

The neurofibromin recruitment factor Spred1 binds to the GAP related domain without affecting Ras inactivation

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Neurofibromatosis type 1 (NF1) and Legius syndrome are related diseases with partially overlapping symptoms caused by alterations of the tumor suppressor genes NF1 (encoding the protein neurofibromin) and SPRED1 (encoding sprouty-related, EVH1 domain-containing protein 1, Spred1), respectively. Both proteins are negative regulators of Ras/MAPK signaling with neurofibromin functioning as a Rasspecific GTPase activating protein (GAP) and Spred1 acting on hitherto undefined components of the pathway. Importantly, neurofibromin has been identified as a key protein in the development of cancer, as it is genetically altered in a large number of sporadic human malignancies unrelated to NF1. Spred1 has previously been demonstrated to interact with neurofibromin via its N-terminal Ena/VASP Homology 1 (EVH1) domain and to mediate membrane translocation of its target dependent on its C-terminal Sprouty domain. However, the region of neurofibromin required for the interaction with Spred1 has remained unclear. Here we show that the EVH1 domain of Spred1 binds to the noncatalytic (GAPex) portion of the GAP-related domain (GRD) of neurofibromin. Binding is compatible with simultaneous binding of Ras and does not interfere with GAP activity. Our study points to a potential targeting function of the GAPex subdomain of neurofibromin that is present in all known canonical RasGAPs.

cancer \mid signal transduction \mid protein–protein interaction \mid RASopathy \mid nontruncating mutations

eurofibromatosis type 1 (NF1) is an autosomal dominant genetic disorder with an incidence of 1 in 3,500 newborns and full penetrance. Patients with NF1 have an increased risk of developing the disease-typical neurofibromas consisting of benign and malignant tumors of the nervous system. Additional symptoms include characteristic pigmentation anomalies (café au lait macules, axillary freckling), melanocytic hamartomas of the iris (Lisch nodules), bone deformations, and learning disabilities, altogether defining a clinical picture that is far from being well understood and for which no cure is available (1). The tumor suppressor gene NF1 encodes the giant signal regulatory protein neurofibromin and is mutated in NF1 patients. The reported alterations (currently >2,000 mutations are listed in the Human Gene Mutation Database) include splice variants, nonsense and missense mutations, insertions and deletions, duplications and rearrangements, as well as repeat variations (www.hgmd.cf.ac.uk/ac/index.php). About 10% of the mutations comprise nontruncating (in-frame) deletions or missense mutations that are in principle compatible with the translation of the protein product (2).

Neurofibromin is a 320-kDa Ras-specific GTPase activating protein (RasGAP) that uses the central GAP-related domain (GRD) to enhance the GTPase activity of the small guanine nucleotide binding protein Ras, thereby down-regulating its biological activity (3–5). The *NF1* gene has gained considerable impact as it is frequently mutated in a variety of very aggressive human malignancies, including glioblastoma, lung adenocarcinoma, acute myeloid leukemia, and ovarian and breast cancers (6–12). Using structural biology approaches, we previously discovered a bipartite module located C-terminal to the GRD that is composed of a

glycerophospholipid binding Sec14-like domain associated with a previously undetected C-terminal pleckstrin homology (PH)-like domain (13–15). Although a number of interaction partners of neurofibromin have been reported, the significance of the underlying protein–protein interaction is not clearly established in many cases, as reviewed in refs. 2 and 16.

The recently described Legius syndrome (LS) shares the milder features with NF1, including café au lait macules, axillary freckling, and sometimes macrocephaly, but does not appear to include the more severe clinical manifestations of NF1 such as neurofibromas, optic pathway gliomas, or osseous lesions (17). LS is caused by germ-line mutations in the SPRED1 gene (17, 18) encoding the Sprouty-related, EVH1 domain-containing protein 1 (Spred1), which has been demonstrated to specifically inhibit the Ras/MAPK pathway in response to growth factor-, cytokine-, and chemokine-induced ERK activation (19-22). In pediatric acute myeloblastic leukemia, Spred1 acts as a tumor suppressor (23). The 50-kDa protein comprises an N-terminal Ena/VASP Homology 1 (EVH1) domain (24) and a C-terminal Sprouty-related domain (SPR) separated by a central c-Kit binding domain (19). EVH1 domains are PH-like protein-protein interaction modules (25, 26) that bind to proline rich sequence stretches of the type FPPPP (27, 28). Most of the reported interaction partners of Spred1 involve binding the C-terminal SPR domain with unclear function or significance (29). Most of the

