the type I receptor is required for this process. However, the existence of TGF-β–unresponsive Mv1Lu cell mutants that lack type I receptors yet have normal levels of type II receptors (*5*) suggests that type I receptors may be needed to mediate these activities of TGF-β through the type II receptor.

Our findings also indicate that the signaling through the type I receptor does not require a functional type II receptor to mediate autonomously its distinct set of TGF-β–induced activities. This is consistent with the fact that 293 cells, which lack detectable levels of type II receptors but have type I receptors (Fig. 1A), are not responsive to the antiproliferative activity of TGF-β yet display a TGF-β–induced synthesis of fibronectin (*20*). These data further support our conclusion that the type II receptor is required for and mediates the antiproliferative effect of TGF-β, whereas the induction of fibronectin synthesis is mediated by the type I receptor. Moreover, the wide variability and lack of correlation between the cell surface levels of the type I and type II receptors suggest that besides possible heterodimers, there may be type I and type II receptors that do not physically interact with each other and that perhaps could function as homodimers.

Finally, the selective functional abolition of the type II receptors results in alterations in the complex response to TGF-β similar to the functional inactivation of pRB by viral transforming proteins such as the SV40 large T antigen, that is, a specific abrogation of the antiproliferative effect of TGF-β (*11, 21*) without affecting the induction of expression of several genes by TGF-β (*11*). This very similar phenotype thus indicates that the role of pRB in the response to TGF-β is specific for and restricted to the signaling pathway associated with the type II receptor. Accordingly, our transfected cells lacking functional type II receptors did not show TGF-β–induced inhibition of pRB phosphorylation, suggesting that type II receptors are required for the effect of TGF-β on the phosphorylation state of pRB.

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25. To generate the truncated type II receptor, we made an Eco RI–Hpa I fragment corresponding to nucleotides −7 through 573 of the type II receptor by polymerase chain reaction (PCR), followed by cleavage at the unique Hpa I site located immediately 3′ of the transmembrane domain sequence. A double-stranded oligonucleotide adaptor for the sequence encoding the epitope tag, FLAG (*22*), was ligated to the 3′ end of the truncated type II cDNA, and the resulting fragment was inserted into the Eco RI and Xba I sites of the expression vector pRK5, thus generating the expression plasmid for the truncated type II receptor. DNA was transfected into the QT6 cells and 293 cells by calcium phosphate precipitation (*23*). Cross-linking analysis was performed as described (*3, 24*), and the cross-linked proteins were separated by denaturing and reducing polyacrylamide (7 5%) gel electrophoresis and subsequent autoradiography.
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Human Sos1: A Guanine Nucleotide Exchange Factor for Ras That Binds to GRB2

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A human complementary DNA was isolated that encodes a widely expressed protein, hSos1, that is closely related to Sos, the product of the *Drosophila* son of sevenless gene. The hSos1 protein contains a region of significant sequence similarity to CDC25, a guanine nucleotide exchange factor for Ras from yeast. A fragment of hSos1 encoding the CDC25-related domain complemented loss of CDC25 function in yeast. This hSos1 domain specifically stimulated guanine nucleotide exchange on mammalian Ras proteins in vitro. Mammalian cells overexpressing full-length hSos1 had increased guanine nucleotide exchange activity. Thus hSos1 is a guanine nucleotide exchange factor for Ras. The hSos1 interacted with growth factor receptor–bound protein 2 (GRB2) in vivo and in vitro. This interaction was mediated by the carboxyl-terminal domain of hSos1 and the Src homology 3 (SH3) domains of GRB2. These results suggest that the coupling of receptor tyrosine kinases to Ras signaling is mediated by a molecular complex consisting of GRB2 and hSos1.

Ras genes encode membrane-bound guanine nucleotide binding proteins that function in the transduction of signals that control cell growth and differentiation. Binding of guanosine triphosphate (GTP) activates Ras proteins, and subsequent hydrolysis of the bound GTP to guanosine diphosphate (GDP) inactivates signaling by these proteins. GTP binding can be catalyzed by guanine nucleotide exchange factors for Ras and GTP hydrolysis can be accelerated by GTPase activating proteins (GAPs) (*1*). The first exchange factor for Ras to be identified was the CDC25 gene

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product of *Saccharomyces cerevisiae* (*2–4*). Genetic analysis indicates that CDC25 is essential for activation of Ras proteins (*2, 5, 6*). In *Drosophila*, the protein encoded by the son of sevenless gene, Sos, contains a domain that shows sequence similarity with the catalytic domain of CDC25 (*7*). Sos may act as a positive regulator of Ras by promoting guanine nucleotide exchange. Recently, murine homologues of CDC25, namely Ras-guanine nucleotide releasing factor (Ras-GRF), mSos1, and mSos2, have been identified (*8–11*). Among these, only Ras-GRF has been shown to possess specific guanine nucleotide exchange activity towards Ras proteins (*8*).

