To evaluate tyrosine phosphorylation of IκBα[30] after treatment of cells with EPO, lysates were immunoprecipitated with anti-IκBα and then probed with anti-phosphotyrosine antibodies. To detect serine phosphorylation of IκBα, lysates were immunoblotted without immunoprecipitation and probed directly with antibodies specific for phosphorylation of IκBα serine residues 32 and 36 (Santa Cruz Biotechnology). For Jak2 *in vitro* kinase assays, after cell lysis Jak2 was immunoprecipitated with 20 μg of a polyclonal antibody (Santa Cruz Biotechnology). Jak2 was then resuspended in kinase buffer (including 25 mM HEPES, 25 mM $MgCl_2$, 0.1 mM Na-orthovanadate and 2 mM dithiothreitol) plus 10 μM ATP and 30 μg full-length recombinant IκBα. After reaction for 30 min, anti-IκBα immune complexes were resolved by SDS–PAGE, probed with anti-phosphotyrosine antibodies, and visualized by ECL (Amersham).

Electrophoretic mobility-shift assays (EMSA)

Nuclear extracts were obtained from cerebrocortical cultures[19]. Binding of NF-κB to DNA was assayed with a double-stranded probe labelled with ^{32}P-dUTP that binds to the consensus sequence (Santa Cruz Biotechnology). Nuclear lysates were incubated with the labelled probe for 2 h at 37 °C, resolved on a 7% native polyacrylamide gel, and exposed to X-ray film[21]. Mutated probe was used as a control. Antibodies specific for p50 and p65 NF-κB subunits were used for supershift analysis. In non-neuronal cells, *S*-nitrosylation (transfer of NO-related species to a critical thiol from *S*-nitrosocysteine or other donors) has been reported to block DNA binding by NF-κB. However, this phenomenon did not affect the EMSA results reported here in neurons because *S*-nitrosocysteine did not prevent EPO-induced binding.

Reporter gene assays

Cerebrocortical cells were transfected with pNFκB-Luc using calcium phosphate precipitation (Stratagene). Two days later, cells were lysed and mixed with luciferase assay reagent (Promega), and the activity was measured in a luminometer. All measurements were normalized against a non-κB-dependent control plasmid, pCIS-CK (Stratagene). Results represent mean of three experiments measured in triplicate.

Received 23 January; accepted 8 May 2001.

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

Acknowledgements

We thank M. Kaul, N. Moayeri, B. Price, M. Cokol and M. Altinoz for insightful discussions or technical advice, and the Genetics Institute, Cambridge, Massachusetts, for supplying the anti-EPOR monoclonal antibodies. The complementary DNA strands for the IκB super-repressor (Ad5IκB) and kinase-negative mutant Jak2 (JAK2.KE) were the gifts of R. R. Ratan and J. Ihle, respectively. This work was supported in part by grants from the National Institutes of Health and American Heart Association (S.A.L.).

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Spred is a Sprouty-related suppressor of Ras signalling

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Cellular proliferation, and differentiation of cells in response to extracellular signals, are controlled by the signal transduction pathway of Ras, Raf and MAP (mitogen-activated protein) kinase. The mechanisms that regulate this pathway are not well known. Here we describe two structurally similar tyrosine kinase substrates, Spred-1 and Spred-2. These two proteins contain a cysteine-rich domain related to Sprouty (the SPR domain) at the carboxy terminus. In *Drosophila*, Sprouty inhibits the signalling by receptors of fibroblast growth factor (FGF) and epidermal growth factor (EGF) by suppressing the MAP kinase pathway[2–7]. Like Sprouty, Spred inhibited growth-factor-mediated activation of MAP kinase. The Ras–MAP kinase pathway is essential in the differentiation of neuronal cells and myocytes. Expression of a dominant negative form of Spred and Spred-antibody microinjection revealed that endogenous Spred regulates differentiation in these types of cells. Spred constitutively associated with Ras but did not prevent activation of Ras or membrane translocation of Raf. Instead, Spred inhibited the activation of MAP kinase by suppressing phosphorylation and activation of Raf. Spred may represent a