Significance

Neurofibromatosis type 1 (NF1) and Legius syndrome (LS) are disorders characterized by deregulation of the Ras/MAPK pathway, which is central to a variety of cellular processes, including growth and differentiation. Both neurofibromin protein and Sprouty-related, EVH1 domain-containing protein 1 (Spred1) inhibit the Ras/MAPK pathway, and loss-of-function alterations in their encoding genes cause NF1 or LS, respectively. Such alterations are also often found in sporadic tumors unrelated to NF1 or LS. An earlier report described a cellular complex containing neurofibromin and Spred1; the details of these interactions remained unknown. Here we show that the EVH1 domain of Spred1 binds to the GTPase activating protein-related domain of neurofibromin without interfering with its catalytic activity. We further describe how a disease associated mutation in neurofibromin prevents Spred1 binding.

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LS-associated mutations found in the SPRED1 gene result in premature stop codons, presumably associated with truncated translation products, and the majority of the identified nontruncating missense mutations are located within the EVH1 domain (18).

Using MS combined with a tandem affinity purification (TAP) approach, neurofibromin was identified as a Spred1-interacting protein (30, 31). Stowe and coworkers performed a detailed analvsis using overexpressed as well as endogenous proteins to suggest a model for the role of Spred1 in Ras/MAPK inhibition, in which the EVH1 domain serves as the binding site for neurofibromin; the complex is then translocated to the plasma membrane via the Sprouty domain of Spred1. However, it was not clear from that study which region of the 2,818 residues comprising neurofibromin is necessary for Spred1 recognition and whether the interaction was direct or mediated by another cellular factor (31).

In this study, we tested binding of purified recombinant Spred1(EVH1) to different segments of neurofibromin that were prepared following recombinant expression in Escherichia coli or in insect cells. We find that the EVH1 domain binds to the GRD of neurofibromin with nanomolar affinity and that the interaction is fully compatible with binding of activated Ras without interfering with RasGAP activity. Furthermore, our study identifies the extra domain (GAPex) of the GRD as the binding site of the Spred1(EVH1) domain. GAPex is present in known canonical RasGAPs but its function has thus far remained unclear. A mutant neurofibromin harboring a disease-associated single residue deletion in the identified Spred1-binding site fails to interact with Spred1 and does not translocate to the plasma membrane but retains GAP activity, underscoring the physiological importance of this interaction.

Results

Spred1 Binds to the GRD of Neurofibromin and Localizes the Domain at the Membrane. To define the Spred1(EVH1) binding site on neurofibromin, we purified recombinant neurofibromin and soluble segments thereof (schematically shown in Fig. 1A) to homogeneity and analyzed their interaction with the EVH1 domain of Spred1 using an in vitro affinity purification approach. Immobilized recombinant Spred1(EVH1) captured all neurofibromin protein variants containing the GRD including the isolated GRD (Fig. 1B). Two LS patient-derived pathogenic missense mutations T102→R and W31→C (18) located on the surface of the EVH1 domain prevented the interaction with GRD-containing fragments (24) (Fig. S1). This result was supported and confirmed by analysis of several Flag-tagged neurofibromin constructs (Fig. 1A) for their ability to coimmunoprecipitate with Spred1 from human embryonic kidney cell lysates overexpressing both proteins (Fig. 1C). The observed failure of the constructs containing the N- and C-terminal portions of neurofibromin to bind but not of those containing the GRD strongly suggests that no other Spred1 binding site is present on neurofibromin. Membrane localization, which is a requirement to inactivate membrane-anchored Ras, is mediated by Spred1/2 (30). To determine whether membrane recruitment of neurofibromin by Spred1 depends on the GRD, we performed biochemical fractionation of human embryonic kidney cells in which either Spred1 or GFP (as a negative control) was expressed together with the various Flag-tagged neurofibromin constructs (Fig. 1D). As expected, Spred1 expression increased the membrane localization of endogenous neurofibromin. For the Flagtagged neurofibromin fragments, Spred1 expression specifically localized the Flag-tagged GRD-containing neurofibromin fragments to the membrane, but not those fragments lacking the GRD. Thus, our experiments define the GRD of neurofibromin as the binding site for the Spred1(EVH1) domain.

