-hemagglutinin (HA) antibody (sc-7392), the polyclonal anti-FRS2 antibody (sc-8318), the polyclonal anti-Raf-1 antibody (sc-133), the monoclonal anti-H-Ras antibody (sc-35), the monoclonal anti-RasGAP antibody (sc-63), the polyclonal anti-Sprouty1 antibody (sc-18599), the polyclonal anti-Sprouty4 antibodies (sc-18607 and sc-18609), and Grb2-agarose (sc-4015AC) were obtained from Santa Cruz Biotechnology. The monoclonal anti-Flag antibody (F3165), the monoclonal anti-phosphoERK1/2 antibody (M8159), and the monoclonal anti-actin antibody (A4700) were from Sigma. The polyclonal anti-Shc antibody (06-203) was from Upstate Biotechnology. The polyclonal anti-Sprouty2 antibody (ab1043) was from Abcam. Glutathione-sepharose 4B was purchased from Amersham Biosciences. PD184352 was synthesized as described previously (Tanimura et al., 2003). Other chemicals and reagents were of the highest purity available.

Plasmids

Full-length cDNAs of mouse Sprouty1 (GenBank Accession Number: AF176903), human Sprouty2 (GenBank: BC004205), human Sprouty3 (NCB: NM_005840), mouse Sprouty4 (GenBank: AF176906), human Grb2 (GenBank: AF498925) and truncated forms of mouse Sprouty1 (nt 1-522 or nt 525-942) were amplified by reverse transcriptase-polymerase chain reaction using poly $(A)^+$ RNA isolated from human TIG3 fibroblasts or mouse brain. Amplified products were subcloned into pEF1/Myc (Invitrogen), pcDNA3.1 (Invitrogen) with a Flag-tag at the N-terminus, or into pcDNA3.1 with an HA-tag at the C-terminus. Sequences were verified by DNA sequencing. The HA-tagged ERK2 expression plasmid was kindly donated by Michael J. Weber (University of Virginia Health Science Center).

Cell culture and transfection

Human embryonic kidney 293T cells and Swiss 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. For transfection experiments, 293T cells were plated at a density of 5×10^5 cells per 35 mm dish and incubated for 12 hours. Cells were transfected for 24 hours with appropriate expression plasmids in LipofectAMINE 2000 reagent (Invitrogen) as described in the manufacture's standard procedure. The total amount of plasmids transfected in each experiment was adjusted to 3 μg by the addition of empty plasmid (pcDNA3.1). In some experiments, cells were serum-starved for 6/24 hours in serum-free medium (DMEM containing 2 mg/ml bovine serum albumin, 1 μg/ml insulin, 2 μg/ml transferrin, 30 nM Na_2SeO_3 and 10 mM Hepes, pH 7.4) (Iwasaki et al., 1999; Tanimura et al., 2002) prior to stimulation with FGF-2 (20 ng/ml), EGF (20 ng/ml) or phorbol 12-myristate-13-acetate (PMA) (10 ng/ml).

Cell lysis and immunoblotting

Cells were scraped off plates into a hypotonic cell lysis buffer (25 mM Tris-HCl, pH 7.4, 25 mM NaCl, 0.5 mM EDTA, 1 mM sodium orthovanadate, 10 mM NaF, 25 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 0.2 mM sodium molybdate, 10 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride and 1% Triton X-100) and lysed by sonication for 60 seconds. Lysates were cleared by centrifugation for 30 minutes at 15,000 ***g***, and protein concentrations were determined by using the BCA protein assay reagent (Pierce). Cell lysates were separated by SDS-PAGE, electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes and probed with respective primary antibody and horseradish peroxidase-conjugated secondary antibody (Promega). Proteins were visualized with the enhanced chemiluminescence system (Amersham Biosciences) (Hoshino et al., 2001).

Co-immunoprecipitation assay

Cells were lysed in IP lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1 mM sodium orthovanadate, 10 mM NaF, 25 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 0.2 mM sodium molybdate, 10 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride and 1% Nonidet P-40). Cell lysates (500 μg of protein) were incubated overnight at 4°C with 1 μg of respective precipitation antibody with gentle rocking. Immuno-complexes were collected on protein A/G plus-agarose (sc-2003, Santa Cruz Biotechnology), washed three times with IP wash buffer (25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5 mM EDTA, 1 mM sodium orthovanadate, 10 mM NaF, 25 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 0.2 mM sodium molybdate, 10 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, and 0.5% Nonidet P-40), heated for 3 minutes at 95°C in SDS-PAGE sample buffer, resolved by SDS-PAGE and then analyzed by immunoblotting as described above.

Pull-down assay

293T cells transfected with the plasmid encoding Myc-tagged Sprouty1 or Sprouty2 were stimulated with 20 ng/ml EGF for 15 minutes and lysed in the IP lysis buffer. Cell lysates (500 μg) were incubated with Grb2-agarose or glutathione-Sepharose 4B beads overnight at 4°C with gentle rocking. Bound complexes were collected by centrifugation, washed three times with the IP wash buffer, boiled in SDS-sample buffer, resolved by SDS-PAGE and analyzed by immunoblotting as described above.

Small interfering RNA (siRNA) 'knockdown' experiment

The following sequences targeting mouse Sprouty1 or Sprouty4 and their respective scrambled sequences as controls were designed to generate Stealth™ siRNA duplex oligoribonucleotides (Invitrogen): for mouse Sprouty1, 5′-GCAGGAAAGGACTCATGAAATCATA-3′ (sense), 5′-GCAGAAATCAGGTACTAAACGGATA-3′ (scrambled); for mouse Sprouty4, 5′-GCCTGGGCTACTTGGTACCATTGTA-3′ (sense), 5′-GCCTCGGTTCAATGGTACCTGTGTA-3′ (scrambled). Swiss 3T3 cells were plated at a density of 6×10^4 cells per 35 mm dish and incubated for 12 hours, and then transfected with appropriate siRNAs (final concentration: 50 nM) for 24 hours using LipofectAMINE 2000 (Invitrogen).

Semi-quantitative reverse transcription (RT)-PCR

Swiss 3T3 cells transfected with appropriate siRNA were serum-starved for 24 hours and then stimulated with 20 ng/ml FGF-2 for 1 hour (for the analysis of Sprouty1 expression) or 2 hours (for the analysis of Sprouty4 expression) (Ozaki et al., 2001). Total RNA was isolated using ISOGEN™ reagent (Nippon Gene, Tokyo). RT-PCR was performed with ThermoScript™ RT-PCR system (Invitrogen) using 2 μg of total RNA. Sets of primers, 5′-GGCCCCCCGGGC-CCTGCC-3′/5′-TTGCCCTGAGCCCTTGAG-3′ or 5′-CCCCGGT-TCCACAGAGCA-3′/5′-CGGAGCTGTCGGGCCTTT-3′, were used to amplify portions of mouse Sprouty1 or Sprouty4 cDNA, respectively, resulting in generation of a 372 or 443 bp fragment. Conditions for PCR cycles were: 94°C for 30 seconds, 58°C for 42 seconds, 72°C for 1 minute, and a final extension step at 72°C for 10 minutes. A portion of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was co-amplified as an internal control with the following primer pair to generate a 250 bp fragment: 5′-GTATGACTCCACTCACGGCA-3′/5′-CAAACATGGGGGCATCG-GCA-3′. Amplified products were separated on 2.0% agarose gels.

Results

Mammalian Sprouty isoforms form homo-/hetero-oligomers through their C-terminal cysteine-rich domains

All four mammalian Sprouty proteins consist of conserved C-terminal cysteine-rich domains and highly divergent N-terminal domains. As cysteine-rich regions participate in oligomer formation of several proteins (Gum, 1992; Wallis and Drickamer, 1999), we first examined whether or not Sprouty isoforms interact with each other to form homo-/hetero-oligomers through their C-terminal domains. For the analysis, we constructed two series of expression vectors encoding Sprouty1-4 with either a Flag-tag at the N-terminus (F1, F2, F3 and F4) or a Myc-tag at the C-terminus (1M, 2M, 3M and 4M), and transfected two of them in combination into 293T cells, i.e. one encoding each of either Flag-tagged Sprouty isoforms and the other encoding each of either Myc-tagged Sprouty isoforms. The amount of Sprouty expression plasmid transfected was fixed to be 0.5 μg/cells in a 35 mm dish; under such conditions, expression level of each Sprouty protein was rather similar to that observed in Swiss 3T3 cells stimulated with FGF-2 (see supplementary Fig. S1).

