Regulation of Son of sevenless by the membrane-actin linker protein ezrin

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Receptor tyrosine kinases participate in several signaling pathways through small G proteins such as Ras (rat sarcoma). An important component in the activation of these G proteins is Son of sevenless (SOS), which catalyzes the nucleotide exchange on Ras. For optimal activity, a second Ras molecule acts as an allosteric activator by binding to a second Ras-binding site within SOS. This allosteric Ras-binding site is blocked by autoinhibitory domains of SOS. We have reported recently that Ras activation also requires the actin-binding proteins ezrin, radixin, and moesin. Here we report the mechanism by which ezrin modulates SOS activity and thereby Ras activation. Active ezrin enhances Ras/MAPK signaling and interacts with both SOS and Ras in vivo and in vitro. Moreover, in vitro kinetic assays with recombinant proteins show that ezrin also is important for the activity of SOS itself. Ezrin interacts with GDP-Ras and with the Dbl homology (DH)/pleckstrin homology (PH) domains of SOS, bringing GDP-Ras to the proximity of the allosteric site of SOS. These actions of ezrin are antagonized by the neurofibromatosis type 2 tumor-suppressor protein merlin. We propose an additional essential step in SOS/Ras control that is relevant for human cancer as well as all physiological processes involving Ras.

ERM proteins | autoinhibition | GEF regulation

The small GTPase Ras (rat sarcoma) regulates essential cellular processes such as proliferation, motility, and differentiation. Activation of Ras by receptor tyrosine kinases (RTKs) is mediated by the guanine nucleotide-exchange factor (GEF) Son of sevenless (SOS). SOS is recruited by activated RTKs and subsequently engages Ras. In recent years, however, it has been recognized that this simple activation process is subject to a complex regulation. A number of regulatory motifs on SOS have been identified: the C-terminal catalytic Ras-binding domain for nucleotide exchange (1), the N-terminal half that carries histone-like sequences rich in positively charged amino acids, a Dbl homology (DH) domain, and a pleckstrin homology (PH) domain (1, 2). The DH/PH domains decrease the catalytic activity of SOS by folding back on the catalytic domain, thereby restricting accessibility to a second Ras-binding site that is distinct from the catalytic site (2). This allosteric Ras-binding site is important for the activation of SOS. Thus, Ras itself is an essential determinant of SOS regulation (2). Finally, lipid interaction contributes to the activation of SOS: The positively charged histone-like sequences interact with the negatively charged plasma membrane (3, 4). Moreover, binding of both phosphoinositides to the PH domain (5) and phosphatidic acid (PA) to the histone-like domain enhances SOS activity by relieving autoinhibition and exposing the allosteric Ras-binding site (6–8).

Our interest in small GTPases was triggered originally by the observation that members of a family of actin-binding proteins—ezrin, radixin, and moesin (ERM)—appear to enhance Ras activity (9). We showed that in response to growth factors ERM proteins form a multiprotein complex at the plasma membrane that comprises Ras, SOS, filamentous actin, and coreceptors such as β1-integrin. Coreceptors focus these complexes to relevant sites of RTK activity at the plasma membrane/F-actin interface. We defined binding sites on ezrin for both Ras and SOS, mutations of which destroy the interactions and inhibit the activation of Ras. Our data revealed that ERM proteins are essential intermediates in the control of Ras activity that fine-tune growth factor signals. In the present study we dissect the level of ERM action in a purified system: In addition to the direct assembly of Ras and SOS, ezrin (the ERM protein prototype) participates in the control of SOS activity by facilitating the encounter of Ras and SOS. We conclude that ezrin mediates the spatiotemporal control of Ras activity by acting as a regulatory scaffold for Ras and SOS.

Results

We demonstrated that SOS precipitated from cells exerts significant nucleotide exchange activity only when bound to ezrin (9). This finding suggests that ezrin not only acts as a scaffold assembling Ras and SOS but also participates in SOS activity control (9).

In this paper we explore the regulatory condition under which ezrin participates in Ras activation. We then characterize the ERM-dependent complex in vitro by examining SOS activity using purified recombinant proteins. This method excludes the possibility that additional components present in the cell-derived ezrin complexes participated in SOS activation. Specifically, we focused

Significance

Activity of the small GTPase Ras (rat sarcoma) needs to be tightly regulated because aberrant activity has a potent oncogenic effect, causing several forms of cancer as well as developmental disorders known as RASopathies. We have identified the ezrin, radixin, and moesin protein family as a previously unknown component in the control of Ras activity. Ezrin functions in the assembly of a protein complex that facilitates the encounter of Ras with its activating enzyme, Son of sevenless. Thus ezrin mediates a spatiotemporal control of Ras activity that helps coordinate and safeguard Ras signaling output.

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The authors declare no conflict of interest.

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on whether ezrin is subject to regulation itself and whether ezrin exerts control over its partners, possibly contributing to the release of SOS from autoinhibition and enabling the presentation of Ras to the allosteric regulatory site of SOS.

Active Ezrin Interacts with both SOS and Ras. In vivo, ezrin can be activated by threonine phosphorylation (10). Threonine phosphorylation of the ERM proteins (ezrinT567, radixinT564, and moesinT558) releases intramolecular and/or intermolecular head-to-tail associations. An open active conformation allows the ERM proteins to link the plasma membrane with the actin cytoskeleton (10). We observed that with the addition of PDGF this threonine-phosphorylated site is enhanced (Fig. 1*A*; see Table S1 for overview about used mutants) and that this amplification correlates with enhanced Ras and MAPK/Erk activation (Fig. 1*A*).

We tested whether this active ezrin can interact with Ras and SOS from cellular lysates in vitro and whether this active ezrin is sufficient for or contributes to Ras activation. GST- growth factor receptor-bound protein 2 (Grb2) pull-down experiments from cell lysates showed that pseudoactive ezrin, unlike wild-type ezrin (ezrinWT), formed a complex with SOS and Ras independent of PDGF stimulation (Fig. 1*B* and Fig. S1*A*, quantitation of exogenous ezrin expression). These data indicate that an active ezrin can interact with both SOS and Ras.