The GRD of Neurofibromin Forms a Ternary Complex with EVH1 and Activated Ras. The GRD is probably the best investigated part of the neurofibromin protein, especially its interaction with and enzymatic activity toward activated Ras (4, 32-36). We were interested in whether the neurofibromin-Spred interaction competes with Ras recognition or whether the GRD harbors an

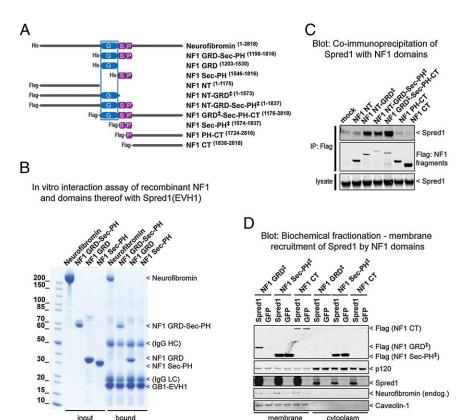


Fig. 1. The GRD of neurofibromin binds to Spred1 and localizes to the membrane. (A) Schematic presentation of the human neurofibromin constructs used to map the binding site of Spred1 on neurofibromin as shown in B and C. (B) In vitro binding study: purified recombinant neurofibromin proteins were incubated with GB1-tagged Spred1(EVH1) fusion proteins immobilized on IgG resin. Bound proteins were eluted and analyzed by SDS/PAGE, and protein bands were visualized by Coomassie staining. Neurofibromin constructs containing the GRD did bind to Spred1(EVH1). (C) Immunoprecipitation of Flag-tagged neurofibromin proteins overexpressed together with Spred1 in 293T cells. Proteins were separated by SDS/PAGE and identified by Western blotting. Only neurofibromin constructs containing the GRD were able to pull down Spred1. (D) Biochemical fractionation of 293T cells coexpressing the Flag-tagged neurofibromin constructs indicated on top of the gel and either Spred1 or GFP as a negative control. Proteins were analyzed as in C. Spred1 expression specifically localized the GRD to the membrane but not the other neurofibromin fragments.

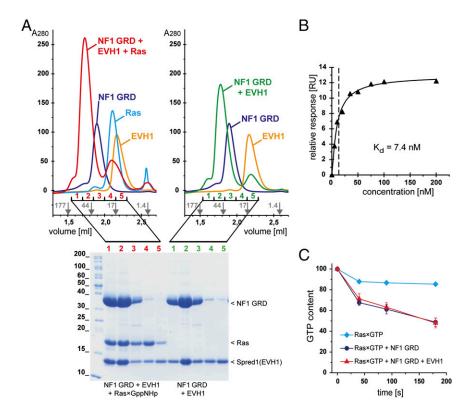


Fig. 2. GRD, Spred1(EVH1), and activated Ras form stable complexes. (A) Separation of a ternary GRD•Spred1(EVH1)•Ras×GppNHp complex (Upper Left; chromatogram in red) and of a binary GRD•Spred1(EVH1) complex (Upper Right; chromatogram in green). Equimolar amounts of the indicated purified recombinant proteins were mixed and analyzed by analytical gel filtration chromatography. Positions of size marker proteins are indicated by gray arrows. The indicated fractions from the gel filtration column were analyzed by SDS/PAGE [15% (wt/vol) acrylamide], and protein bands were visualized by Coomassie staining. (B) Surface plasmon resonance supported quantification of the GRD-Spred1(EVH1) affinity. Spred1(EVH1) (at concentrations between 5 and 200 nM) was injected over a surface loaded with 25 nM GRD. Relative responses at saturation were plotted against the Spred1(EVH1) concentration. The K_d corresponds to the Spred1(EVH1) concentration at 50% of the maximum intensity (saturation). (C) Enzymatic GAP activity of the GRD toward RasxGTP in the presence and absence of Spred1(EVH1). RasxGTP was incubated at room temperature with (blue circles) or without GRD (cyan diamonds); the former experiment was repeated in the presence of an excess of Spred1(EVH1) (red triangles). Samples from the reactions were taken at the indicated time points, and the nucleotides were qualified and quantified by HPLC. SEM values were calculated for the average of three independent experiments and displayed in the diagram (SEM < 0.01 is not visible).