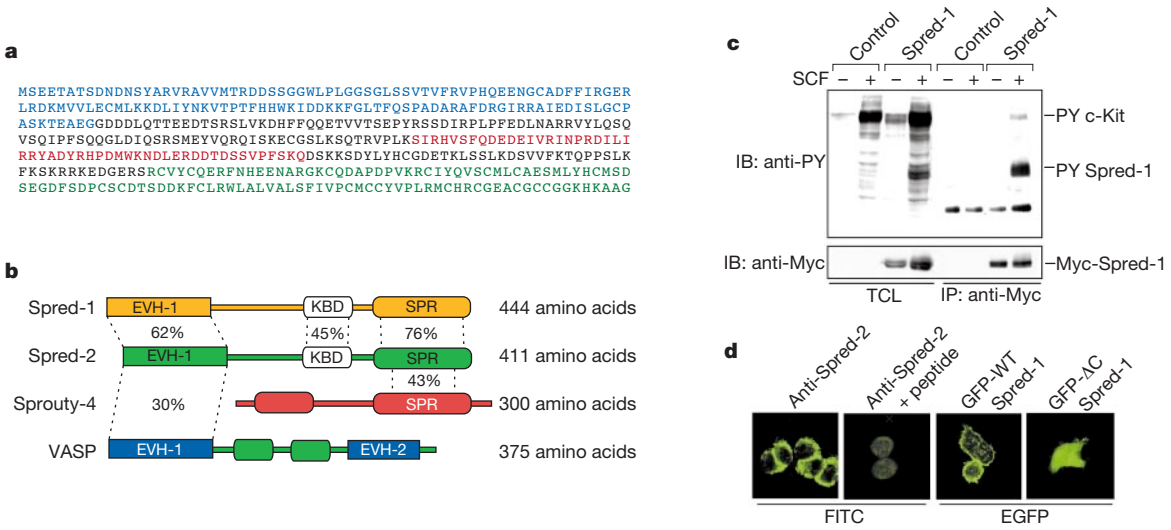

Figure 1 Characterization of Spred molecules. **a**, Amino acid sequence of Spred-1. The EVH-1 domain is shown in blue, the KBD in red, and the SPR domain in green. **b**, Comparison of the domain structure of murine Spred-1 and -2 with that of murine Sprouty-4 and VASP. **c**, Tyrosine phosphorylation (PY) of Myc-tagged Spred-1 expressed in 293 cells in response to SCF. IB, immunoblot; IP, immunoprecipitate; TCL, total cell lysates. **d**, Subcellular localization of Spred. The endogenous Spred-2 protein in PC12 cells was detected using immunofluorescence microscopy without or with antigen peptide. FITC, fluorescein isothiocyanate. The two right panels show a fluorescent microscopic view of wild-type (GFP-WT) and C-terminal truncated (GFP-ΔC) Spred-1 fused to EGFP in PC12 cells.