Immunoprecipitation with anti-Myc/anti-Flag antibody and immunoblotting with anti-Flag/anti-Myc antibody showed that all Sprouty isoforms interacted with each other, most probably to form hetero- as well as homo-oligomers (Fig. 1A,B) (see Discussion). Furthermore, expression of Myc-tagged Sprouty1, 2, 3 or 4 in combination with either Flag-tagged Sprouty1 N-terminal fragment (residues 1-174; F1n) or Flag-tagged Sprouty1 C-terminal fragment (residues 175-313; F1c) followed by co-immunoprecipitation experiments revealed that C-terminal cysteine-rich domains were responsible for oligomer formation of the Sprouty isoforms (Fig. 1C,D, and data not shown).

Sprouty1 interacts specifically with Grb2, whereas Sprouty4 interacts with Sos1

As the N-terminal domains of the four Sprouty isoforms differ significantly in their structure, it is conceivable that each Sprouty protein could interact with a distinct signaling molecule to interrupt the RTK pathway. To address this possibility, we examined by co-immunoprecipitation assay whether or not each Sprouty isoform could associate specifically with Grb2, Sos1, Ras, Ras-GAP or Raf-1. These proteins are known to function in the growth factor-induced ERK activation downstream of RTK. Furthermore, their possible interaction with Sprouty proteins has been reported (Hanafusa et al., 2002; Tefft et al., 2002; Sasaki et al., 2003).

293T cells were transfected with Flag-tagged Sprouty1, Sprouty2, Sprouty3 or Sprouty4, stimulated with EGF or FGF, immunoprecipitated with anti-Grb2 antibody, and then probed with anti-Flag antibody. As shown in Fig. 2A, a specific association of Grb2 with Sprouty1 but not with Sprouty2, Sprouty3 or Sprouty4 was observed. Although a small amount of Sprouty1 was detected in the immunoprecipitates obtained from unstimulated control cells, it was markedly increased by stimulation of the cells with EGF or FGF-2. Furthermore, a pull-down assay using Grb2-agarose beads, performed on the lysates of EGF-stimulated cells transfected with Myc-tagged Sprouty1 or Sprouty2, confirmed that Grb2 specifically interacted with Sprouty1 but not with Sprouty2 (Fig. 2B).

Similar experiments using anti-Sos1 antibody revealed that Sprouty4, but not other Sprouty isoforms, was co-precipitated with Sos1. Unlike the interaction between Grb2 and Sprouty1, association of Sprouty4 with Sos1 appeared to be constitutive and was not affected significantly by stimulation of the cells with EGF or FGF-2 (Fig. 2A). On the contrary, co-immunoprecipitation assays using anti-Ras, anti-RasGAP or anti-Raf-1 antibody did not reveal any specific association of Sprouty isoforms with these signaling molecules, regardless of whether or not the cells were stimulated with EGF or FGF-2 (Fig. 2A, and data not shown).

When the experiments were performed on 293T cells transfected with Myc-tagged Sprouty1 and either Flag-tagged Sprouty2, Sprouty3 or Sprouty4 in combination, anti-Grb2 antibody immunoprecipitates contained not only Sprouty1 but also the other respective Sprouty isoform. Similarly, anti-Sos1 antibody immunoprecipitates contained not only Sprouty4 but also the other respective Sprouty isoform when the experiments were performed on 293T cells transfected with Myc-tagged Sprouty4 and either Flag-tagged Sprouty1, Sprouty2 or Sprouty3 in combination (Fig. 2C). Such observed interaction between Grb2 and Sprouty2/3/4, or that between Sos-1 and Sprouty1/2/3, however, was considered not to be direct but through Sprouty1 or Sprouty4, respectively; all Sprouty isoforms form hetero-oligomers with other Sprouty isoforms through their C-terminal domains in the cells (Fig. 1).

Co-expression of Sprouty1 and Sprouty4 efficiently suppresses the ERK activation induced by FGF-2, but not by EGF or PMA

We examined the inhibitory effect of three major Sprouty isoforms on the mitogen-induced ERK activation. Sprouty3 is rather a minor isoform whose expression level has been found to be only limited in a variety of mammalian cells (see supplementary Fig. S2) and thus we have not examined further the functional significance of Sprouty3. The transfection efficiency of any gene into 293T cells was at most ~50% under our experimental conditions, which made it difficult to accurately determine the effect of exogenously expressed Sprouty proteins on the activation of endogenous ERK1/2. Thus, 293T cells were transfected with either Flag-tagged Sprouty 1, 2 or 4 together with HA-tagged ERK2, and stimulated for 15 minutes with EGF, FGF-2 or PMA. Immunoblot analysis on the anti-HA antibody-immunoprecipitates with the use of anti-phospho ERK1/2 revealed that all of these Sprouty isofoms efficiently suppressed the ERK activation induced by FGF-2. On the contrary, none of them significantly inhibited the ERK activation induced by EGF or PMA (Fig. 3A). Inability of Sprouty2/4 to interfere with the EGF-induced activation of the ERK pathway has also been reported by other investigators (Impagnatiello et al., 2001; Sasaki et al., 2001).

We next examined the possibility that hetero-oligomerization of Sprouty isoforms might enhance the inhibitory effect of each Sprouty isoform. As shown in Fig. 3B, co-expression of Sprouty1 and Sprouty4 (1:1) in 293T cells clearly caused a more efficient suppression of FGF-2-induced ERK activation than the expression of either Sprouty1 or Sprouty4 alone. Co-expression of Sprouty1 and Sprouty2 also suppressed the ERK activation slightly more effectively than that observed with the expression of each Sprouty isoform alone. However, co-expression of Sprouty1 and Sprouty4 did not inhibit the EGF- or PMA-induced ERK activation in the cells (Fig. 3, and data not shown).

Co-expression of Sprouty1 and Sprouty4 efficiently inhibits the FGF-2-induced association of Grb2 to FRS2

We examined the molecular mechanism by which co-expression of Sprouty1 and Sprouty4 efficiently suppresses the FGF-2-induced ERK activation. Unlike EGF receptor, FGF receptor lacks binding sites for the SH2 domain of Grb2. Consequently, FGF-2-induced ERK activation is mediated by the recruitment of Grb2/Sos to the plasma membrane through FRS2. FRS2 is a lipid-anchored docking protein and is phosphorylated at multiple tyrosine residues in response to FGF receptor activation, providing the binding site for the SH2 domain of Grb2 (Kouhara et al., 1997; Hadari et al., 1998). Furthermore, involvement of other adaptor proteins, Shc