additional vet unknown protein binding site. To answer this question, we prepared recombinant Spred1(EVH1), neurofibromin (GRD), and Ras bound to the nonhydrolysable GTP analog GppNHp (referred to as RasxGppNHp). The integrity of the purified recombinant proteins was analyzed by MS, revealing masses of 40,126 (GRD, calculated $M_r = 40,125$), 13,363 [Spred1(EVH1), calculated $M_r = 13,494$], and 18,852 (H-Ras, calculated $M_r = 13,494$) 18,853) confirming the expected masses for the GRD and for H-Ras and indicating cleavage of the N-terminal methionine $(\Delta m = 132)$ of Spred1(EVH1). Using these proteins, we performed analytical gel filtration experiments, and we were able to isolate a stable ternary complex consisting of the GRD, RasxGppNHp, and the EVH1 domain of Spred1 [termed GRD•RasxGppNHp•Spred1(EVH1)], as well as a binary complex of the GRD and the EVH1 domain of Spred1 [termed GRD•Spred1(EVH1)] (Fig. 24 and Fig. S2). The formation of the previously characterized GRD•Ras×GppNHp complex was used as a positive control (4, 33). Thus, both activated Ras and the Spred1(EVH1) domain can simultaneously bind to the GRD. Using a multiple angle light scattering (MALS) detector, we determined the molecular masses for the GRD, Spred1(EVH1), and the binary GRD•Spred1(EVH1) complex eluting in one peak from a size exclusion column, suggesting a 1:1 stoichiometry of the GRD to Spred1(EVH1).

To quantify the Spred1–neurofibromin affinity, we performed surface plasmon resonance experiments injecting Spred1(EVH1), activated Ras (RasxGppNHp), or inactive Ras [Ras bound to GDP (referred to as RasxGDP), as a negative control] over the GRD immobilized to surfaces (Fig. 2*B* and Fig. S3). Fitting the response curve for a 1:1 interaction model, as determined by the light scattering analysis, allowed the calculation of a dissociation constant (K_d) of 7.4 nM for the GRD•Spred1(EVH1) complex and of 6.2 μ M for the GRD•RasxGppNHp complex.

Spred1(EVH1) Binding to the GRD Does Not Affect RasGAP Activity. The GTPase reaction of Ras has been widely studied in vivo and in vitro. Because we observed binding of EVH1 to the GRD•Ras complex, we aimed to analyze potential influences on the catalytic property of the GRD. We set up an in vitro GAP reaction

and monitored the GRD-catalyzed Ras×GTP hydrolysis by HPLC analysis. Comparison of the reaction rates in the absence and presence of Spred1(EVH1) did not reveal significant differences (Fig. 2C), suggesting that Spred does not modulate the catalytic activity of the GRD. Consistently, we could isolate a stable transition state mimicking complex of the GRD and Ras×GDP in the presence of aluminum fluoride (32) binding to Spred1(EVH1) (Fig. S4). These data suggest that the interaction site of Spred on the GRD is distant to and independent of the Ras binding site.