Ras proteins are required to propagate signals from receptor tyrosine kinases (*12–16*) and the stimulation of various receptor tyrosine kinases promotes the accumulation of the active GTP-bound form of Ras (*17–20*). Thus Ras appears to function downstream of receptor tyrosine kinases in mammalian cells. Studies of mutations that affect development in nematodes and flies have identified two proteins that may have an important role in relaying signals from receptor tyrosine kinases to Ras. In *Drosophila*, the sevenless receptor tyrosine kinase and Ras are essential for normal development of the *Drosophila* compound eye. Genetic evidence indicates that the putative guanine nucleotide exchange factor Sos is a key element in the activation of Ras by sevenless. Studies of vulval development in *Caenorhabditis elegans* indicate that *sem*-5, a gene that encodes a protein with Src homology 2 (SH2) and Src homology 3 (SH3) domains, functions in the same signaling pathway as the Let-23 receptor tyrosine kinase and Let-60, a Ras homolog (*21*). Because SH2 domains interact with specific phosphotyrosine containing sites on activated receptors (*22*), Sem-5 has been postulated to participate in signaling downstream from receptor tyrosine kinases. A mammalian homologue of Sem-5, GRB2, has been identified (*23*). GRB2 can associate with activated growth factor receptors and microinjection studies demonstrated that GRB2 participates in mitogenic signaling by Ras (*23*). These observations suggest