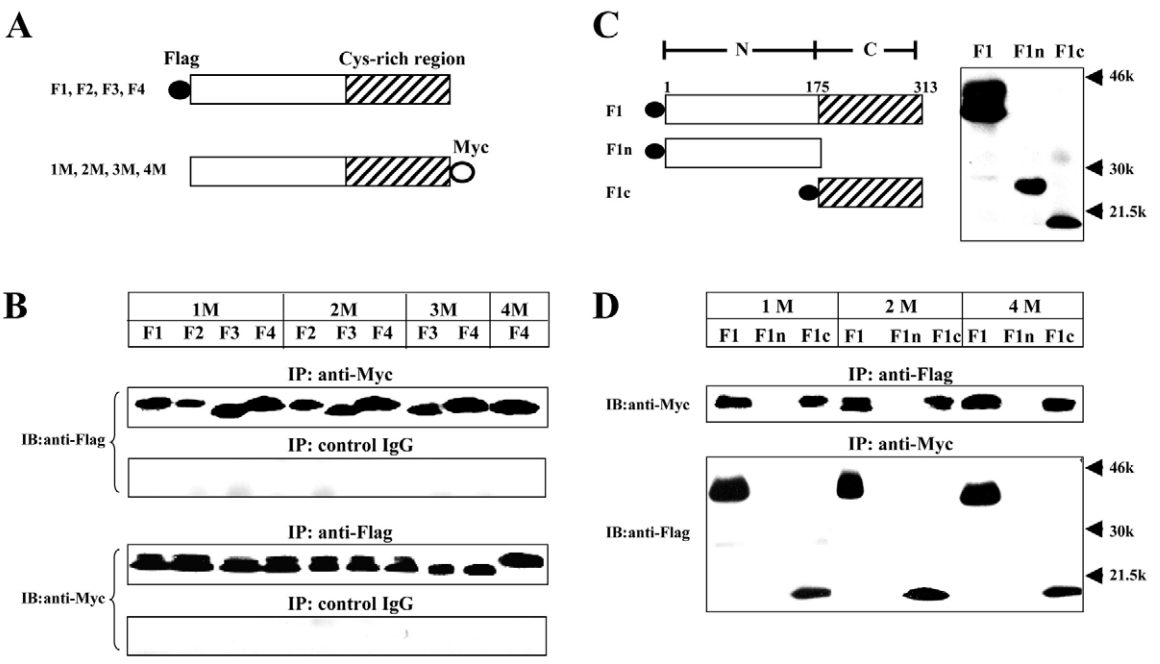

Fig. 1. Mammalian Sprouty isoforms form homo-/hetero-oligomers through their C-terminal domains. (A) Expression constructs of Sprouty1-4, either Flag-tagged at their N-terminus (F1-F4) or Myc-tagged at their C-terminus (1M-4M), are illustrated. Hatched boxes indicate cysteine-rich C-terminal domains. (B) 293T cells were co-transfected with two expression plasmids (0.5 μg each), one encoding one of the Flag-tagged Sprouty isoforms and the other encoding one of the Myc-tagged Sprouty isoforms as indicated. Cell lysates (500 μg protein) were subjected to immunoprecipitation (IP) using anti-Myc/anti-Flag antibody, followed by immunoblotting (IB) with anti-Flag/anti-Myc antibody. Rabbit non-immune IgG was used as a control. (C) F1, F1n, and F1c represent constructs encoding Flag-tagged full-length, N-terminal domain (residues 1-174), and C-terminal domain (residues 175-313) of Sprouty1, respectively. Expression of these constructs in 293T cells was assured by immunoblot analysis with anti-Flag antibody. (D) 293T cells were co-transfected with two plasmids (0.5 μg each), one encoding one of the Myc-tagged Sprouty isoforms and the other encoding Flag-tagged full-length, N-terminal domain or C-terminal domain of Sprouty1 as indicated. Cell lysates (500 μg protein) were subjected to immunoprecipitation using anti-Flag/anti-Myc antibody, followed by immunoblotting with anti-Myc/anti-Flag antibody. Data shown in B and D are representative of three separate experiments that gave essentially the same results.

isoforms, in the recruitment of Grb2/Sos to the plasma membrane has been reported in some cell systems (Wennstom and Downward, 1999). Therefore, we next analyzed the FGF-2/EGF-induced interaction between Grb2 and FRS2/Shc isoforms and further the possible interference of Sprouty isoforms with this interaction.

As discussed above, the transfection efficiency of any gene into 293T cells was at most ~50%. To avoid the effect of interaction between Grb2 and FRS2/Shc isoforms that would occur in cells not expressing exogenous Sprouty proteins, we utilized a plasmid encoding HA-tagged Grb2 as a transfection/expression marker. Thus, 293T cells were transfected with Flag-tagged Sprouty1, Sprouty4, or Sprouty1 and Sprouty4 in combination, together with HA-tagged Grb2, and then stimulated with FGF-2 or EGF for 15 minutes. The results of a co-immunoprecipitation assay using anti-HA antibody revealed that FGF-2 stimulation clearly induced the association of Grb2 to FRS2 in the cells (Fig. 4). Although expression of Sprouty1 or Sprouty4 suppressed the FGF-2-induced interaction between Grb2 and FRS2, co-expression of these two Sprouty isoforms apparently inhibited the association of Grb2 to FRS2 even more markedly. These

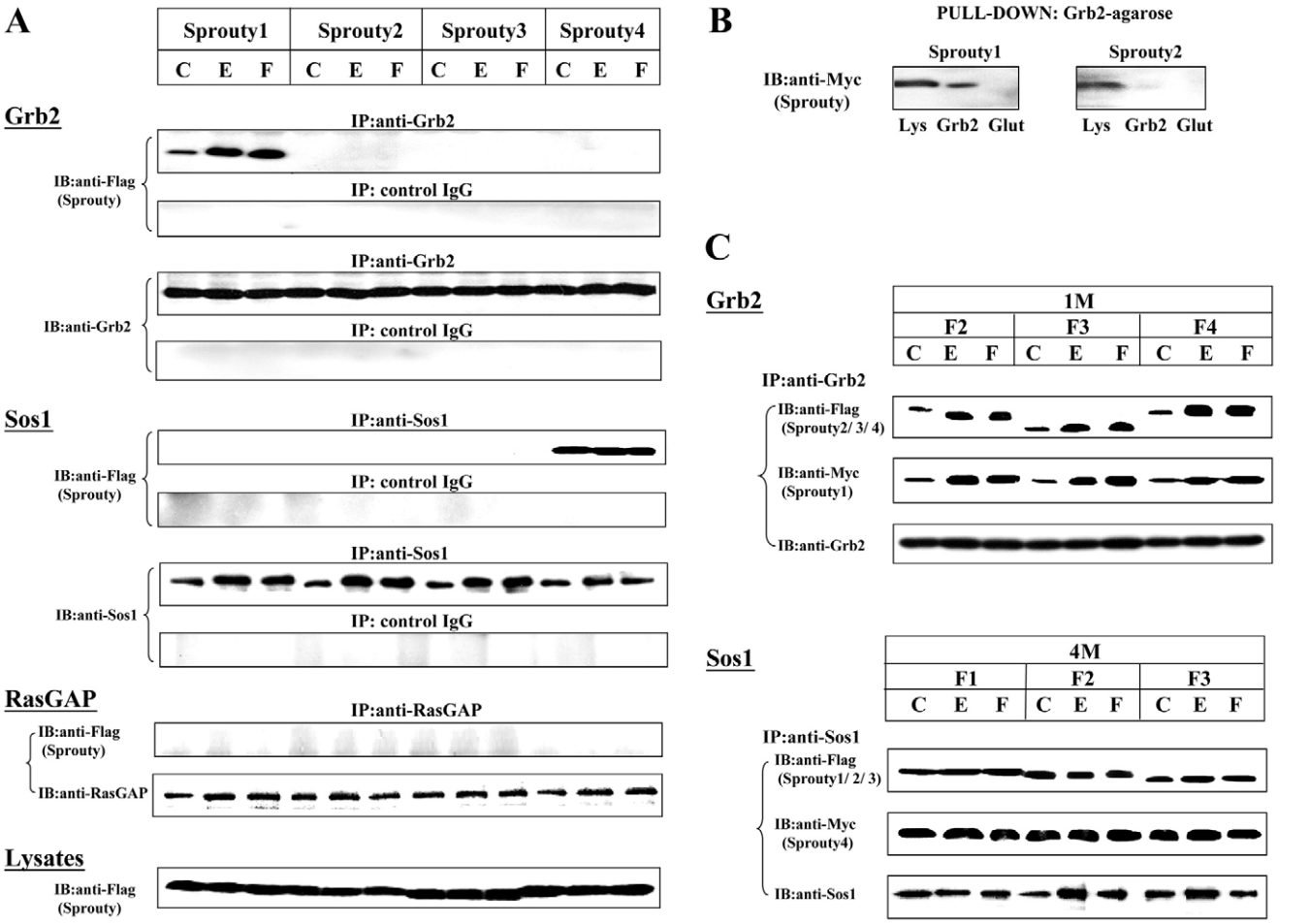

Fig. 2. Specific association between Grb2 and Sprouty1 or Sos1 and Sprouty4. (A) 293T cells were transfected with the expression plasmid encoding either Flag-tagged Sprouty1, Sprouty2, Sprouty3 or Sprouty4 (0.5 μg). After 24 hours, cells were serum-starved for 6 hours and then mock-treated (C) or treated with 20 ng/ml EGF (E) or 20 ng/ml FGF-2 (F) for 15 minutes. The cell lysates (500 μg protein) were subjected to immunoprecipitation (IP) using anti-Grb2 antibody, anti-Sos1 antibody, or anti-RasGAP antibody, followed by immunoblotting with anti-Flag antibody (for Sprouty proteins), anti-Grb2 antibody, anti-Sos1 antibody or anti-RasGAP antibody. Rabbit non-immune IgG was used as a control. Total cell lysates (50 μg protein) were subjected to immunoblot analysis with anti-Flag antibody to show the expression levels of Sprouty proteins. (B) 293T cells transfected with the expression plasmid encoding Myc-tagged Sprouty1 or Sprouty2 were treated with 20 ng/ml EGF for 15 minutes. Cell lysates (500 μg protein) were subjected to a pull-down assay using Grb2-agarose beads (Grb2) or glutathione-sepharose 4B beads (Glut), followed by immunoblotting with anti-Myc antibody (for Sprouty1/2). Total cell lysates (50 μg protein) were subjected to immunoblot analysis with anti-Myc antibody to show the expression levels of exogenous Sprouty1/2 (Lys). (C) 293T cells were co-transfected with expression plasmids encoding Myc-tagged Sprouty1 and each of either Flag-tagged Sprouty2, Sprouty3 or Sprouty4, or co-transfected with expression plasmids encoding Myc-tagged Sprouty4 and each of either Flag-tagged Sprouty1, Sprouty2 or Sprouty3 as indicated (0.5 μg each). After 24 hours, the cells were serum-starved for 6 hours and then mock-treated (C) or treated with 20 ng/ml EGF (E) or 20 ng/ml FGF-2 (F) for 15 minutes. Cell lysates (500 μg protein) were subjected to immunoprecipitation using anti-Grb2 antibody or anti-Sos1 antibody, followed by immunoblotting with anti-Flag antibody, anti-Myc antibody, anti-Grb2 antibody, or anti-Sos1 antibody. Similar results were obtained in three independent experiments.