The Extra Domain of the GRD Contains the Binding Site of Spred1 (EVH1). The simultaneous binding of Spred1(EVH1) and activated Ras to the GRD raises the question of where the EVH1 binding region is located on the GRD. Proteolysis experiments and structural analysis of a 333-residue neurofibromin GRD (NF1-333) revealed a bipartite module consisting of a 230-residue central domain exhibiting full GAP activity (termed NF230, GAPc, or GRDmin) and an extra domain (GAPex), formed by residues located N- and C-terminally to GRDmin (Nex and Cex, respectively) as schematically outlined in Fig. 3A (33, 37). Because the Spred1 interaction with the GRD neither influences Ras binding nor its catalytic GAP activity, we suspected that the location of the EVH1 binding site may be on the extra domain. Overexpression of deletion mutants of the GRD either lacking the N-terminal or the C-terminal part or the entire GAPex in an embryonic kidney cell line and immunoprecipitation, as well as biochemical fractionation experiments, revealed that the extra domain of the GRD is necessary for interaction with Spred1 and for membrane recruitment of neurofibromin (Fig. 3 B and C). Thus, EVH1 binds to the extra domain of the GRD as schematically shown in Fig. 3D. In addition, a neurofibromin protein carrying a patient-derived single residue deletion in the N-terminal part of GAPex (\Delta M1215) (38) failed to coimmunoprecipitate with Spred1 and to localize to the membrane upon Spred1 overexpression (Fig. 3 E and F). Although the NF1 Δ M1215 mutant could not be localized to the membrane by Spred, sufficient overexpression of this mutant, such that it was localized throughout the cell, showed that NF1 Δ M1215 retains GAP

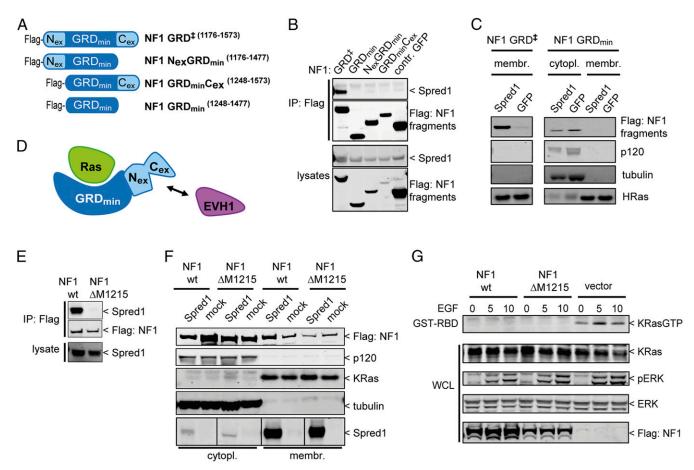


Fig. 3. An intact GAPex is required for Spred1(EVH1) binding. (A) Schematic presentation of the constructs used in this experiment outlining the location of the N- and C-terminal parts forming the extra domain of the GRD. (B) Untagged Spred1 and Flag-tagged GRD constructs or GFP as a negative control were expressed in 293T cells. Immunoprecipitation with anti-Flag beads using the indicated lysates shows that Spred1 does not associate with GRD proteins lacking the N-terminal, C-terminal, or both N- and C-terminal portions forming GAPex. (C) Spred1 or GFP (negative control) was expressed along with Flag-tagged GRD or Flag-tagged GRDmin in 293T cells. Cells were subject to biochemical fractionation of membrane and cytoplasmic compartments. Proteins in the fractions were separated by SDS/PAGE and identified by subsequent Western blotting. Expression of Spred1 moved the GRD to the membrane but did not alter the localization of the GRD lacking the extra domain (GRDmin). (D) Cartoon of the presumed ternary complex formed by the GRD, Ras, and Spred1(EVH1) according to our mapping data. (E) Same experiment as in B but using full-length Flag-tagged neurofibromin, either as WT protein or harboring a single residue deletion in the N-terminal part of the extra domain (NF1 Δ M1215). WT neurofibromin (NF1 wt) coimmunoprecipitated with Spred1, whereas the Δ M1215 mutation in the extra domain prevented the interaction. (F) Similar fractionation experiment as in C but following the transfection of plasmids encoding full-length Flag-tagged neurofibromin, either as WT protein (NF1 wt) or carrying the Δ M1215 mutation or an empty vector as a negative control together with Spred1. Overexpression of Spred1 enhanced the localization of WT neurofibromin to the membrane but not of the Δ M1215 mutant. (G) Overexpression of the neurofibromin Δ M1215 mutant in 293T cells suppressed EGF-induced GTP loading of Ras.

activity in human embryonic kidney cells by suppressing basal and EGF-induced Ras \times GTP loading (Fig. 3G).