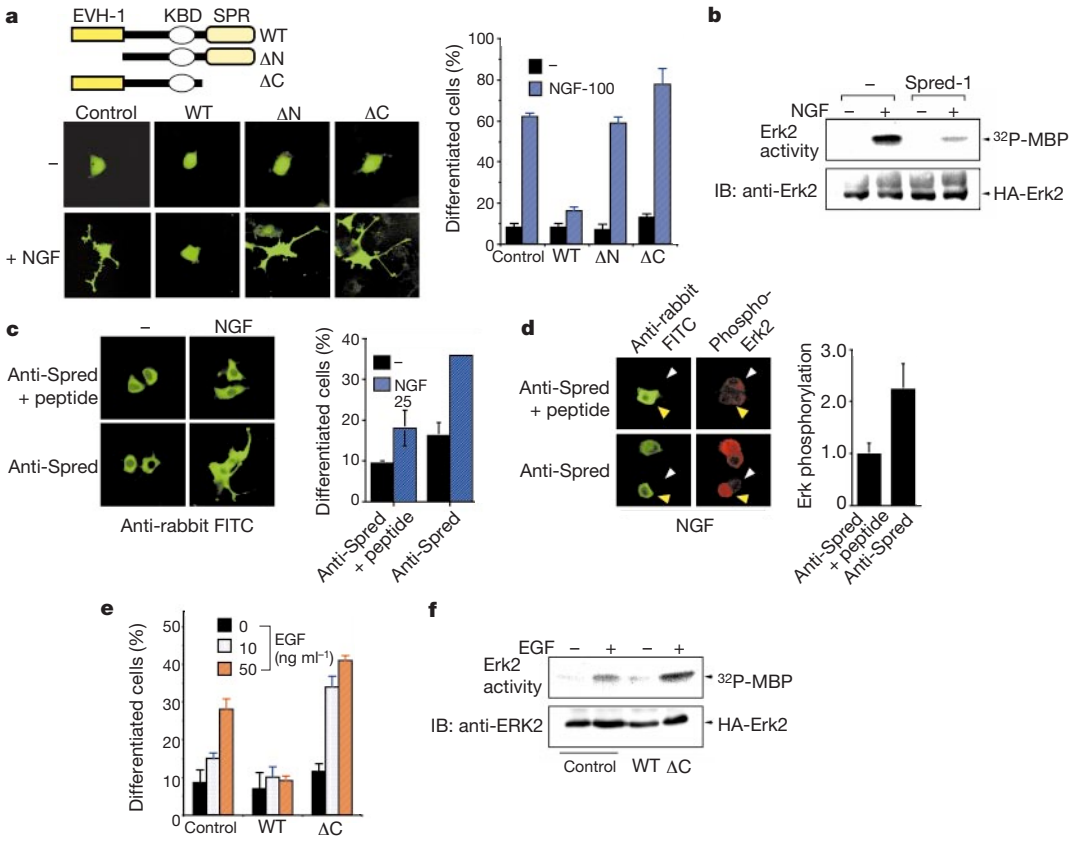

Figure 2 Effects of Spred on differentiation and Erk2 activity of PC12 cells. **a**, Wild-type (WT) and N-terminal- (ΔN) or C-terminal-truncated (ΔC) Spred-1 constructs were expressed in PC12 cells with EGFP, then incubated with 100 ng ml^{-1} NGF for 3 d. **b**, PC12 cells transfected with expression vectors encoding wild-type Spred-1 and HA-tagged Erk2 were stimulated with NGF for 30 min. HA-Erk2 was immunoprecipitated and an *in vitro* kinase assay was performed with MBP as the substrate. IB, immunoblot. **c**, **d**, PC12 cells were microinjected with a mixture of affinity-purified anti-Spred-1 and -2 antibodies, with or without antigen peptide, and then treated with 25 ng ml^{-1} NGF for 2 d (**c**) or 5 min (**d**). Activated Erk was visualized with a monoclonal anti-phosphorylated-Erk antibody and quantified. The *y* axis shows relative fluorescence intensity of each cell. In **d**, yellow arrowheads indicate injected cells, and white arrowheads uninjected cells. **e**, **f**, Effects of dominant negative Spred. Cells were co-transfected with ΔC-Spred-1 mutant and EGF receptor, then treated with 10 or 50 ng ml^{-1} EGF for 3 d (**e**) or 30 min (**f**).

class of proteins that modulate Ras–Raf interaction and MAP kinase signalling.

From an osteoclast complementary DNA library, we isolated a tyrosine-kinase-binding protein by a yeast two-hybrid system using the c-Kit and c-Fms tyrosine kinase domains as bait. Full-length cDNA of this gene encodes a protein that contains a C-terminal SPR domain and an amino-terminal Ena/Vasodilator-stimulated phosphoprotein (VASP) homology-1 (EVH-1) domain[1] (Fig. 1a). Thus, we named this gene *Spred-1*: Sprouty-related protein with EVH-1 domain. We found a very similar gene (*Spred-2*) in the database and cloned its full-length cDNA (Fig. 1b). Using various deletion mutants, we identified a c-Kit-binding domain (KBD) composed of about 50 amino acids (codon 234–286) (see Supplementary Information Fig. 1). This region is not related to any previously identified tyrosine kinase interaction domains such as SH2, PTB or c-Met-binding domain. Spred-1 was tyrosine phosphorylated in response to stem cell factor (SCF), platelet-derived growth factor (PDGF) and EGF (Fig. 1c and data not shown), and efficient phosphorylation of Spred-1 required the KBD region (Supplementary Information Fig. 2). Using immunofluorescence microscopy, we detected localization of endogenous Spred-2 to the plasma membrane (Fig. 1d). Membrane localization of Spred was confirmed by exogenously expressed Spred fused to enhanced green fluorescent protein (EGFP) (Fig. 1d). The C-terminal SPR domain was essential for plasma membrane localization, as a deletion mutant lacking SPR domain (GFP-ΔC) localized in the cytoplasm (Fig. 1d).