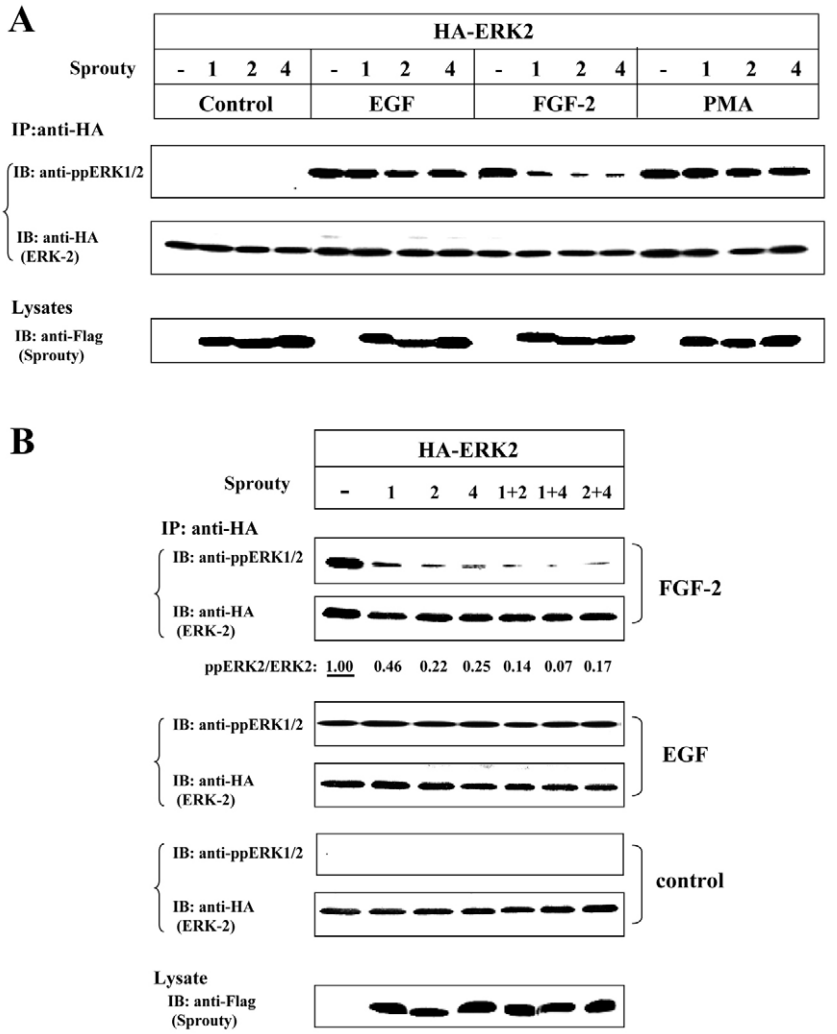

Fig. 3. Co-expression of Sprouty1 and Sprouty4 efficiently suppresses ERK activation induced by FGF-2. (A) 293T cells were co-transfected with expression plasmids encoding HA-tagged ERK2 (0.5 μg) and each of either vector control (–), Flag-tagged Sprouty1, Sprouty2 or Sprouty4 (0.5 μg). After 24 hours, cells were serum-starved for 6 hours and then mock-treated (C) or treated with 20 ng/ml EGF, 20 ng/ml FGF-2 or 10 ng/ml PMA for 15 minutes. Cell lysates (500 μg protein) were subjected to immunoprecipitation (IP) using anti-HA antibody, followed by immunoblotting (IB) with anti-ppERK1/2 antibody. An anti-HA blot demonstrates equal amounts of immunoprecipitated ERK2. Total cell lysates (50 μg protein) were subjected to immunoblot analysis with anti-Flag antibody to show the expression levels of exogenous Sprouty1/2/4. (B) 293T cells were co-transfected with expression plasmids encoding HA-tagged ERK-2 (0.5 μg) and vector control (–), Flag-tagged Sprouty1 (1 μg), Flag-tagged Sprouty2 (1 μg), Flag-tagged Sprouty4 (1 μg), Flag-tagged Sprouty1 and Sprouty2 in combination (0.5 μg each), Flag-tagged Sprouty1 and Sprouty4 in combination (0.5 μg each), or Flag-tagged Sprouty2 and Sprouty4 in combination (0.5 μg each) as indicated. ERK activation induced by FGF-2 or EGF was analyzed as described above. The relative intensity of phosphorylated HA-ERK2 band, as compared with that of the respective HA-ERK2 signal, was determined by using the Multi Gauge software, version 3.0 (Fuji Photo Film, Tokyo), and normalized to 1.00 for control cells without exogenous Sprouty protein(s) (ppERK2/ERK2). Total cell lysates (50 μg protein) were subjected to immunoblot analysis with anti-Flag antibody to show the expression levels of exogenous Sprouty1/2/4. Similar results were obtained in three independent experiments.

results are consistent with the observations that co-expression of Sprouty1 and Sprouty 4 induced a more efficient suppression of FGF-2-induced ERK activation than that observed with the expression of Sprouty1 or Sprouty4 alone (Fig. 3B)

Association of Shc isoforms (p46 and p52 and p66) with Grb2 was observed in unstimulated cells, which was not affected by stimulation of the cells with FGF-2. Although EGF-stimulation of the cells enhanced the interaction between Shc isoforms and Grb2, this interaction was not affected at all by the expression of Sprouty1, Sprouty4, or Sprouty1 and Sprouty4 in combination. Inability of Sprouty1/4 to interfere with the EGF-induced association between Shc isoforms and Grb2 is in agreement with the fact that these Sprouty isoforms did not inhibit the ERK activation induced by EGF (Fig. 3).

FGF-2-stimulation induces the expression and hetero-oligomerization of Sprouty isoforms in Swiss 3T3 cells

We next examined whether or not endogenous Sprouty isoforms actually form hetero-oligomers after their expression is induced by mitogen-stimulation of cells. FGF-2-stimulation of Swiss 3T3 cells induced the expression of Sprouty1, 2 and 4 proteins in an ERK-dependent manner (Fig. 5A). The time course profiles for the induction of Sprouty protein differed significantly among the isoforms; expression of Sprouty2 was observed as early as 1 hour after stimulation of the cells with FGF-2, which was followed by the expression of Sprouty1 and then by Sprouty4. Such a delayed expression of Sprouty4 as compared with the expression of other Sprouty isoforms in response to mitogen-stimulation of cells is consistent with our previous findings on Sprouty gene expression analyzed by northern blotting (Ozaki et al., 2001). FGF-2-stimulation induced a rather sustained activation of ERK1/2 in Swiss 3T3 cells, the down-regulation of which became apparent after 3 hours stimulation of the cells. In addition, FGF-2-stimulation induced a significant retardation of motility of Sos1 on SDS-PAGE, reflecting its phosphorylation (Langlois et al., 1995), in an ERK-dependent manner.

Cell lysates of the FGF-2-stimulated Swiss 3T3 cells were then subjected to co-immunoprecipitation using anti-Sprouty4 antibody. As shown in Fig. 5B, the immunoprecipitates obtained from the cells that had been stimulated with FGF-2 for 3-4 hours contained not only Sprouty4 but also Sprouty1 and Sprouty2. Furthermore, these immunoprecipitates also contained Sos-1 as well as Grb2. These results suggest that Sprouty4 actually formed hetero-oligomers with Sprouty1/Sprouty2 in FGF-2-stimulated Swiss 3T3 cells.