Discussion

In this study, we show that neurofibromin interacts with Spred1 using its GAP-related domain. In detail, the interaction occurs via the noncatalytic extra domain of the GRD, is compatible with Ras binding, and does not affect GAP catalysis. Our study defines GAPex, the function of which has been unclear, as a protein-interaction module. The observation that the Spred1 binding region is close to but apparently not overlapping with the Ras-binding site may point to a Ras-targeting function of the Spred1-neurofibromin interaction, which is in agreement with our previous studies, where we showed that Spred1 binding enables neurofibromin to act on Ras in cells, presumably by bringing neurofibromin into proximity to Ras at the plasma membrane. Indeed, we showed that loss of function mutations in Spred1's C-terminal Sprouty domain could be rescued by targeting the mutant Spred1 protein to the plasma membrane with sequences derived from the Ras CAAX box (30).

We were surprised to discover that Spred1 binds at the GRD region of neurofibromin because another RasGAP, p120GAP (RASA1) is recruited to the plasma membrane through a very different mechanism: SH2 domains bind to phospho-tyrosines on receptor tyrosine kinases, and so position p120GAP in proximity to Ras×GTP (39). We therefore might have expected that Spred1 would bind to a domain elsewhere in the neurofibromin protein to recruit it to the plasma membrane. However, Spred proteins are thought to be intimately related to regulating signal transduction from receptor tyrosine kinases, such as the FGF receptor (40, 41). In contrast, neurofibromin-related proteins are well known in organisms such as Saccharomyces cerevisiae, which, of course, lack tyrosine kinase signaling altogether. The yeast IRA1 and IRA2 proteins are the best studied neurofibromin related proteins in distantly related organisms (42). Therefore, neurofibromin is likely to play important and highly conserved roles in aspects of cell physiology that are unrelated to tyrosine kinase signaling, and, in this sense, it would be surprising if neurofibromin and IRA proteins shared a domain devoted to Spred1 binding, as S. cerevisiae lack Spred proteins.

Sequence comparisons and structural studies of canonical RasGAPs, including p120GAP, synGAP, and neurofibromin, identify the presence of a GAPex domain that is located next to the central catalytic GAP domain and forms a conserved structure (33, 43, 44). Secondary structure prediction analyses of the IRA1/2 proteins suggest that the extra domain might be conserved in yeast. We speculate that GAPs may interact with proteins that perform functions similar to that of Spred1, and we are actively attempting to identify such novel partners and to determine their role in GAP and Ras regulation. Spred1 appears to play a role in positioning neurofibromin at the membrane, where it can interact with Ras. The localization of Ras to specific types of membranes is likely cell type dependent, and it is certainly possible that the regulation of Spred1 recruitment to different types of membranes has an effect on the regulation of Ras signaling, which will be addressed in future studies. However, the mechanism by which Spred1 associates with the membrane is not clear. The Sprouty domain is thought to be palmitoylated and necessary for membrane anchoring (21). The kit-binding domain may direct the Spred • neurofibromin complex to specific receptors within the membrane, and, perhaps, binding of Spred1 to the GAPex domain brings neurofibromin to Ras in a much more specific manner than by plain membrane recruitment. Our data represent a step toward a better understanding of the precise molecular mechanism by which Ras proteins are regulated. Defining the mechanistic details of the underlying protein-protein interactions will have to await the molecular structures of the complexes identified in this study.