Because Spred contains a Sprouty-related domain, we examined the effect of Spred on the Ras–MAP kinase pathway. First, we examined the differentiation of PC12 pheochromocytoma cells, which is induced by nerve growth factor (NGF) and dependent on MAP kinase[8]. As shown in Fig. 2a, overexpression of Spred-1 or Spred-2 strongly inhibited NGF-induced differentiation of PC12 cells. Both EVH-1 and SPR domains were essential for the suppression of differentiation (Fig. 2a, see ΔN and ΔC). Spred-1 suppressed NGF-induced activation of Erk2 MAP kinase (Fig. 2b). Next, we inhibited endogenous Spred by microinjecting affinity-purified anti-Spred-1 and -Spred-2 antibodies. The microinjection augmented neurite outgrowth of PC12 cells treated with a low concentration of NGF (Fig. 2c) as well as NGF-induced Erk2 activation (Fig. 2d). These results indicate that the endogenous Spred proteins suppress growth-factor-induced activation of MAP kinase and reduce the threshold of growth factor sensitivity for differentiation in PC12 cells. We noticed that the C-terminal deletion mutant augmented NGF-induced neurite outgrowth of PC12 cells (Fig. 2a), suggesting that the ΔC mutant may function as a dominant negative form against endogenous Spred proteins. Indeed, overexpression of the ΔC mutant augmented differentiation of PC12 cells treated with a low concentration (10 ng ml^{-1}) of EGF (Fig. 2e) and enhanced EGF-induced activation of Erk2 (Fig. 2f).

The negative effects of Spred on the Ras–MAP kinase pathway were confirmed in a different system. C2C12 cells differentiated into promyocytes when cultured in 2% horse serum (HS) (differentiation medium) for 3–5 days. MAP kinase activity has been shown to drop rapidly after switching to differentiation medium, but recovers after 3 days[9,10]. This decrease in MAP kinase activity in differentiation medium is essential for myotube formation. The levels of Spred-1 in C2C12 cells had increased by day 2 and dropped sharply on day 3 (Fig. 3a), and were inversely correlated to the levels of MAP kinase activity[10]. As shown in Fig. 3b and c, forced expression of dominant negative Ras (N17-Ras) induced morphological changes in C2C12 cells and reduced MAP kinase activity, even under normal growth conditions (20% FBS). Forced expression of wild-type Spred-1 and -2 exhibited similar effects (Fig. 3b, c). In contrast, C2C12 cells transfected with the constitutively activated form of Raf (ΔN-Raf) or dominant negative mutants (ΔC) of Spred-1 and -2 did not differentiate into myotubes even in differentiation medium, and a higher level of MAP kinase activity was maintained (Fig. 3d, e). Thus, ΔC mutants inhibited C2C12 cell differentiation probably by augmenting MAP kinase signalling, supporting the hypothesis that endogenous Spred proteins function as suppressors of the MAP kinase pathway in various systems.

Next, we investigated the molecular mechanism by which Spred suppresses the Ras–MAP kinase pathway. As one of the nuclear targets of MAP kinase is Elk-1, a transcription factor of the Ets family, EGF-induced activation of MAP kinase can be monitored by measuring the rate of Elk-1-dependent transcription[11]. In 293 cells, forced expression of Spred-1 or -2 dose-dependently suppressed EGF-dependent Elk-1 activation (Supplementary Information Fig. 3a). The negative effect of Spred-1 and -2 was comparable to that of Ras GTPase activating protein (rasGAP) and N17-Ras, and Spred-1 and -2 were more potent inhibitors than was murine Sprouty-4 or the Raf kinase inhibitor protein (RKIP)[12]. Both EVH-1 and SPR domains were necessary for the suppression of Elk-1 activation. Replacement of the EVH-1 domain of Spred-1 with that of Wiskott–Aldrich syndrome protein (WASP) abolished the inhibitory activity of Spred-1 (Supplementary Information Fig. 3c), suggesting that the EVH-1 domain of Spred-1 may interact with a specific target required for suppression of the MAP kinase pathway. In contrast, the KBD region was not essential but required for efficient suppression of the MAP kinase pathway (Supplementary Information Figs 2b and 3c).