According to our previous data, this interaction with ezrinT567D should increase the GTP loading of Ras. Although the pseudoactive ezrin can complex with SOS and Ras before growth factor stimulation, ezrinT567D expression in NIH 3T3 fibroblasts showed enhanced and prolonged activation of Ras (determined as GTP-Ras) and MAPK/Erk activation only upon PDGF stimulation (Fig. 1*C* *Right*, compared with ezrinWT, *Left*). A quantification of GTP-Ras and phospho-Erk over total Ras illustrates an enhanced and prolonged signaling activation (graph in Fig. S1*B*). Apparently ezrin action on Ras is specific, because the pseudoactive ezrin had no significant effect on other small GTPases (e.g., Rac and Rho) (Fig. S1*C*). As a consequence of an increase in Ras activity, the proliferation rate of ezrinT567D-expressing cells was increased as compared with cells expressing ezrinWT (Fig. 1*D*).

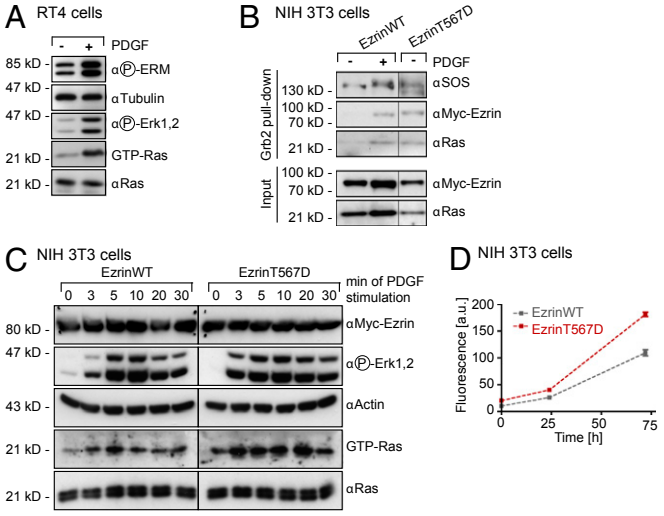

Fig. 1. Impact of active ezrinT567D on Ras activity and proliferation. (*A*) Ras activation correlates with ERM protein phosphorylation. Low-density RT4 cells were serum starved and PDGF stimulated (3 min). Ras activity was determined by GTP-Ras pull-down with GST-Raf1-Ras binding domain and coprecipitated proteins immunoblotted with a Ras antibody. Lysates immunoblotted as indicated. (*B*) EzrinT567D is in a complex with SOS and Ras even in the absence of PDGF stimulation. EzrinWT or ezrinT567D expressing stable clones of NIH 3T3 cells were serum starved and PDGF stimulated (1 min), and GST-Grb2 pull-downs of SOS were performed. Immunoblots as indicated, (*C*) EzrinT567D elevates the GTP-Ras level in NIH 3T3 cells. Cells of *B* were serum starved overnight and PDGF stimulated for the indicated time periods. Ras activity was determined as described in *A* (see quantification in Fig. S1*B* *Upper*). Lysates immunoblotted as indicated ($n = 3$). (*D*) The proliferation rate of NIH 3T3 ezrinWT cells is lower than that of ezrinT567D cells (Presto Blue assay; results are shown as mean ± SD; $n \geq 3$). All immunoblots are assembled from the same gels and taken at the same exposure time.

Active Ezrin Forms a Complex with SOS and Ras in Vitro. To ensure that it is the active ezrinT567D that binds its partner proteins and that no other components were required, we analyzed the association of recombinant ezrinT567D with SOS and Ras in vitro. Recombinant SOS containing the histone, DH, PH, and catalytic Rem-Cdc25 domains (SOS HDPcat) (see Table S1 for construct overview; see Fig. 3*A* for SOS domain organization) bound to ezrinT567D, but not to ezrinWT (shown by His pull-down in Fig. 2*A*), suggesting that activation of ezrin is a prerequisite for ezrin–SOS interaction. EzrinT567D bound directly most strongly to the PH domain of SOS, indicating that the PH domain is at least one docking site for ezrin (GST pull-downs in Fig. S2*A*). In the ezrin–Ras interaction, ezrinT567D, like ezrinWT (9), interacted directly with GDP-Ras in vitro (Fig. 2*B*), as demonstrated by the concentration-dependent increase of fluorescence when ezrinT567D was added to fluorescent 2′-/3′-O-(*N*′-Methylanthraniloyl)GDP (mantGDP)-Ras (Fig. 2*B* and *Material and Methods*). Size-exclusion chromatography further supported the finding that Ras interacts directly with ezrinT567D (Fig. S2*B*; ezrin–Ras interaction in fraction D1).

The critical test is whether ezrinT567D can form a complex with both Ras and SOS in vitro. To avoid nucleotide exchange that would disturb the fluorescence measurements, we used the fluorescent mantGDP loaded onto the Ras mutant Y64A that preferentially interacts with the allosteric Ras-binding site of SOS (11, 12). We could show clearly that SOS interacts with RasY64A and that ezrinT567D interacts with RasY64A, inducing an increase in fluorescence when added to fluorescent mantGDP-labeled Ras (Fig. 2*C*). Mixing all three proteins together further enhanced the fluorescence (Fig. 2*C*), suggesting that ezrinT567D, Ras, and SOS form a complex in vitro.

Finally we showed that, unlike wild-type Ras (RasWT), Ras containing the D38A mutation in the switch 1 region (13) could not interact with ezrin (Fig. 2*D*), indicating that GDP-Ras interacts with ezrin through the Ras switch I region. We conclude that active ezrin can recruit both SOS and Ras and thus serves as a scaffold, which is required for Ras activation in vivo.