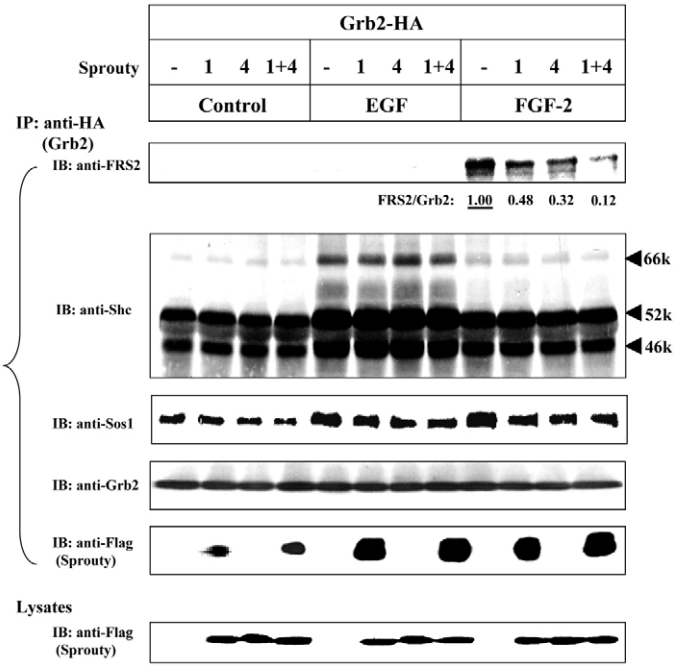

Fig. 4. Co-expression of Sprouty1 and Sprouty4 efficiently inhibits the FGF-2-induced association of Grb2 with FRS2. 293T cells were co-transfected with expression plasmids encoding HA-tagged Grb2 (0.5 μg) and vector control (–), Flag-tagged Sprouty1 (1 μg), Flag-tagged Sprouty4 (1 μg), or Flag-tagged Sprouty1 and Sprouty4 in combination (0.5 μg each) as indicated. After 24 hours, cells were serum-starved for 6 hours and then mock-treated (Control) or treated with 20 ng/ml EGF or 20 ng/ml FGF-2 for 15 minutes. Cell lysates (500 μg protein) were subjected to immunoprecipitation (IP) using anti-HA antibody, followed by immunoblotting with anti-FRS2 antibody, anti-Shc antibody, anti-Grb2 antibody or anti-Flag antibody (for Sprouty1/4). The relative intensity of FRS2 band, as compared with that of the respective Grb2 signal, was determined by using the Multi Gauge software, and normalized to 1.00 for control cells without exogenous Sprouty protein(s) (FRS2/Grb2). Total cell lysates (50 μg protein) were subjected to immunoblot analysis with anti-Flag antibody to show the expression levels of exogenous Sprouty1/4. Similar results were obtained in three independent experiments.

Then, such formed Sprouty1-Sprouty4 hetero-oligomer would have efficiently sequestered Grb2/Sos-1, leading to the down-regulation of the ERK signaling pathway.

siRNA knockdown of either Sprouty1 or Sprouty4 induces a prolonged activation of ERK1/2 in FGF-2-stimulated Swiss 3T3 cells

We finally examined the effect of siRNA knockdown of endogenous Sprouty1 and/or Sprouty4 on the FGF-2-induced activation of ERK1/2 in Swiss 3T3 cells. Semi-quantitative RT-PCR analyses revealed that transfection of Swiss 3T3 cells with Sprouty1 siRNA or Sprouty4 siRNA efficiently and specifically suppressed the FGF-2-induced expression of Sprouty1 or Sprouty4, respectively (Fig. 6A). Western blot analysis showed that the FGF-2-induced expression of Sprouty4 was markedly reduced in the Sprouty4 siRNA-tansfected cells (see supplementary Fig. S3).

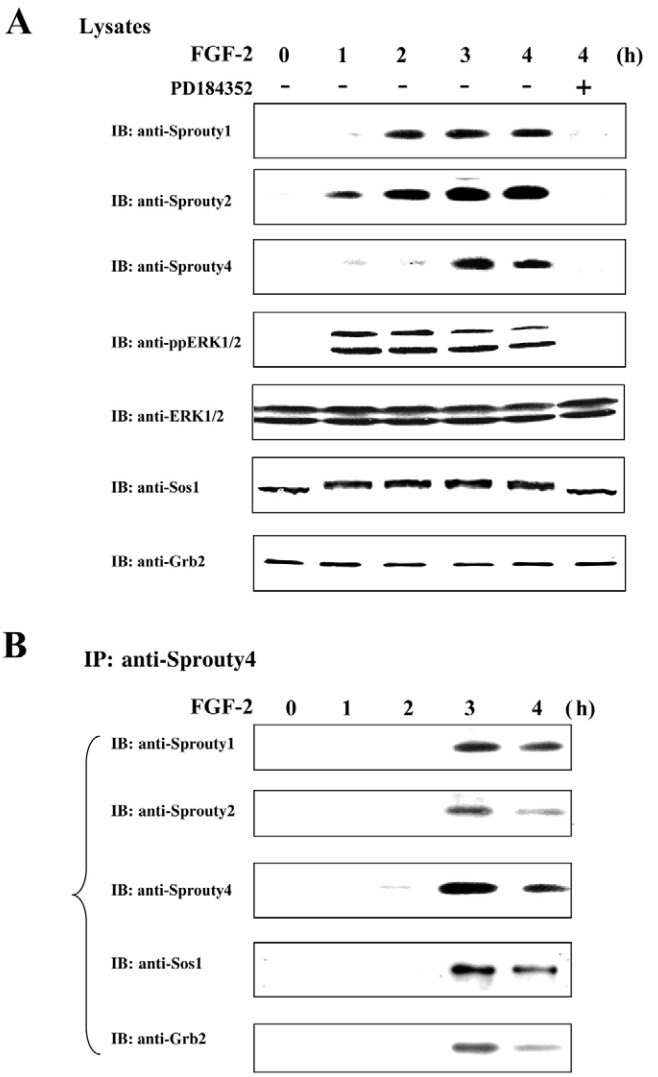

Fig. 5. Hetero-oligomer formation of Sprouty1/2 and Sprouty4 in FGF-2-stimulated Swiss 3T3 cells. (A) Swiss 3T3 cells were serum-starved for 24 hours and then stimulated with 20 ng/ml of FGF-2 for the indicated periods of time. In some experiments, the cells were pretreated with 10 μM PD184352 (PD) for 30 minutes and then stimulated with FGF-2. Total cell lysates (50 μg protein) were subjected to immunoblot analysis with anti-Sprouty1 antibody, anti-Sprouty2 antibody, anti-Sprouty4 antibody, anti-ppERK1/2 antibody, anti-ERK1/2 antibody, anti-Sos1 antibody or anti-Grb2 antibody. (B) Swiss 3T3 cells were serum-starved for 24 hours and then stimulated with FGF-2 (20 ng/ml) for the indicated periods of time. Cell lysates (500 μg protein) were then subjected to immunoprecipitation (IP) using the anti-Sprouty4 antibody (2 μg each of sc-18607 and sc-18609), following by immunoblotting with anti-Sprouty1 antibody, anti-Sprouty2 antibody, anti-Sprouty4 antibody (sc-18607), anti-Sos1 antibody, or anti-Grb2 antibody. Similar results were obtained in three independent experiments.

Knockdown of Sprouty1 or Sprouty4 in Swiss 3T3 cells induced a more prolonged activation of ERK1/2 in response to FGF-2-stimulation, as compared with that observed in the scrambled RNA-transfected control cells (Fig. 6B). Sustained ERK1/2 activation observed in Sprouty1 siRNA-transfected cells was most prominent 2-4 hours after FGF-2-stimulation of