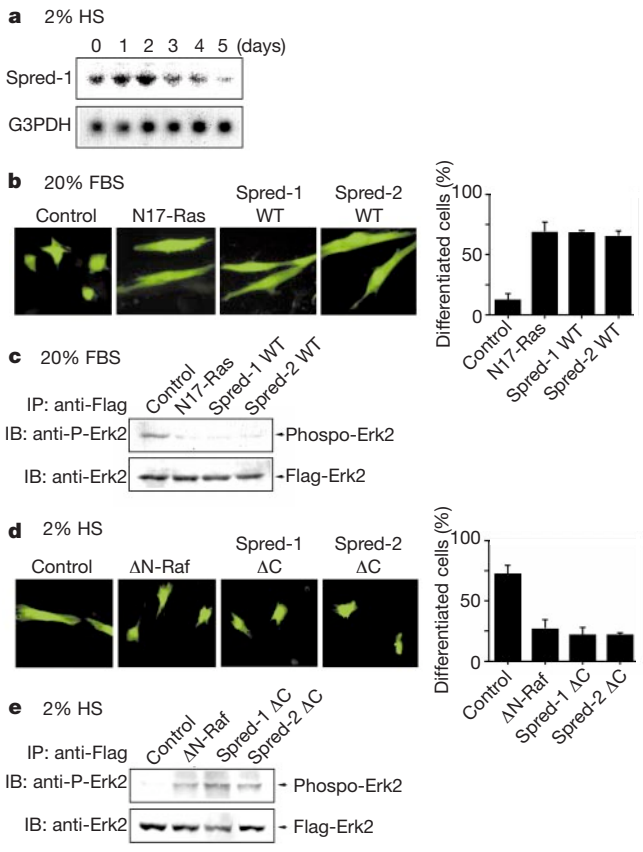

Figure 3 Effects of wild-type (WT) and C-terminal-truncated (ΔC) Spred proteins on differentiation of C2C12 cells and Erk2 activation. **a**, C2C12 cells were cultured in differentiation medium (2% HS) for the indicated periods and Spred-1 mRNA was measured. **b**, **c**, Wild-type Spred-1, Spred-2 and N17-Ras constructs were transfected with EGFP (**b**) or Flag-tagged Erk2 (**c**) then cultured in the growth medium (20% FBS) for 5 d (**b**) or 2 d (**c**). Cell morphology (**b**) and Erk2 phosphorylation (**c**) were measured. **d**, **e**, ΔC Spred-1 and Spred-2 constructs and constitutively activated Raf (ΔN-Raf) were transfected with EGFP (**d**) or Flag-tagged Erk2 (**e**). Cells were cultured in differentiation medium (2% HS) for 5 d (**d**) or 2 d (**e**).

We determined which component of the Ras–MAP kinase pathway is suppressed by Spred. Ras directly interacts with and activates Raf. Raf phosphorylates and activates MEK, which in turn phosphorylates and activates MAP kinases. Spred inhibited activation of Elk-1 induced by active Ras (V12-Ras), but not that induced by active MEK or active Raf (ΔN-Raf) (Supplementary Information Fig. 3b). Therefore, the target of Spred is probably located between Ras and Raf. To test this hypothesis, we examined the effect of Spred-1 on EGF-induced Ras and Raf activation (Fig. 4a). Interestingly, Spred sustained Ras activation, whereas it inhibited Raf activation, as measured by autophosphorylation (Fig. 4a) and by *in vitro* kinase assay (Fig. 4b). Furthermore, like rasGAP, Spred inhibited the phosphorylation of Raf on Ser 338, which is required for Raf activation, but not on Ser 259, which is not (refs 13, 14; Fig. 4c). Thus, Spred inhibits MAP kinase activity by suppressing Raf activation. In contrast, Spred did not affect EGF- or V12-Ras-dependent Akt activation, or EGF-dependent tyrosine phosphorylation of phospholipase-Cγ and membrane ruffling induced by the small GTP-binding protein Rac[15] (Supplementary Information Fig. 4). These data suggest that Spred specifically suppresses the Ras–Raf signalling pathway.