Materials and Methods

Expression Plasmids. The expression constructs encoding recombinant human NF1 GRD-Sec-PH and NF1 Sec-PH have been previously described (45). A synthetic gene encoding human neurofibromin (45) was inserted into pACEBac1 finally encoding an N-terminally His-tagged protein. NF1 GRD used for in vitro experiments was expressed from a pET21 derivative in which a synthetic gene encoding residues D1203-H1530 of human neurofibromin preceded by a (His)₆ tag was inserted. Flag-tagged neurofibromin constructs used for transfection experiments in HEK293T cells encoded residues M1-T1175 (NF1 NT), M1-Y1573 (NF1 NT-GRD[‡]), M1-A1837 (NF1 NT-GRD-Sec-PH[‡]), Q1574-A1837 (NF1 Sec-PH[‡]), R1176-V2818 (NF1 GRD[‡]-Sec-PH-CT), K1724-V2818 (NF1 PH-CT), L1838-V2818 (NF1 CT), R1176-Y1573 (NF1 GRD[‡]), R1176-F1477 (NF1 N_{ex}GRD_{min}), D1248-Y1573 (NF1 GRD_{min}C_{ex}), and D1248-F1477 (NF1 GRD_{min}) in a pcDNA3.1 vector. Numbering corresponds to neurofibromin isoform 2 (NP_000258.1). Note: the domain boundaries of the GRD and the Sec-PH used for transfections differs slightly from the recombinant proteins (NF1 GRD and NF1 Sec-PH) described below and were therefore labeled NF1 GRD[‡] and NF1 Sec-PH[‡]. Human Spred1 and GFP were expressed from a pcDNA3.1 vector as described previously (30). A plasmid encoding untagged Spred1(EVH1) (residues S13-S130) was generated by inserting the corresponding synthetic gene into pET21d. The same cDNA was also cloned in pET21d downstream of a gene encoding the IgG binding protein G B1 domain (46, 47). Point mutations were introduced to generate the pathogenic mutated (T102→R, W31→C) EVH1 variants of the GB1-fusion proteins. H-Ras (M1-H166) was expressed from a construct previously described (48).

Preparation of Recombinant Proteins. The expression and purification of NF1 GRD-Sec-PH and NF1 Sec-PH have been previously described (45). Human neurofibromin was expressed in insect cells (SF21). NF1 GRD used for in vitro experiments was expressed in *E. coli* strain BL21 Star (DE3). All recombinant neurofibromin proteins were purified by metal chelate affinity and preparative size exclusion chromatography. Spred1(EVH1) and mutated forms (T102→R, W31→C) were expressed in *E. coli* strain BL21 Star (DE3). The introduction of the point mutation was verified by MS. Untagged Spred1 (EVH1) was purified by cation exchange and preparative size exclusion chromatography. H-Ras (M1-H166) was expressed as previously described (49) and purified by anion exchange and preparative size exclusion chromatography, yielding Ras in the GDP bound state (named RasxGDP). The integrity of the recombinant proteins used for the in vitro experiments described below was analyzed by electrospray ionization MS.

Ras loaded with guanosine 5'- $[\beta\gamma$ -imido]triphosphate (named Ras×GppNHp) was prepared following the concept described previously (50) but with the following modifications: Mg²+ was withdrawn from recombinant Ras×GDP (0.5 mM) by treatment with EDTA (1 mM) to enhance the nucleotide exchange

reaction (49), and released GDP was hydrolyzed by the addition of alkaline phosphatase in the presence of 200 mM (NH $_4$)₂SO $_4$ while GppNHp (added in a 1.5-fold molar excess) was incorporated. After GDP degradation was completed, as monitored by HPLC analysis, Ras×GppNHp was purified from the reaction mixture by size exclusion chromatography. Ras in its activated state (Ras×GTP) was prepared as follows: Ras×GDP was incubated with a molar excess of EDTA in the presence of GTP (100-fold molar excess) at 273 K for 1 h. The Ras protein was purified from the excess of nucleotide by size exclusion chromatography, yielding between 50% and 74% Ras×GTP.

In Vitro Interaction Assay. Bacterial lysates generated from GB1-Spred1(EVH1) expression cultures were incubated with IgG-sepharose. The resins were washed in 20 mM Tris buffer (pH 8.0) containing 300 mM NaCl, 2 mM MgCl₂, 2 mM DTT, and 2% (vol/vol) glycerol and purified recombinant neurofibromin proteins were added. After a 30-min incubation at 273 K, the resins were washed again using the same buffer. Bound proteins were eluted in SDS sample buffer and analyzed by SDS/PAGE [4–12% (wt/vol) acryl amide gradient].