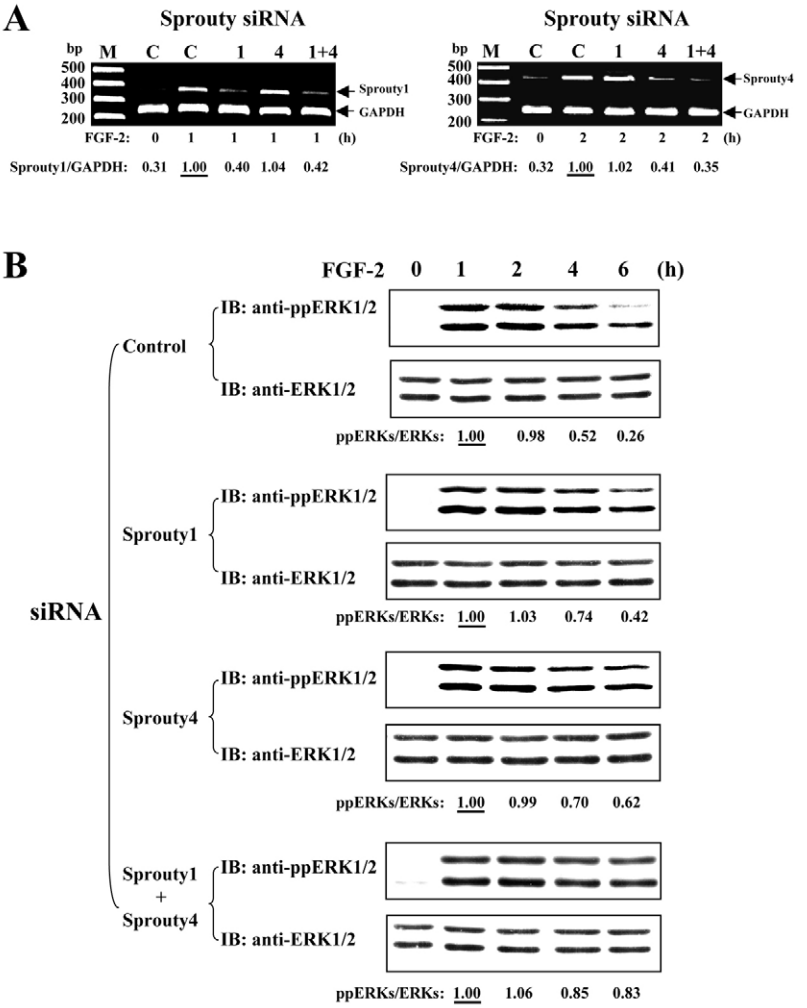

Fig. 6. siRNA knockdown of Sprouty1/Sprouty4 induces a prolonged activation of ERK1/2 in FGF-2-stimulated Swiss 3T3 cells. (A) Swiss 3T3 cells were transfected with Sprouty1 siRNA (1), Sprouty4 siRNA (4), Sprouty1 siRNA and Sprouty4 siRNA in combination (1+4), or matched scrambled RNAs to Sprouty1 siRNA and Sprouty4 siRNA in combination (C). After 24 hours, cells were serum-starved for 24 hours and then stimulated with FGF-2 (20 ng/ml) for 1 hour (for the analysis of Sprouty1 expression) or 2 hours (for the analysis of Sprouty4 expression). Total RNA was isolated and RT-PCR (27 cycles) was performed for Sprouty1 or Sprouty4 to generate 372 bp fragment or 443 bp fragment, respectively. A portion of GAPDH was co-amplified as an internal control (250 bp). The relative intensity of Sprouty1/4 band, as compared with that of the respective GAPDH signal, was determined by using the Multi Gauge software, and normalized to 1.00 for the respective scramble RNA-transfected control cells stimulated with FGF-2 for 0.5 hours. M, DNA ladder markers. (B) Swiss 3T3 cells were transfected with Sprouty1 siRNA (Sprouty1), Sprouty4 siRNA (Sprouty4), Sprouty1 siRNA and Sprouty4 siRNA in combination (Sprouty1 + Sprouty4), or matched scrambled RNAs to Sprouty1 siRNA and Sprouty4 siRNA in combination (Control). After 24 hours, cells were serum-starved for 24 hours and then stimulated with FGF-2 (20 ng/ml) for the indicated periods of time. Total cell lysates (10 μg protein) were subjected to immunoblot analysis with anti-ppERK1/2 antibody or anti-ERK1/2 antibody. The relative intensity of phosphorylated ERK1 and ERK2 bands, as compared with that of the respective ERK1 and ERK2 signals, was determined by using the Multi Gauge software, and normalized to 1.00 for the respective Swiss 3T3 cells stimulated with FGF-2 for 1 hour (ppERKs/ERKs). Similar results were obtained in two independent experiments.

the cells, while that observed in Sprouty4 siRNA-transfected cells was apparent 4 hours after the stimulation, which is consistent with the differential time course profile of their expression (Fig. 5A). Furthermore, FGF-2-stimulation induced a markedly prolonged ERK1/2 activation when Swiss 3T3 cells were co-transfected with Sprouty1 siRNA and Sprouty4 siRNA to knockdown both of these Sprouty isoforms. These results indicate that Sprouty1 and Sprouty4 actually function as negative regulators of the ERK pathway, and that their co-operative interaction would lead to a more efficient suppression of the mitogen-induced ERK activation.

Discussion

In this report, we have shown that four mammalian Sprouty isoforms interact with each other, which is observed in 293T cells transfected with expression vectors encoding any of Flag-tagged Sprouty isoforms and any of Myc-tagged Sprouty isoforms in combination (Fig. 1) and also in Swiss 3T3 cells stimulated with FGF-2 (Fig. 5). Co-immunoprecipitation of Sprouty isoforms by itself, however, dose not necessary indicate that such immunoprecipitated Sprouty isoforms interact directly to form oligomers; it could be a result of indirect interaction among Sprouty isoforms through some other protein such as Grb2 or Sos1 (Fig. 2C). In this respect, Sprouty1 C-terminal fragment, which lacks N-terminal domain through which each Sprouty protein interacts with respective signaling molecule, can interact with any of Sprouty isoforms (Fig. 1D). Furthermore, Sprouty2 has been reported to form homodimer through its C-terminal region (residues 209-238) (Hanafusa et al., 2002), to which residues 212-242 of Sprouty1, residues 178-207 of Sprouty3, and residues 190-220 of Sprouty4 are very similar (43% identical residues and additional 27% similar amino acid residues). Considering all these results into account, it seems very probable that four mammalian Sprouty isoforms interact directly with each other to form hetero- as well as homo-oligomers through their C-terminal domains.

The four Sprouty isoforms have highly variable N-terminal domains, through which each Sprouty protein can interact with a specific signaling molecule. Accordingly, we have shown in this report that Sprouty1 interacts specifically with Grb2 and Sprouty4 with Sos1. These results were obtained by analyzing 293T cells in which gene(s) encoding the respective Sprouty protein(s), but not other signaling molecules, were expressed (Fig. 2). Furthermore, we employed suboptimal conditions for the transfection of Sprouty expression plasmids into the cells, which made the expression level of Sprouty proteins not too high but rather similar to those observed under physiological conditions (supplementary Fig. S1). In this respect, specific

interaction of *Drosophila* Sprouty with Grb2 and RasGAP (Casci et al., 1999), *Xenopus* Sprouty1 and mammalian Sprouty2 with Grb2 and Shp2 (Hanafusa et al., 2002; Hanafusa et al., 2004), Sprouty2 with Raf-1, Grb2, FRS2 and Shp2 (Tefft et al., 2002), Sprouty2 with c-Cbl (Wong et al., 2001; Hall et al., 2003), Sprouty2 with PTP-1B (Yigzaw et al., 2003), Sprouty4 with Raf-1 (Sasaki et al., 2003), and Sprouty1 and Sprouty2 with Caveolin-1 but not with Grb2 (Impagnatiello et al., 2001) have been reported. All of these results were obtained in mammalian cell lines in which the respective *Sprouty* gene together with genes encoding other signaling molecules, such as Grb2 and Raf1, and even genes encoding RTKs in some experiments (Hanafusa et al., 2002; Wong et al., 2002; Yusoff et al., 2002; Hanafusa et al., 2004), were over-expressed. Under such conditions, the relative concentrations of Sprouty proteins versus other signaling molecules in the cells would differ significantly from the endogenous ones and also vary depending on the experimental conditions. This might explain why similar, but not identical, results have been obtained by different investigators. However, taking all of these results into consideration, it seems very probable that four Sprouty proteins are functionally distinct from each other and that each Sprouty protein interacts with some specific signaling molecule through its N-terminal domain to interrupt the RTK pathway.

Grb2 is an adaptor protein and consists of two SH3 domains surrounding one SH2 domain. The SH2 domain of Grb2 recognizes phosphorylated tyrosine residues of several proteins such as EGF receptor, Shc and FRS2. In this respect, work by several groups has shown that Spouty proteins are phosphorylated on tyrosine residues upon RTK stimulation, thereby generating docking sites for the SH2 domain of Grb2 (Hanafusa et al., 2002). We have confirmed that EGF- or FGF-stimulation of 293T cells transfected with Sprouty1/4 induces tyrosine phosphorylation of Sprouty proteins (data not shown). Furthermore, EGF- or FGF-stimulation of the cells clearly enhanced the binding of Sprouty1 to Grb2 (Fig. 2A). All these results support the notion that interaction between Sprouty1 and Grb2 is, at least in part, through the binding of phosphorylated tyrosine residue of Sprouty1 to the SH2 domain of Grb2.