We then examined the interaction between Raf, Ras and Spred in C2C12 cells. After immunoprecipitation, endogenous Spred-2 was identified as a protein with a relative molecular mass of 40,000 (M_r 40K) that co-immunoprecipitated with endogenous Ras but not Raf (Fig. 5a). Furthermore, Ras and Spred co-localized at the plasma membrane, independent of EGF stimulation (Fig. 5b). Unexpectedly, Raf was translocated into the plasma membrane even in the presence of Spred-1 and co-localized with Spred-1 (Fig. 5b), thus indicating that inhibition of Raf activation by Spred is not due to a simple masking of the effector domain of Ras. We therefore examined the effect of Spred on the interaction between Raf and activated Ras (V12-Ras). As shown in Fig. 5c, the amount of Raf co-precipitated with active Ras was increased by Spred-1 co-expression. Augmentation of the interaction between Raf and Ras by Spred was confirmed by monitoring the time course of EGF-induced translocation of Raf to the plasma membrane. Raf was retained longer in the plasma membrane by overexpression of Spred-1 or Spred-2 (Supplementary Information Fig. 5). Thus Spred proteins potentiate Ras–Raf interaction, and Raf may not be accessible to Raf kinase

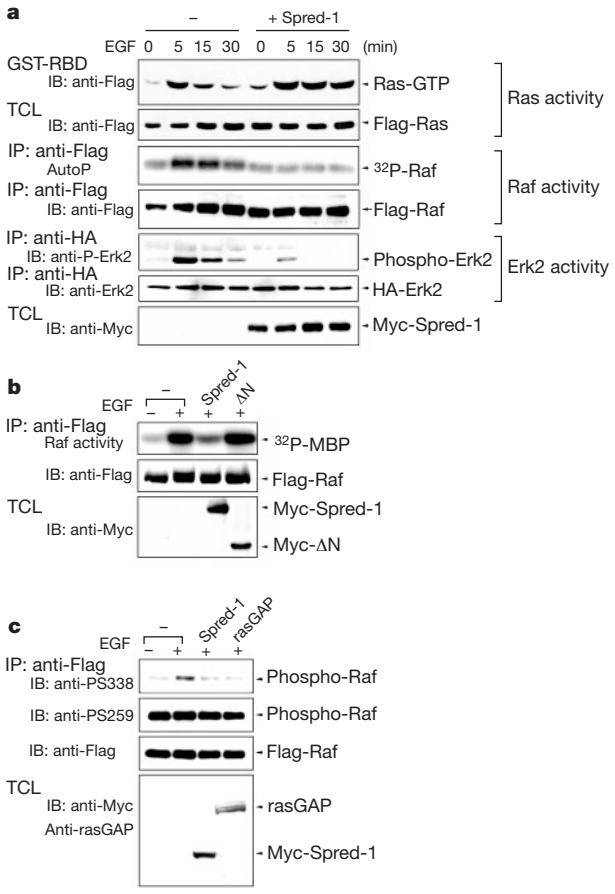

Figure 4 Spred inhibits the activation of MAP kinase by suppressing Raf activation. **a**, Time course of Erk2, Ras and Raf activation in 293 cells transfected with HA-Erk2, wild-type Flag-Ras and Flag-Raf with or without Spred-1. Activated Ras was precipitated with GST-Ras binding domain (GST-RBD) beads, and Raf activation was assessed by an *in vitro* autophosphorylation (AutoP) assay. **b**, Suppression of Raf kinase activity confirmed by an *in vitro* kinase assay using MAP kinase cascade components and MBP. **c**, Effect of Spred-1 on Raf phosphorylation. Flag-Raf was immunoprecipitated (IP) and blotted (IB) with anti-phospho-Ser 338 (PS338) or Ser 259 (PS259) Raf antibodies.