Active Ezrin Stimulates SOS Activity in Vitro. The DH/PH domains impede SOS activation by shielding the allosteric Ras-binding site (2). Our identification of the PH domain as a direct interaction site for ezrin suggests that ezrin might facilitate SOS activation either by removing this DH/PH shield and/or bringing GDP-Ras to the allosteric site on SOS, that is essential for SOS activity (2). Therefore we investigated the action of ezrin on SOS activity in vitro by monitoring nucleotide exchange activity via the release of fluorescently labeled GDP (i.e., mantGDP) from preloaded Ras (made visible as a time-dependent reduction of fluorescence intensity; Fig. 3*B* and Fig. S3). Recombinant SOS (autoinhibited SOS HDPcat) exhibited low activity in vitro compared with an N-terminally truncated form of SOS (SOScat) that is not autoinhibited because it lacks the DH/PH and histone-like domains but still carries the allosteric site (a comparison of the nucleotide-exchange activity of different SOS constructs in the in vitro GEF assay is shown in Fig. S3) (2). Interestingly, the addition of ezrinT567D caused a 41% increase in SOS HDPcat activity (Fig. 3*B*), indicating that ezrin alone is a modulator of SOS activity. Because Ras activation in a cellular system requires ezrin's direct interaction with Ras (9), the Ras-binding site mutation in ezrin (R40L) (9) was introduced in the T567D background and tested for relevance in the purified system. Compared with the stimulating effect of ezrinT567D, the phospho-mimicking double mutant ezrinR40L/T567D, which lacked the ability to bind to Ras

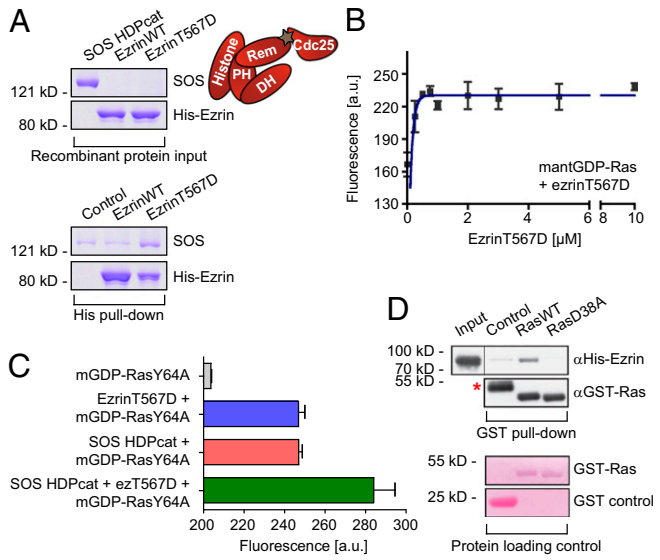

Fig. 2. Active ezrinT567D associates with Ras and SOS in vitro. (*A*) His pull-down studies using His-ezrinWT or His-ezrinT567D and SOS HDPcat recombinant protein. Immunoblots as indicated. (*B*) EzrinT567D binds Ras in solution as shown by an increase in fluorescence of mantGDP-Ras (1 μM) incubated with ezrinT567D (0.25–10 μM). Results are represented as mean ± SD ($n = 3$). (*C*) EzrinT567D, SOS HDPcat, and mantGDP-Ras (mGDP-Ras) form a complex in vitro as demonstrated by a higher maximal fluorescence than seen with ezrinT567D/mantGDP-RasY64A and SOS HDPcat/mantGDP-RasY64A (results are shown as mean ± SD; $n = 3$). (*D*) Recombinant ezrinT567D associates with GST-RasWT but not with GST-RasD38A switch mutant protein in a GST pull-down assay; the red asterisk marks an unspecific band. The input of GST fusion proteins was stained with Ponceau S ($n = 3$).

by its N terminus, could not increase SOS HDPcat activity (Fig. 3*C*); this result clearly indicates that the Ras-binding to ezrin is essential for ezrin's effect on SOS activation. Taken together, these data show that in a purified system the nucleotide exchange activity of SOS is increased significantly when bound to active ezrin.

The lack of stimulating SOS activity could result from the failure of the ezrinR40L/T567D mutant to act as a scaffold bringing SOS and Ras into the complex and releasing autoinhibition. We also considered the possibility that ezrin might bring GDP-Ras closer to the allosteric site of SOS. This allosteric site drives nucleotide exchange and is subject to positive feedback control by nucleotide-bound Ras (2). The rate of nucleotide exchange by SOS is dependent on the concentration and on the nucleotide species bound to allosteric Ras (2): GTP-Ras is more potent than GDP-Ras. Generally, high concentrations of both GDP-Ras and GTP-Ras also will enhance the occupancy of the allosteric site and thereby increase the catalytic activity of SOS. To see whether ezrin indeed promotes the association of Ras with the allosteric site of SOS, we first exploited the unique property of the RasY64A mutant as a potent allosteric stimulator in a purified system with the self-masking and low-activity SOS HDPcat. The addition of the RasY64A stimulator at a high concentration increased nucleotide exchange significantly (46%) compared with a low concentration (19%) (Fig. 3*D*). Interestingly, the addition of the low concentration of RasY64A stimulator in combination with ezrinT567D increased SOS activity by 52%, mimicking the effect of the high concentration of stimulator alone (Fig. 3*D*). However, disruption of ezrinT567D's GDP-Ras binding (R40L/T567D) abolished its SOS-enhancing ability (Fig. 3*D*). These results suggest that part of ezrin's action might be to bring Ras to the proximity of the allosteric site on SOS.

Interaction of the Ezrin–Ras Complex with the Allosteric Site of SOS Enhances SOS Activity. The notion that phosphorylated, Ras-bound ezrin stimulates SOS activity was analyzed further in a second experimental approach. SOScat composed of the Ras exchange motif (Rem) and the Cdc25 domains interacted with the ezrinT567D mutant when isolated together with ezrin from cell lysates (coimmunoprecipitation in Fig. 4*A*). The interaction with ezrinWT was weaker than that with ezrinT567D (Fig. 4*A*), perhaps because of insufficient activation of the bulk of transfected ezrin. Disruption of the allosteric site by mutation (SOScat W729E; Table S1) (2) completely inhibited the interaction with ezrin (Fig. 4*A*). These data indicate that the phospho-mimicking ezrin can interact with SOScat. EzrinT567D and SOScat might interact directly when stabilized by other cellular components, or ezrinT567D may bind SOScat indirectly through Ras at the allosteric Ras-binding site.