Specific interaction of Sprouty4 with Sos has been observed, and this is not affected by stimulation of the cells with EGF/FGF-2 (Fig. 2A). Under physiological conditions, however, expression of four Sprouty isoforms is induced only after activation of the ERK pathway, which ensures the negative feedback role of Sprouty proteins against the ERK pathway (Ozaki et al., 2001; Sasaki et al., 2001). The mechanism by which Sprouty4 interacts specifically with Sos1 remains to be determined.

Expression of Sprouty1, 2 or 4 efficiently inhibited the ERK activation induced by FGF-2 (Fig. 3A). As discussed above, Sprouty1 associates specifically with Grb2 and Sprouty4 with Sos1, thereby disturbing the interaction between Grb2 and Sos1 and furthermore their interaction with other signaling molecules, such as FRS2 (Fig. 4). All of these protein-protein interactions are prerequisites to induce the activation of Ras (Li et al., 1993; Rozakis-Adcock et al., 1993). Sprouty2 would also interact with certain signaling molecule(s); however, we have not yet identified them in our experimental system. In this respect, we have previously shown that Sprouty2 interrupts the RTK pathway upstream of Ras (Ozaki et al., 2001). Thus, by interacting with and sequestering several signaling molecules, Sprouty proteins intercept the FGF receptor-signaling pathway upstream of Ras, leading to the suppression of ERK activation. Results of the siRNA knockdown studies (Fig. 6B) have confirmed the negative feedback role of Sprouty1 and Sprouty4 against the FGF2-induced activation of the ERK pathway.

By binding to Grb2, Sos1 is recruited to the plasma membrane and activates Ras by GTP loading. Interaction of these two molecules appears to be constitutive and the majority of them appear to form Grb2-Sos1 complexes in the cells (Fig. 4). However, a population of each of these molecules can be free from the other: the affinity of these molecules may be high but cannot be infinite. In this situation, Sprouty1 homodimers, for example, bind to and sequester Grb2-Sos1 complex as well as free Grb2, and Sprouty4 homodimers bind to and sequester Grb2-Sos1 complex as well as free Sos1, thereby suppressing the reciprocal association of Grb2 and Sos1 and further the association of Grb2-Sos1 complex with other signaling molecules considerably, such as FRS2. On the contrary, Sprouty1-Sprouty4 heterodimers bind to and sequester free Grb2, free Sos1 and Grb2-Sos1 complex, which would result in a more efficient blockade of the interaction between Grb2-Sos1 complex and FRS2 (see supplementary Fig. S4). Consistent with this idea, we have shown that co-expression in the cells of Sprouty1 and Sprouty4 inhibits the association of Grb2-Sos1 complex with FRS2 more markedly than that observed with the expression of each Sprouty isoform alone, and furthermore suppresses the FGF-2-induced ERK activation almost completely (Figs 3, 4).

Co-expression of Sprouty1 and Sprouty2 also suppressed the FGF-2-induced ERK activation more efficiently than that observed with the expression of each Sprouty isoform alone (Fig. 3). The molecular mechanism of this suppression remains to be elucidated. The enhanced suppression of ERK activation observed by co-expression of Sprouty isoforms could have biological significance. The majority of our experiments were performed by utilizing 293T cells in which limited amount of Sprouty expression plasmid had been transfected in an effort to make their expression levels not too high but rather similar to those observed in FGF-2-stimulated Swiss 3T3 cells (supplementary Fig. S1). Under physiological conditions, however, expression of Sprouty proteins is rather transient (Fig. 5A). Under such conditions, limited amounts of Sprouty isoforms would be able to efficiently suppress the growth factor-induced activation of the ERK pathway by forming appropriate hetero-oligomers. Strongly supporting this idea, we provide evidence that Sprouty1/2 and Sprouty4 form hetero-oligomer in FGF-2-stimulated Swiss 3T3 cells (Fig. 5B). The Sprouty1-Sprouty4 hetero-oligomer thus formed is suggested to sequester Grb2/Sos1 effectively, which would result in the efficient suppression of the ERK signaling pathway under physiological conditions.

Excessive or inappropriate activation of the ERK pathway has been associated with carcinogenesis of human neoplasms (Oka et al., 1995; Hoshino et al., 1999; Hoshino et al., 2001; Kohno and Pouyssegur, 2003). Thus, negative feedback inhibition of the ERK pathway, after growth factor-stimulation, is essential for maintaining balanced cell growth in multi-cellular organisms. In this respect, mammalian cells would probably have developed several systems for the very strict

spatial and temporal regulation of the ERK activity. For example, activation of the ERK pathway induces the expression of MAP kinase phosphatases, which directly dephosphorylate and inactivate ERKs (Camps et al., 1999; Farooq and Zhou, 2004). Alternatively, ERK activation induces the expression of four Sprouty isoforms. Although each Sprouty isoform binds to and sequesters its respective signaling molecule to interfere with the growth factor-induced activation of the ERK pathway, their cooperation, i.e. formation of hetero-oligomers among them, apparently suppresses the ERK activation more efficiently (Fig. 3). The markedly prolonged ERK1/2 activation observed in FGF-2-stimulated Swiss 3T3 cells in which Sprouty1 siRNA and Sprouty4 siRNA were transfected to knockdown both of them (Fig. 6B) would support the importance of cooperative interaction among Sprouty isoforms in the negative feedback inhibition of the ERK pathway. This could explain, at least in part, why mammalian cells express four Sprouty isoforms.

In conclusion, we have demonstrated that four mammalian Sprouty isoforms form hetero- as well as homo-oligomers through their C-terminal domains. Many of these hetero-oligomers, especially that formed between Sprouty1 and Sprouty4, suppress the FGF-2-induced ERK activation more efficiently than the corresponding homo-oligomers. This could represent a more advanced system for strict regulation of the activation state of the ERK signaling pathway in mammalian cells.

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References

Blume-Jensen, P. and Hunter, T. (2001). Oncogenic kinase signaling. *Nature* **411**, 355-365.

Camps, M., Nichols, A. and Arkinstall, S. (1999). Dual specificity phosphatases: a gene family for control of MAP kinase function. *FASEB J.* **14**, 6-16.

Casci, T., Vinos, J. and Freeman, M. (1999). Sprouty, an intracellular inhibitor of Ras signaling. *Cell* **96**, 655-665.

Christofori, G. (2003). Split personalities: the agonistic antagonist Sprouty. *Nat. Cell Biol.* **5**, 377-379.

Egan, J. E., Hall, A. B., Yatsula, B. A. and Bar-Sagi, D. (2002). The bimodal regulation of epidermal growth factor signaling by human Sprouty proteins. *Proc. Natl. Acad. Sci. USA* **99**, 6041-6046.

Farooq, A. and Zhou, M. M. (2004). Structure and regulation of MAPK phosphatases. *Cell Signal.* **16**, 769-779.

Gross, I., Bassit, B., Benezra, M. and Licht, J. D. (2001). Mammalian sprouty proteins inhibit cell growth and differentiation by preventing ras activation. *J. Biol. Chem.* **276**, 46460-46468.

Gum, J. R. J. (1992). Mucin genes and the proteins they encode: structure, diversity, and regulation. *Am. J. Respir. Cell Mol. Biol.* **7**, 557-564.

Hacohen, N., Kramer, S., Sutherland, D., Hiromi, Y. and Krasnow, M. A. (1998). *sprouty* encodes a novel antagonist of FGF signaling that patterns apical branching of the *Drosophila* airways. *Cell* **92**, 253-263.

Hadari, Y. R., Kouhara, H., Lax, I. and Schlessinger, J. (1998). Binding of Shp2 tyrosine phosphatase to FRS2 is essential for fibroblast growth factor-induced PC12 cell differentiation. *Mol. Cell. Biol.* **18**, 3966-3973.

Hall, A. B., Jura, N., DaSilva, J., Jang, Y. J., Gong, D. and Bar-Sagi, D. (2003). hSpry2 is targeted to the ubiquitin-dependent proteasome pathway by c-Cbl. *Curr. Biol.* **13**, 308-314.

Hanafusa, H., Torii, S., Yasunaga, T. and Nishida, E. (2002). Sprouty1 and Sprouty2 provide a control mechanism for the Ras/MAPK signaling pathway. *Nat. Cell Biol.* **4**, 850-858.