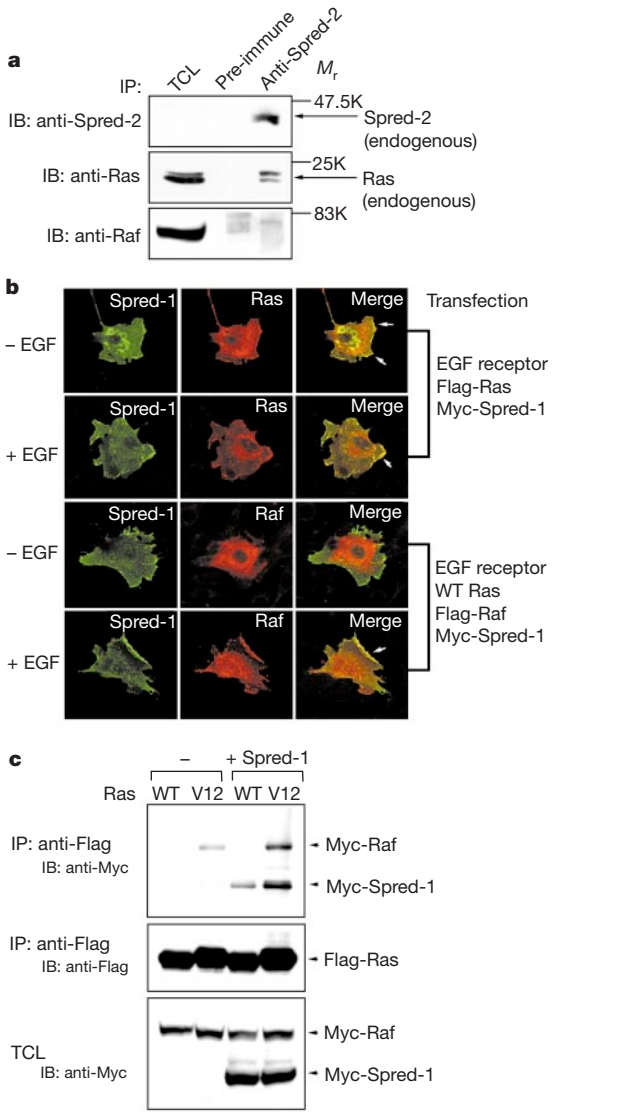

Figure 5 Interaction of Spred with Ras and Raf. **a**, Anti-Spred-2 immunoprecipitates (IP) from C2C12 cells were blotted (IB) with the indicated antibodies. TCL, total cell lysates. **b**, C2C12 cells were transfected with the EGF receptor, Myc-Spred-1 and Flag-Ras, or Myc-Spred-1, Flag-Raf and wild-type (WT) Ras. After stimulation with EGF for 5 min, Myc-tagged and Flag-tagged proteins were stained with secondary antibodies conjugated with FITC (green) and Cy3 (red), respectively. Arrows indicate co-localization. **c**, C2C12 cells were transfected with Flag-tagged wild-type (WT) or constitutively activated (V12) Ras, Myc-tagged Spred and Myc-tagged Raf. Anti-Flag immunoprecipitates were blotted with anti-Myc and anti-Flag antibodies.

when associated with the Spred–Ras complex, thereby preventing its activation.

Drosophila Sprouty is induced by FGF and inhibits Ras–Raf activation by interacting with rasGAP and Drk/Grb2 (ref. 3). The Spred orthologue of *Drosophila* is AE33, which was cloned as a probable target of the *rough* transcription factor that regulates photoreceptor cell development[16]. Thus, Spred and Sprouty are evolutionarily conserved, and several isoforms have been identified in mammals. Spred and Sprouty may negatively regulate the Ras–MAP kinase pathway in response to different stimuli in different tissues or organs. □

Methods

Screening and cloning of Spred-1 and -2

Randomly primed cDNA synthesized from mouse osteoclast messenger RNA was cloned into λ phage pASV3A vector and packaged. Plasmids were recovered by infecting phage into *Escherichia coli* BNN-132 (ref. 17). The yeast two-hybrid screen was done as described[18].