We also tested whether ezrin could enhance the activity of SOScat and performed fluorescent GEF activity measurements analogous to the experiments with SOS HDPcat shown in Fig. 3. Interestingly, biexponential fitting of the kinetics data revealed two

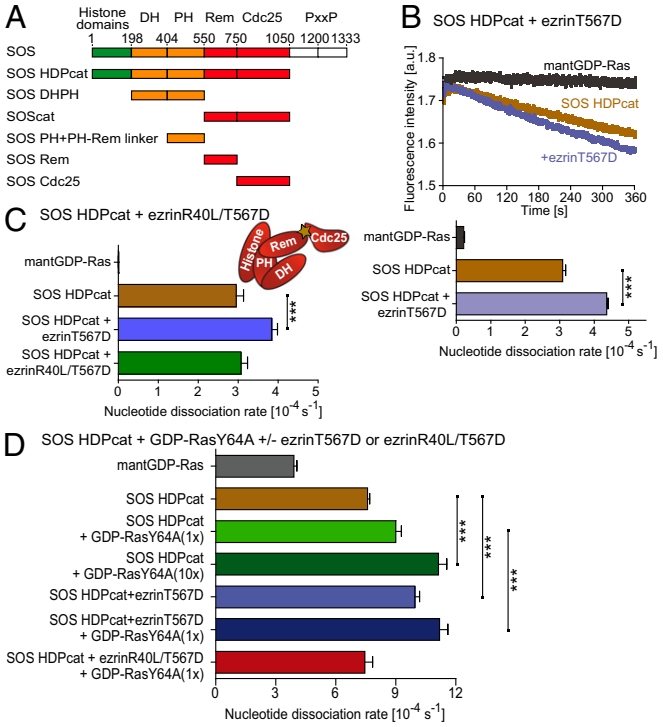

Fig. 3. Active ezrinT567D stimulates SOS HDPcat GEF activity in a purified system. (*A*) Schematic representation of the domain organization of different SOS constructs. The core catalytic unit consists of the Cdc25 domain and the Ras exchange motif (Rem). The very C-terminal proline-rich region (PxxP) is required for binding the SH3 domain of the protein Grb2. (*B*) EzrinT567D increases the SOS HDPcat exchange reaction (41%). Fluorescent mantGDP-Ras was incubated with unlabeled GDP in the absence (mantGDP-Ras, black) or presence of 1 μM SOS HDPcat (brown) or 1 μM SOS HDPcat plus 250 nM ezrinT567D (blue), and release of labeled nucleotide was measured. (*Upper*) Raw data are shown. (*Lower*) Raw data were subjected to linear fitting. Apparent nucleotide dissociation rates are illustrated (***$P < 0.001$ using the Mann–Whitney *u* test). Quantitative results represent mean ± SD ($n = 3$). (*C*) EzrinR40L/T567D (green bar), unlike ezrinT567D (blue bar), cannot stimulate the SOS HDPcat exchange reaction (250 nM of ezrin). Nucleotide exchange as described in *B*. Calculated apparent nucleotide dissociation rates are shown (linear fitting). The masked allosteric site of autoinhibited SOS HDPcat is illustrated by a brown star. (*D*) A combination of 250 nM ezrinT567D and 1×GDP-RasY64A (1× indicates a level equal to substrate mantGDP-Ras) stimulates SOS HDPcat exchange reaction more efficiently (dark blue, 52%) than 1×GDP-RasY64A alone (light green, 19%) and comparably to 10×GDP-RasY64A (dark green, 46%). A combination of 250 nM ezrinR40L/T567D and 1×GDP-RasY64A (red) cannot stimulate the SOS HDPcat exchange reaction at all. Nucleotide exchange is as described in *B*. Calculated apparent nucleotide dissociation rates are shown (linear fitting, ***$P < 0.001$ using the Mann–Whitney *u* test).

components describing the exchange of mantGDP-Ras by SOScat: a faster and a slower rate acting at timescales of seconds and minutes, respectively (Fig. S3). We propose that the two kinetics components might reflect different conformational states of SOScat acting at different exchange rates. Addition of the allosteric stimulator RasY64A further enhanced both rate constants in a concentration-dependent manner (Fig. S4*A* and Table S2); increasing concentrations of GDP- and GTP-RasY64A support the faster exchange, with GTP-RasY64A being more potent, demonstrating the moderate binding affinity of GDP-RasY64A (Fig. S4*C*). In addition, GTP-RasY64A shifted the relative contributions of the two rates (Table S2). In the presence of GDP, 30% of the nucleotide exchange is related to fast-acting SOScat, whereas in the presence of increasing concentrations of GTP-RasY64A the contribution of fast-acting SOScat increased progressively up to 80% (Table S2). Mutation of the allosteric site (SOScat W729E) completely eliminated the fast-kinetics component (Fig. S4 *B* and *D*), demonstrating the importance of allosteric regulation of SOS.

These data set the stage for testing the effect of ezrin on the allosteric site of SOScat. A mixture of ezrinT567D, SOScat, and fluorescent mantGDP-Ras (substrate as well as stimulator) (raw data are shown Fig. 4*B*) significantly stimulated both the fast-kinetics component (52% increase of the nucleotide dissociation rate) (Fig. 4*C*, *Left*) and the slow-kinetics component (36% increase) (Fig. 4*C*, *Right*) compared with mantGDP-Ras and SOScat in the absence of ezrin. Disruption of ezrinT567D's GDP-Ras binding (R40L/T567D) abolished this stimulation of SOScat activity of both kinetics components (Fig. 4*D*), indicating that ezrin–Ras interaction is required to elevate SOScat activity. Disruption of the allosteric site in SOScat (SOScat W729E) also abolished the stimulation by ezrin–Ras (Fig. 4*E*). We also studied ezrin action at different SOScat concentrations (Fig. S5*A*). Remarkably, ezrin does not potentiate nucleotide exchange at low and high concentrations of SOScat (Fig. S5 *B*, *C*, and *E*) as compared with moderate SOScat concentrations (0.5 μM) (Fig. S5*D*). We investigated this behavior in more detail by analyzing the relative contribution of the fast and slow components of the exchange reaction (Fig. S5*F*). At low SOScat concentrations, the slow rate dominates the reaction; at high SOScat concentrations, the fast rate takes over. We reason that ezrin acts predominantly on the mechanism that is responsible for the fast rate of nucleotide exchange, thus explaining why ezrin is less effective at low SOScat concentrations with little contribution of the fast rate. At very high concentrations of SOScat, the exchange reaction saturates, and ezrin again is less effective. In conclusion, ezrin-dependent stimulation of SOS requires an intact allosteric site and suggests that ezrin could bring GDP-Ras to the allosteric site on SOS more efficiently than free GDP-Ras.