Immunochemical Analysis and Biochemical Fractionation. HEK293T cells were grown in DMEM with 10% (wt/vol) bovine calf serum and grown to 70% confluency at the time of transfection. Cells were transfected with Lipofectamine 2000 and cultivated for another 24-48 h. For immunoprecipitation experiments, cells were lysed in a buffer containing 20 mM Tris (pH 7.5), 100 mM NaCl, 20 mM MgCl₂, 1% (vol/vol) Triton-X100, 1 mM DTT, and protease and phosphatase inhibitors. For biochemical fractionation, cells were resuspended in hypotonic lysis buffer [10 mM Tris (pH 7.5), 1 mM EDTA, 1 mM DTT, supplemented with protease and phosphatase inhibitors], kept on ice for 20 min, and then passaged through a 25-G needle 15 times. Nuclei and unbroken cells were then removed by centrifugation at 100 \times g for 3 min; the NaCl concentration was adjusted to 100 mM, and the supernatant was then centrifuged at 10,000 \times g for 15 min to pellet the membrane fraction. The resulting supernatant was then centrifuged at $100,000 \times g$ for 30 min to clear the cytoplasmic fraction. Membrane pellets were resuspended in SDS/PAGE sample buffer. The generated fractions were analyzed by Western blotting. The Spred1 antibody was from Abcam; the neurofibromin, p120GAP, H-Ras, and tubulin antibodies were from Santa Cruz Biotechnology; and the FLAG, ERK, and K-Ras antibodies were from Sigma.

Analytical Gel Filtration. GRD, RasxGppNHp, and Spred1(EVH1) were each diluted to 100 μM and injected into a Superdex 200 increase 5/150 analytical SEC column (GE Healthcare) equilibrated in 20 mM Tris buffer (pH 8.0) containing 150 mM NaCl and 2 mM MgCl₂. Proteins were UV detected at 280 nm, and additionally, the molecular weight of the eluted species was determined by multiple angle light scattering (MALS) using a miniDAWN TREOS detector (WYATT). For analysis of complex formation, proteins were mixed at 100 μM each in the same total volume and analyzed under identical conditions. A similar experiment was performed using RasxGDP in the presence of an equimolar amount of aluminum ions (Al³⁺) and a 330-fold excess of fluoride together with the GRD and Spred1(EVH1). The protein content of the collected fractions was analyzed by SDS/PAGE [15% (wt/vol) acrylamide] and subsequent Coomassie staining. Blue dextran and a protein standard mixture were run under the same conditions to determine the void volume v_0 of the size exclusion column and the elution volume of the size reference proteins.

Surface Plasmon Resonance Experiments. A Ni²⁺-loaded NTA sensor chip in a BiacoreX100 system (GE Healthcare) was coated with the His-tagged GRD in 10 mM Hepes buffer (pH 7.4) containing 150 mM NaCl and 0.05% (vol/vol) polysorbate 20. For experiments including Ras, the buffer was supplemented with 1 mM MgCl $_2$. The second flow cell with immobilized Ni²⁺ ions was used as a reference surface. Dilution series in the range between 5 and 200 nM of Spred1(EVH1) and 700 nM and 50 μ M of RasxGppNHp (positive control) or RasxGDP (negative control) were prepared and injected. The dissociation constant K_d was determined by nonlinear fitting of the sensorgrams to a 1:1 interaction model using Biacore X100 software (version 2.0.1). At least two independent titration experiments were performed, and results were averaged.

GAP Assays. RasxGTP samples (20–100 μ M RasxGTP in 50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 10 mM MgCl₂, and 2 mM DTT) were incubated at 293 K in the absence or presence of the GRD (molar ratio of GRD to RasxGTP 1:2,000) or the GRD together with an 100-fold excess of Spred1(EVH1) relative to the GRD. Samples were taken after 40, 90, and 180 s and heat denatured for 60 s at 95 °C. The released nucleotide was qualified by reversed phase HPLC as

previously described (51). For HPLC quantification, standards with GDP and GTP concentrations between 1 and 100 μM and protein samples from the GAP reactions were analyzed in triplicates. GAP activity was determined in three independent experiments for each of the experimental setups. The initial loading state of RasxGTP was set to 100%. The SEM was calculated using the formula SEM = $\sqrt{|\sum (x - \overline{x})^2|/n/\sqrt{n}}$ with n = 3. EGF stimulation and measurement of GAP activity from 293T lysates was performed as described previously (30).

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