Plasmid construction

Mouse Spred-1 and -2, human Ras, Raf and MEK, and murine Sprouty-4 cDNAs were cloned into pcDNA3 with a six-repeated Myc tag or pCMV2 with a Flag tag at the N terminus. Spred-1 and -2 were also cloned into pEGFP (Clontech) to introduce the EGFP tag at the N terminus. Erk2 MAP kinase tagged with haemagglutinin (HA) and Flag was a gift of Y. Goto. WASP cDNA was donated by H. Miki and T. Takenawa. Deletion, substitution and chimaeric mutants were generated by standard polymerase chain reaction, as described[19].

Differentiation of PC12 and C2C12 cells

Rat pheochromocytoma-derived PC12 cells were maintained in DMEM supplemented with 10% FBS and 5% HS. We transfected PC12 cells seeded in plates coated with poly-L-lysine with EGFP-tagged Spred-1, EGF receptor and HA-tagged Erk2, using TransFast (Promega). Myc-tagged Spred-1 was introduced into PC12 cells using a retrovirus vector (pMX-IRES-EGFP) carrying Spred-1 cDNA and IRES-EGFP[20]. From 48 h after transfection or infection, PC12 cells were cultured in the presence of NGF or EGF for 72 h, then examined by fluorescence microscopy. Cells with processes longer than 1.5 times the diameter of the cell body were considered to be positive for neurite outgrowths[8]. Affinity-purified Spred antibodies with or without antigen peptides were microinjected with Eppendorf transjector 5246 or 5171 and examined by inverted microscopy (Zeiss), as described[21]. *In situ* Erk phosphorylation assay was performed as described[12].

Differentiation of C2C12 cells and transfection of Spred-1 were as described[9]. Cells grown in 24-well plates were co-transfected with pEGFP empty vector and Flag-tagged Spred proteins or their deleted mutants, Myc-tagged N17-Ras or ΔN-Raf, using LipofectAMINE (Gibco BRL). After transfection, cells were cultured for 5 d in 20% FBS or 2% HS, then examined by fluorescence microscopy. The elongated and multinucleated myotube-like cells were considered to be differentiated.

Immunochemical analysis

Immunoprecipitation and immunoblotting were performed with anti-Myc (9E10), anti-Flag (M2), anti-Ras (Calbiochem), anti-Erk2 (Santa Cruz), anti-HA (12CA5), anti-phosphorylated-Erk2 (Promega), anti-phospho-Ser 338 (Upstate) or Ser 259 Raf (New England) antibodies, as described[22]. The Ras-GTP form was precipitated with the Raf N-terminal Ras-binding domain fused to GST, as described[23]. Anti-Spred-1 or -2 antibody was prepared by immunizing rabbits with the KLH-conjugated peptides FKSKRRKEDG ERSRC or IKTQPPRAKSRRRKENGEC, respectively.

Kinase assays

In vitro kinase assay for Erk2 and Raf was performed using myelin basic protein as a substrate according to methods described[12].

Received 17 April; accepted 20 June 2001.

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

Acknowledgements

The first two authors contributed almost equally to this work. We thank H. Ohgusu, M. Sasaki and N. Tanaka for technical assistance; T. Sato and Y. Kaziro for wild-type and mutant Ras; H. Miki for the *Drosophila* cDNA library; Y. Hiromi for *Drosophila* Sprouty cDNA; P. Chambon, R. Denton, M. Yokouchi and J.-M. Garnier for library construction; Y. Sako and S. Minoguchi for valuable discussions; and M. Ohara for critical comments on the manuscript and for language assistance. This work was supported in part by grants from the Ministry of Education, Science, Technology, Sports, and Culture of Japan, Japan Research Foundation for Clinical Pharmacology, Fukuoka Cancer Society, Uehara Memorial Foundation and the Mitsubishi Foundation. Murine Spred-1 and Spred-2 are deposited in the nucleotide sequence databases under accession numbers AB063495 and AB063496, respectively.

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General transcription factors bind promoters repressed by Polycomb group proteins

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To maintain cell identity during development and differentiation, mechanisms of cellular memory have evolved that preserve transcription patterns in an epigenetic manner. The proteins of the Polycomb group (PcG) are part of such a mechanism, maintaining gene silencing. They act as repressive multiprotein complexes that may render target genes inaccessible to the transcriptional machinery[1,2], inhibit chromatin remodelling[3,4], influence chromosome domain topology[5] and recruit histone deacetylases (HDACs)[6]. PcG proteins have also been found to