We also show that ezrin is a regulatory scaffold for SOS activity control in vivo. We exploited another SOS mutant, the Noonan mutant M269R. Noonan syndrome is caused by a mutation that eliminates SOS autoinhibition (14, 15). Therefore this mutant behaves similarly to SOScat, permitting Ras access to the allosteric site. Cells expressing the Noonan mutation displayed elevated levels of activated Ras and MAPK/ERK (Fig. 4*F*, lanes 1 and 2). Down-regulation of ERM proteins inhibited Ras activation, demonstrating the dependence of SOS M269R on ERM proteins (Fig. 4*F*, lanes 2 and 4). However, a mutated Ras with high affinity to the allosteric site (D38E/A59G) (16) stimulated SOS activity even in the absence of the ERM proteins (Fig. 4*F*, *Right*), indicating that the need for the ERM proteins can be overcome by stronger Ras binding to the allosteric site. In conclusion, ezrin brings GDP-Ras to the proximity of the allosteric site on SOS both in vitro and in vivo.

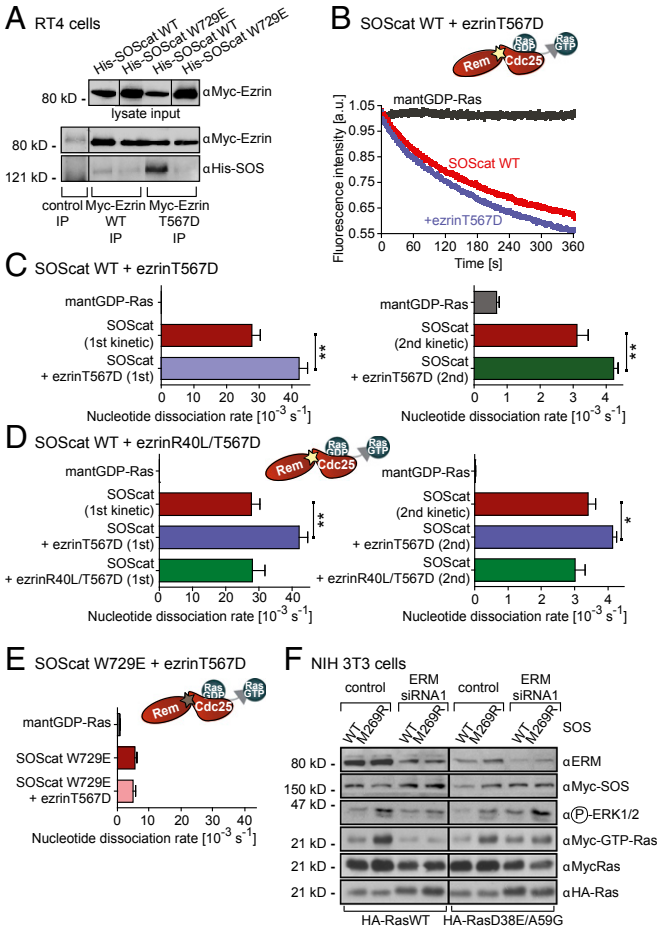

Fig. 4. Active ezrinT567D enhances SOScat activity by assisting with allosteric site engagement. (*A*) RT4 cells were cotransfected with constructs expressing Myc-ezrinWT or Myc-ezrinT567D and His-SOScat WT or His-SOScat W729E. Proteins coimmunoprecipitated with Myc-ezrin were immunoblotted. Mouse IgG was used as the control immunoprecipitant (IP). Immunoblotted as indicated and assembled from the same gels and taken at the same exposure time. (*B* and *C*) EzrinT567D (250 nM) stimulates the first (blue, 52%; *C Left*) and second (green, 36%; *C Right*) kinetic. In vitro GEF activity as described in Fig. 3*B*. (*B*) Raw data are shown. A yellow star indicates the unmasked allosteric site of SOScat. (*C*) A biexponential function was fitted to the data: apparent nucleotide dissociation rates are shown; $^{**}P < 0.01$ using the Mann–Whitney *u* test. Quantitative results represent mean ± SD, $n = 3$. (*D*) Measurement of in vitro GEF activity and calculations as described in Figs. 3*B* and 4*C* (biexponential fitting); $^{*}P < 0.05$ using Mann–Whitney *u* test. (*E*) A gray star indicates the mutated allosteric site of SOScat W729E. Measurement of in vitro GEF activity and calculations was as described in Fig. 3*B* (single exponential fitting). (*F*) Released Noonan SOS M269R is still dependent on ERM proteins. Low-density NIH 3T3 cells treated with ERM-targeting siRNA SMARTpools followed by cotransfection of Myc-SOS WT or the Myc-SOS Noonan mutation M269R with either HA-RasWT or HA-RasD38E/A59G. Ras activity (based on transfected Myc-Ras) was as described in Fig. 1*A* ($n \geq 3$).

The Lipid Environment Contributes to Full SOS and Ras Activation. Ras activation in vivo occurs at the cytoplasmic side of the plasma membrane. In vitro a highly structured artificial surface mimicking a membrane enhances SOS activity (5, 6). Moreover, binding of phosphoinositides to the PH domain and the binding of PA to the histone-like domain also modulate SOS autoinhibition (5–8). Therefore we considered whether other cellular components might be missing in vitro that contribute to full SOS activation in vivo. The addition of PA to SOS HDPcat in the assay with recombinant proteins indeed led to an increased activity (17%) (Fig. 5*A*). A combination of ezrinT567D and PA further enhanced SOS activity (63%) (Fig. 5*A*). Although the recombinant proteins do not fully recapitulate the in vivo conditions, they do demonstrate that ezrin has a decisive role in the regulation of SOS activity and that ezrin and PA cooperate.