Hanafusa, H., Torii, S., Yasunaga, T., Matsumoto, K. and Nishida, E. (2004). Shp2, an SH2-containing protein-tyrosine phosphatase, positively regulates receptor tyrosine kinase signaling by dephosphorylating and inactivating the inhibitor Sprouty. *J. Biol. Chem.* **279**, 22992-22995.

Hanahan, D. and Weinberg, R. A. (2000). The hallmarks of cancer. *Cell* **100**, 57-70.

Hoshino, R., Chatani, Y., Yamori, T., Tsuruo, T., Oka, H., Yoshida, O., Shimada, Y., Ari-I, S., Wada, H., Fujimoto, J. et al. (1999). Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors. *Oncogene* **18**, 813-822.

Hoshino, R., Tanimura, S., Watanabe, K., Kataoka, T. and Kohno, M. (2001). Blockade of the extracellular signal-regulated kinase pathway induces marked G1 cell cycle arrest and apoptosis in tumor cells in which the pathway is constitutively activated: up-regulation of $p27^{Kip1}$. *J. Biol. Chem.* **276**, 2686-2692.

Hunter, T. (2000). Signaling-2000 and beyond. *Cell* **100**, 113-127.

Impagnatiello, M. A., Weitzer, S., Gannon, G., Compagni, A., Cotton, M. and Christofori, G. (2001). Mammalian sprouty-1 and -2 are membrane-anchored phosphoprotein inhibitors of growth factor signaling in endothelial cells. *J. Cell Biol.* **152**, 1087-1098.

Iwasaki, S., Iguchi, M., Watanabe, K., Hoshino, R., Tsujimoto, M. and Kohno, M. (1999). Specific activation of the p38 mitogen-activated protein kinase signaling pathway and induction of neurite outgrowth in PC12 cells by bone morphogenetic protein-2. *J. Biol. Chem.* **274**, 26503-26510.

Kohno, M. and Pouyssegur, J. (2003). Pharmacological inhibitors of the ERK signaling pathway: application as anticancer drugs. *Prog. Cell Cycle Res.* **5**, 219-224.

Kouhara, H., Hadari, Y. R., Spivak-Kroizman, T., Schilling, J., Bar-Sagi, D., Lax, I. and Schlessinger, J. (1997). A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. *Cell* **89**, 693-702.

Kramer, S., Okabe, M., Hacohen, N., Krasnow, M. A. and Hiromi, Y. (1999). Sprouty: a common antagonist of FGF and EGF signaling pathways in *Drosophila*. *Development* **126**, 2515-2525.

Langlois, W. J., Sasaoka, T., Saltiel, A. R. and Olefsky, J. M. (1995). Negative feedback regulation and desensitization of insulin- and epidermal growth factor-stimulated $p21^{ras}$ activation. *J. Biol. Chem.* **270**, 25320-25323.

Lee, S. H., Schloss, D. J., Jarvis, L., Krasnow, M. A. and Swain, J. L. (2001). Inhibition of angiogenesis by a mouse sprouty protein. *J. Biol. Chem.* **276**, 4128-4133.

Li, N., Batzer, A., Daly, R., Yajnik, V., Skolnik, E., Chardin, P., Bar-Sagi, D., Margolis, B. and Schlessinger, J. (1993). Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling. *Nature* **363**, 85-88.

Minowada, G., Jarvis, L. A., Chi, C. L., Neubuser, A., Sun, X., Hacohen, N., Krasnow, M. A. and Martin, G. R. (1999). Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed. *Development* **126**, 4465-4475.

Oka, H., Chatani, Y., Hoshino, R., Ogawa, O., Kakehi, Y., Terachi, T., Okada, Y., Kawaichi, M., Kohno, M. and Yoshida, O. (1995). Constitutive activation of mitogen-activated protein (MAP) kinases in human renal cell carcinoma. *Cancer Res.* **55**, 4182-4187.

Ozaki, K., Kadomoto, R., Asato, K., Tanimura, S., Itoh, N. and Kohno, M. (2001). ERK pathway positively regulates the expression of Sprouty genes. *Biochem. Biophys. Res. Commun.* **285**, 1084-1088.

Reich, A., Sapir, A. and Shilo, B. (1999). Sprouty is a general inhibitor of receptor tyrosine kinase signaling. *Development* **126**, 4139-4147.

Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T. and Bowtell, D. (1993). The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. *Nature* **363**, 83-85.

Sasaki, A., Taketomi, T., Wakioka, T., Kato, R. and Yoshimura, A. (2001). Identification of a dominant negative mutant of Sprouty that potentiates fibroblast growth factor-but not epidermal growth factor-induced ERK activation. *J. Biol. Chem.* **276**, 36804-36808.

Sasaki, A., Taketomi, T., Kato, R., Saeki, K., Nonami, A., Sasaki, M., Kuriyama, M., Saito, N., Shibuya, M. and Yoshimura, A. (2003). Mammalian Sprouty4 suppresses Ras-independent ERK activation by binding to Raf1. *Nat. Cell Biol.* **5**, 427-432.

Schlessinger, J. (2000). Cell signaling by receptor tyrosine kinase. *Cell* **103**, 211-225.

Simon, M. A. (2000). Receptor tyrosine kinases: specific outcomes from general signals. *Cell* **103**, 13-15.

Tanimura, S., Nomura, K., Ozaki, K., Tsujimoto, M., Kondo, T. and

Kohno, M. (2002). Prolonged nuclear retention of activated extracellular signal-regulated kinase 1/2 is required for hepatocyte growth factor-induced cell motility. *J. Biol. Chem.* **277**, 28256-28264.

Tanimura, S., Asato, K., Fujishiro, S. and Kohno, M. (2003). Specific blockade of the ERK pathway inhibits the invasiveness of tumor cells: down-regulation of matrix metalloproteinase-3/-9/-14 and CD44. *Biochem. Biophys. Res. Commun.* **304**, 801-806.

Tefft, J. D., Lee, M., Smith, S., Leinwand, M., Zhao, J., Bringas, P. J., Crowe, D. L. and Warburton, D. (1999). Conserved function of mSpry-2, a murine homolog of *Drosophila* sprouty, which negatively modulates respiratory organogenesis. *Curr. Biol.* **9**, 219-222.

Tefft, J. D., Lee, M., Smith, S., Crowe, D. L., Bellusci, S. and Warburton, D. (2002). mSprouty2 inhibits FGF10-activated MAP kinase by differentially binding to upstream target proteins. *Am. J. Physiol. Lung Cell Mol. Physiol.* **283**, L700-L706.

Voas, M. G. and Rebay, I. (2004). Signal integration during development: insights from the *Drosophila* eye. *Dev. Dyn.* **229**, 162-175.

Wallis, R. and Drickamer, K. (1999). Molecular determinants of oligomer formation and complement fixation in mannose-binding proteins. *J. Biol. Chem.* **274**, 3580-3589.

Wennstrom, S. and Downward, J. (1999). Role of phosphoinositide 3-kinase in activation of ras and mitogen-activated protein kinase by epidermal growth factor. *Mol. Cell. Biol.* **19**, 4279-4288.

Wong, E. S., Lim, J., Low, B. C., Chen, Q. and Guy, G. R. (2001). Evidence for direct interaction between Sprouty and Cbl. *J. Biol. Chem.* **276**, 5866-5875.

Wong, E. S., Fong, C. W., Lim, J., Yusoff, P., Low, B. C., Langdon, W. Y. and Guy, G. R. (2002). Sprouty2 attenuates epidermal growth factor receptor ubiquitylation and endocytosis, and consequently enhances Ras/ERK signalling. *EMBO J.* **21**, 4796-4808.

Yigzaw, Y., Cartin, L., Pierre, S., Scholich, K. and Patel, T. B. (2001). The C terminus of sprouty is important for modulation of cellular migration and proliferation. *J. Biol. Chem.* **276**, 22742-22747.

Yigzaw, Y., Poppleton, H. M., Sreejayan, N., Hassid, A. and Patel, T. B. (2003). Protein-tyrosine phosphatases-1B (PTP1B) mediates the anti-migratory actions of Sprouty. *J. Biol. Chem.* **278**, 284-288.

Yusoff, P., Lao, D. H., Ong, S. H., Wong, E. S., Lim, J., Lo, T. L., Leong, H. F., Fong, C. W. and Guy, G.R. (2002). Sprouty2 inhibits the Ras/MAP kinase pathway by inhibiting the activation of Raf. *J. Biol. Chem.* **277**, 3195-3201.

Zwick, E., Bange, J. and Ullrich, A. (2002). Receptor tyrosine kinases as targets for anticancer drugs. *Trends Mol. Med.* **8**, 17-23.