If membrane targeting and lipid association in vivo are critical components for this unique signaling complex, we speculated that

artificially tethering ezrin to the plasma membrane would lead to activation of Ras. Interestingly, tethering of ezrin to the membrane by the Src membrane-association domain was sufficient to induce Ras activation spontaneously (Fig. 5*B Left*; without FCS stimulation) and cell proliferation (Fig. S6; without FCS stimulation). The membrane-anchored ezrin was more heavily phosphorylated than overexpressed WT ezrin (Fig. 5*C*) and induced MAPK/ERK activity (Fig. 5*C*) and cellular transformation (determined as soft-agar colony formation) (Fig. 5*D*). Despite increased phosphorylation, the membrane-anchored ezrin mutated in the Ras-binding site (R40L) (9) could not induce cellular transformation and carried a reduced MAPK/Erk activity (Fig. 5 *C* and *D*). Taken together, these results suggest that the in vivo targeting of ezrin to the plasma membrane and its direct interaction with Ras and SOS are essential for full activation of SOS.

Physiological Regulation of Ezrin Activity. Merlin, the neurofibromatosis type 2 tumor-suppressor protein, can counteract ezrin by competing for the same binding sites on coreceptors (17). Merlin activation at high cell density antagonizes the formation of the SOS–ezrin complex (18). Neither activated merlin from high cell density cells (18) nor an active merlin mutant (S518A) interacted with SOS (Fig. S7*A*). However, we did observe a complex between merlin and Ras (Fig. S7 *B* and *C*). Purified Ras loaded with GDP pulled down merlin from cellular lysates (Fig. S7*B*). Also incubation with fluorescent mantGDP-Ras indicated that merlin interacted directly with GDP-Ras (Fig. S7*C*), likely through the N-terminal 4.1 protein/ezrin/radixin/moesin (FERM) domain also found in ezrin. Because merlin cannot interact with SOS, we reasoned that, unlike ezrin, merlin is unable to bring GDP-Ras to the allosteric site. Indeed, a mixture of recombinant merlin and SOS HDPcat (Fig. S7*D*) showed no increase in SOS activity. Moreover purified merlin added to SOScat significantly inhibited nucleotide exchange activity in vitro (Fig. S7*E*), indicating that merlin may bind and sequester fluorescent mantGDP-Ras from both allosteric and the catalytic sites. Furthermore, overexpression and activation of merlin in transformed RT4 cells at high cell density (17) counteracted ezrinWT- and PDGF-dependent Ras/Erk activation (Fig. S7*F*) and transformation (Fig. S7*G*; three independent clones). Enhancing the level of ezrin (T567D mimicking the activated state of ezrin at high cell density) abolished these effects of merlin (Fig. S7 *F* and *G*). These data demonstrate the counteraction of ERM proteins and merlin, showing that the ERM protein-dependent regulatory step of Ras activation can be modulated by the physiological regulator merlin.

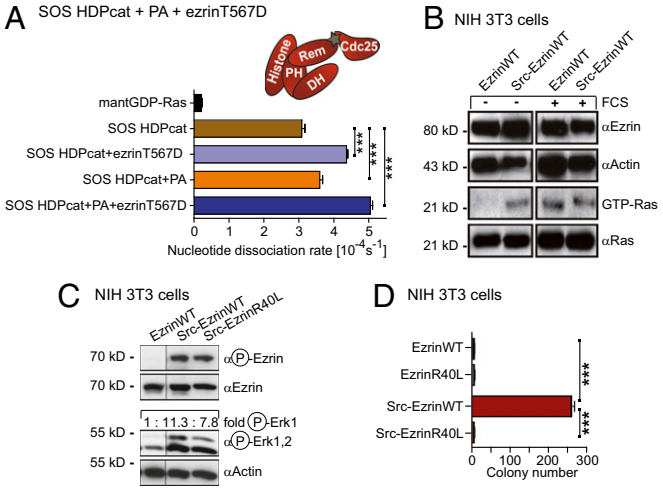

Fig. 5. The lipid environment contributes to SOS and Ras activation. (*A*) PA-containing liposomes were incubated with SOS HDPcat WT in the absence (orange, 17%) or presence of 250 nM ezrinT567D (dark blue, 63%), and nucleotide exchange was measured as described in Fig. 3*B*. Apparent nucleotide dissociation rates are shown (linear fitting, $***P < 0.001$ using the Mann–Whitney *u* test). Quantitative results represent mean ± SD ($n = 3$). (*B*) EzrinWT or Src-ezrin–expressing stable clones of NIH 3T3 cells were either serum starved overnight or maintained in normal medium (+FCS). Ras activity was determined as described in Fig. 1*A*. Lysate controls were immunoblotted as indicated ($n \geq 3$). (*C*) Cells of *B* were seeded overnight, kept in suspension for 40 min, and stimulated with PDGF during the last 3 min. Immunoblots as indicated. Quantification of P-Erk1 increase was carried out using ImageJ. Signals were normalized to actin levels. (*D*) Only the NIH 3T3 cells expressing Src-ezrin, and not cells expressing either ezrinWT or the Ras-binding mutant Src-ezrinR40L, formed colonies in a soft-agar assay. (Results are shown as mean ± SD, $n \geq 3$, $***P < 0.001$ using Student *t* test.)

Discussion

Ras is an essential signaling protein that cycles between inactive GDP-bound and active GTP-bound states. Triggering nucleotide exchange and thereby activating Ras through RTKs is a crucial step in mediating multiple cellular functions. Downstream of RTKs, the GEF SOS is a necessary intermediate in Ras control. This step of SOS-mediated Ras stimulation includes several regulatory actions to coordinate and safeguard biological output. SOS, a large molecule with several domains, contains two binding sites for Ras, the catalytic site and an allosteric site. GDP-Ras or GTP-Ras binding to the allosteric site drives the catalytic function of SOS. The DH/PH domains block the access to the allosteric site (2) and therefore must dissociate from the allosteric site, a process promoted by the interaction with lipids of the plasma membrane (2, 5, 7).

Our results add to the complexity of the SOS-Ras regulation. Phosphorylated ezrin (the ERM protein prototype) directly binds Ras and SOS. In addition, we show that active ezrin elevates the nucleotide exchange activity of SOS (model in Fig. 6), probably by facilitating the encounter of Ras with the allosteric regulatory site of SOS. Our conclusions are based on several experiments, which included the disruption of ezrinT567D binding GDP-Ras, thereby inhibiting ezrin's ability to enhance SOS. Because the affinity of GDP-Ras for the allosteric site is weaker than that of GTP-Ras (12), ezrin appears to stimulate predominantly the initial allosteric regulation through its preferential interaction with GDP-Ras. To exert these functions, ezrin must be activated at least by phosphorylation of threonine 567, a conclusion supported by the experiments with the phospho-mimicking ezrin mutant (T567D).

We have demonstrated recently and in this study that the FERM domain of ezrin interacts with Ras (9). The recent structure determination of the FERM domain of Krev interaction trapped 1 (KRIT1) with Ras-related protein 1 (Rap1) revealed the mode of FERM:GTPase binding (Fig. S8*B*). Superposition of the structures of ezrin FERM and GDP-Ras onto KRIT1 and Rap1 of the complex structure, respectively, illustrates the probable interaction of ezrin with Ras (Fig. S8). The lack of ezrinT567D/R40L binding to GDP-Ras and of GDP-RasD38A binding to ezrinT567D is consistent with ezrin R40 and Ras D38 being part of the resulting interface. In addition, a Ras switch region is involved in binding, in agreement with the observed nucleotide-specific binding of ezrin to GDP-Ras. Interestingly, according to the superposition, GDP-Ras binds to ezrin with a surface area resembling those that are used by Ras to bind to the allosteric site of SOS (12), suggesting that ezrin might not simply transfer GDP-Ras directly to the SOS allosteric target site. Therefore a possible mechanism of SOS activation could be that SOS binding to ezrin might evoke subtle conformational changes in ezrin leading to the release of GDP-Ras and rebinding of Ras to the allosteric regulatory site of SOS. Alternatively, the interaction of GDP-Ras with phosphorylated ezrin might be necessary for full opening of the C-terminal and middle domain of ezrin (Fig. S8*A*) (19), thereby enabling the productive interaction of ezrin with SOS and contributing to the release of SOS autoinhibition (Fig. 6). Obtaining high-quality structures and interactions of SOS–ezrin and Ras–ezrin would be the ultimate goal in achieving a complete mechanistic understanding of how ezrin contributes to the control of SOS activity.

The assembly of the ezrin–SOS–Ras complex using recombinant and purified components indeed proves the regulatory interactions. Although the stimulation is modest in vitro, it still is

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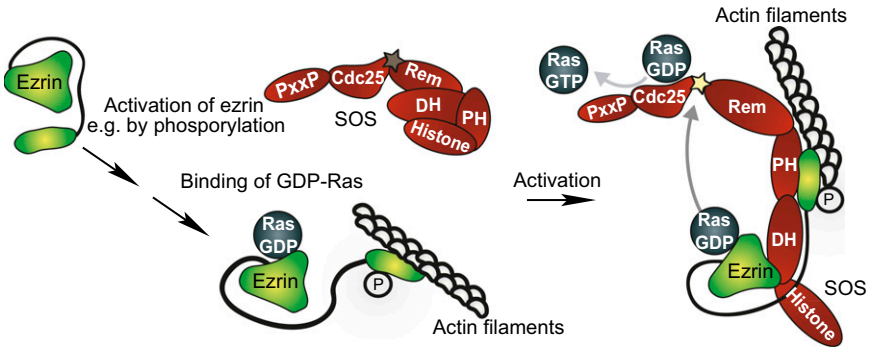

Fig. 6. Model of ezrin-mediated activation of SOS. In cells SOS is kept in an autoinhibited state in which the Rem and Cdc25 domain, required for nucleotide exchange on Ras, as well as the allosteric Ras-binding site (gray star) are not accessible. The very C-terminal proline-rich region (PxxP) is responsible for targeting SOS to sites of activation, e.g., via the adapter protein Grb2. The DH/PH module keeps SOS in the autoinhibited state. By phosphorylation of a conserved threonine residue within the C terminus of ezrin, the C-terminal region dissociates from the N-terminal FERM domain of ezrin (Fig. S7*A*) and binds to F-actin. The FERM domain of phosphorylated ezrin interacts with GDP-Ras. Activated ezrin can bind to SOS and possibly contribute to the release of SOS autoinhibition. Ezrin bound to GDP-Ras docks to the PH domain of SOS, facilitating the encounter of GDP-Ras to the now accessible allosteric Ras-binding site of SOS (yellow star).

significant. In our assay ezrin by itself is not an SOS allosteric stimulator but rather assists its direct partner Ras to localize efficiently to this allosteric Ras-binding site in SOS. Nevertheless, ERM-dependent Ras activation in vivo is obviously more efficient than the stimulation by active ezrin in our purified system (9). The assembly at the inner leaflet of the lipid membrane is one obvious component. A modest increase in SOS activity was observed in vitro by the addition of lipids. A combination of PA and ezrinT567D produced an additive stimulatory effect on SOS activation, suggesting that indeed the in vivo targeting of active ezrin to the plasma membrane and its direct interaction with SOS are both essential features for the full activation of SOS.

Scaffold proteins play a critical role in regulating diverse signaling events by organizing biochemical reactions at the right time and place. Because Ras activity must be tightly controlled in all cells, misregulation of such scaffolds (e.g., ERM proteins) also could contribute to human pathology. Here two disease-relevant results are worth stressing: The neurofibromatosis type 2 tumor-suppressor protein merlin interferes with SOS activation both in vivo and in the complex with recombinant proteins in vitro. Ezrin fused to a membrane anchor and thus constitutively bound to the inner phase of the plasma membrane exerts tumorigenic properties. ERM proteins often are overexpressed in cancer. Our findings suggest that elevated expression of ezrin (20, 21), radixin (22), and moesin (23) may contribute to cancer progression and metastasis by increasing Ras activity. We propose an additional level of SOS/Ras control involving a family of actin-binding proteins as a molecular step that is relevant in Ras-dependent physiological processes.

Materials and Methods

Detailed information about cell lines, plasmids, antibodies, siRNA sequences, purification of recombinant proteins, preparation of ezrin- and merlin-expressing cell lines, and pull-down methods can be found in *SI Materials and Methods*.

Proliferation Assay. Proliferation assays were performed using the PrestoBlue Cell viability reagent (Invitrogen) following the manufacturer's instructions. Details are given in *SI Materials and Methods*.

Soft Agar Assay. Stably transduced NIH 3T3 cells or doxycycline-inducible RT4tetNf2 cells were used based on the principles described (21). Details are given in *SI Materials and Methods*.

Fluorescent GEF Activity Measurements in Vitro. Nucleotide loading and nucleotide exchange measurements for Ras were performed as described previously (12).

Protein Interaction Studies. Protein interaction was analyzed in vitro based on the 2′-/3~-O-(*N*′-Methylanthraniloyl) (mant) fluorophore. Upon binding to a protein, the fluorescence quantum yield of the mant fluorophore increases, thus leading to an increase in fluorescence (24). Details are given in *SI Materials and Methods*.

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1. Boriack-Sjodin PA, Margarit SM, Bar-Sagi D, Kuriyan J (1998) The structural basis of the activation of Ras by Sos. *Nature* 394(6691):337–343.
2. Sondermann H, et al. (2004) Structural analysis of autoinhibition in the Ras activator Son of sevenless. *Cell* 119(3):393–405.
3. Aronheim A, et al. (1994) Membrane targeting of the nucleotide exchange factor Sos is sufficient for activating the Ras signaling pathway. *Cell* 78(6):949–961.
4. Sondermann H, Nagar B, Bar-Sagi D, Kuriyan J (2005) Computational docking and solution x-ray scattering predict a membrane-interacting role for the histone domain of the Ras activator son of sevenless. *Proc Natl Acad Sci USA* 102(46): 16632–16637.
5. Gureasko J, et al. (2008) Membrane-dependent signal integration by the Ras activator Son of sevenless. *Nat Struct Mol Biol* 15(5):452–461.
6. Gureasko J, et al. (2010) Role of the histone domain in the autoinhibition and activation of the Ras activator Son of Sevenless. *Proc Natl Acad Sci USA* 107(8):3430–3435.
7. Yadav KK, Bar-Sagi D (2010) Allosteric gating of Son of sevenless activity by the histone domain. *Proc Natl Acad Sci USA* 107(8):3436–3440.
8. Zhao C, Du G, Skowronek K, Frohman MA, Bar-Sagi D (2007) Phospholipase D2-generated phosphatidic acid couples EGFR stimulation to Ras activation by Sos. *Nat Cell Biol* 9(6):706–712.
9. Sperka T, et al. (2011) Activation of Ras requires the ERM-dependent link of actin to the plasma membrane. *PLoS ONE* 6(11):e27511.
10. Tsukita S, Yonemura S, Tsukita S (1997) ERM proteins: Head-to-tail regulation of actin-plasma membrane interaction. *Trends Biochem Sci* 22(2):53–58.
11. Hall A (1998) Rho GTPases and the actin cytoskeleton. *Science* 279(5350):509–514.
12. Margarit SM, et al. (2003) Structural evidence for feedback activation by Ras.GTP of the Ras-specific nucleotide exchange factor SOS. *Cell* 112(5):685–695.
13. Herrmann C, Martin GA, Wittinghofer A (1995) Quantitative analysis of the complex between p21ras and the Ras-binding domain of the human Raf-1 protein kinase. *J Biol Chem* 270(7):2901–2905.
14. Roberts AE, et al. (2007) Germline gain-of-function mutations in SOS1 cause Noonan syndrome. *Nat Genet* 39(1):70–74.
15. Tartaglia M, et al. (2007) Gain-of-function SOS1 mutations cause a distinctive form of Noonan syndrome. *Nat Genet* 39(1):75–79.
16. Boykevisch S, et al. (2006) Regulation of ras signaling dynamics by Sos-mediated positive feedback. *Curr Biol* 16(21):2173–2179.
17. Morrison H, et al. (2001) The NF2 tumor suppressor gene product, merlin, mediates contact inhibition of growth through interactions with CD44. *Genes Dev* 15(8):968–980.
18. Morrison H, et al. (2007) Merlin/neurofibromatosis type 2 suppresses growth by inhibiting the activation of Ras and Rac. *Cancer Res* 67(2):520–527.
19. Fehon RG, McClatchey AI, Bretscher A (2010) Organizing the cell cortex: The role of ERM proteins. *Nat Rev Mol Cell Biol* 11(4):276–287.
20. Nestl A, et al. (2001) Gene expression patterns associated with the metastatic phenotype in rodent and human tumors. *Cancer Res* 61(4):1569–1577.
21. Hunter KW (2004) Ezrin, a key component in tumor metastasis. *Trends Mol Med* 10(5): 201–204.
22. Valastyan S, Benaich N, Chang A, Reinhardt F, Weinberg RA (2009) Concomitant suppression of three target genes can explain the impact of a microRNA on metastasis. *Genes Dev* 23(22):2592–2597.
23. Abiatari I, et al. (2010) Moesin-dependent cytoskeleton remodelling is associated with an anaplastic phenotype of pancreatic cancer. *J Cell Mol Med* 14(5):1166–1179.
24. Rojas RJ, Kimple RJ, Rossman KL, Siderovski DP, Sondek J (2003) Established and emerging fluorescence-based assays for G-protein function: Ras-superfamily GTPases. *Comb Chem High Throughput Screen* 6(4):409–418.