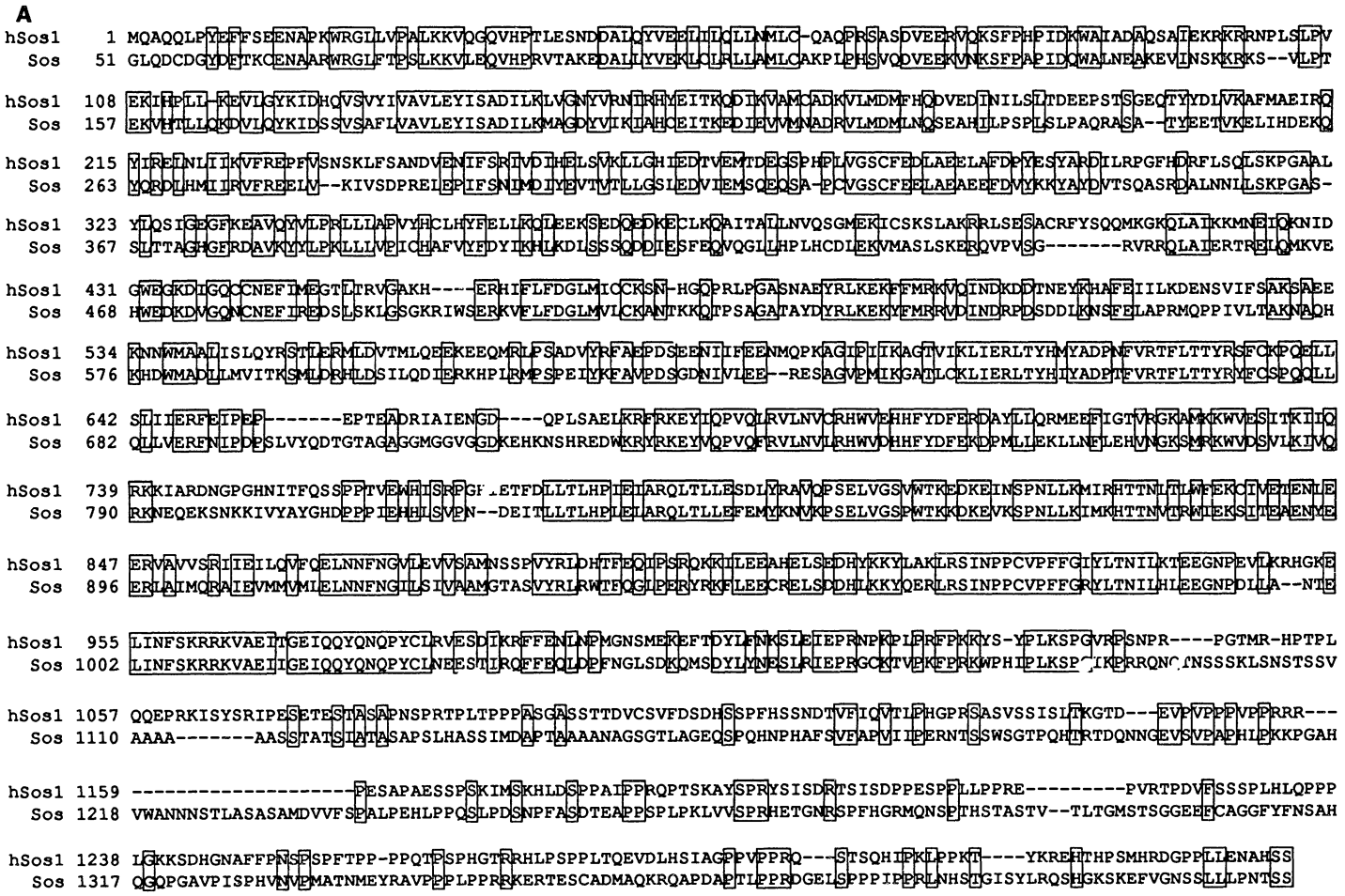

Fig. 1. Comparison of amino acid sequences of hSos1 and related proteins. (**A**) Alignment of the predicted amino acid sequences of hSos1 (residues 1 to 1333) and Sos from *Drosophila* (Sos, residues 51 to 1420). Boxes show residues that are identical in the two sequences. (**B**) Schematic presentation of the similarity between different Ras exchange factors. The black rectangles delineate the highly conserved catalytic domain. The hatched rectangles represent the NH_2-terminal region of similarity between members of the Sos family. COOH-terminal regions containing several proline rich motifs are indicated by the dotted rectangles. Alignments were made with the Pileup program (GCG). Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

a role for GRB2 in linking receptor tyrosine kinases to Ras signaling.

A set of degenerate oligonucleotides corresponding to the coding sequence of a highly conserved region among guanine nucleotide exchange factors for yeast Ras (CDC25, SDC25, and STE6) and Sos was used to screen a rat brain cDNA library (*24*). Sequence analysis of cDNAs of strongly hybridizing clones identified two distinct cDNAs, termed rSos1 and rSos2, highly similar (62% identity) to residues 816 to 1090 of Sos. We used these probes to screen several human cDNA libraries (*24*), and multiple overlapping cDNA clones were isolated. Sequence analysis of these clones identified two cDNAs, hSos1 and hSos2. The amino acid sequence of hSos1 as deduced from the nucleic acid sequence (GenBank accession number L13857) is shown in Fig. 1A. The predicted molecular size of this protein is approximately 150 kD. The two proteins share 69% overall amino acid identity. The lowest degree of similarity is found in the COOH-terminal region of the proteins (residues 1080 to 1309). The hSos1 protein shares 98% identity with mSos1 (*10*).

The NH_2-terminal domain of hSos1 (residues 1 to 1040) is similar (46% identity) to the NH_2-terminal domain of Sos, whereas no similarity is observed between the NH_2-terminal domains of CDC25 and SDC25 (Fig. 1B). The most highly conserved region of hSos1 and Sos is a central domain of approximately 420 residues that is similar (25 to 30% identity) to the corresponding portions of CDC25, SDC25, and Ras-GRF. This region constitutes the catalytic domain of CDC25, SDC25, and Ras-GRF. At the COOH-terminal ends, the similarity between Sos and hSos1 is scattered and the conserved regions are mostly proline-rich motifs. Northern (RNA) analysis revealed that hSos1 is expressed in a wide range of tissue types (*25*), consistent with a role as positive regulators of the ubiquitously expressed *ras* genes.

We tested whether the central domain of hSos1 could complement the CDC25 gene in *S. cerevisiae*. Yeast cells expressing the mutant allele of CDC25, cdc25-5, exhibit a thermosensitive phenotype. At the restrictive temperature, cdc25-5 cells are arrested at the G_0/G_1 stage of the cell cycle (*26*). A region within the central domain of hSos1 (hSos1-cd; residues 707 to 1059) was cloned into the yeast expression vector pBFG1, creating pBFG1-hSos1-cd (*27*). A cdc25-5 strain was transformed with pBFG1, pBFG1-hSos1-cd, or pCDC25 (a Yep13 plasmid carrying the *S. cerevisiae* CDC25 gene) and the resulting transformants were tested for thermosensitivity (*27*). The thermosensitive phenotype of the cdc25-5 strain was suppressed by the expression of hSos1-cd or by CDC25 (Fig. 2A). The cdc25-5 strain carrying only the vector pBFG1 remained thermosensitive. Expression of hSos1-cd also suppressed the lethality of a $cdc25^-$ strain (*25*), but expression of hSos1-cd did not suppress a deficiency in Ras function in the thermosensitive strain STS1 (ras1::URA3, $ras2^{ts}$) at restrictive temperature. These results indicate that the proposed catalytic domain of hSos1 can functionally replace a guanine nucleotide exchange factor for Ras in yeast.

The fragment of hSos1 encoding the putative catalytic domain was cloned into pTrcHisA and expressed as a fusion protein with an NH_2-terminal oligohistidine (*28*). The efficient induction of the His-hSos1-cd fusion protein by isopropyl-β-D-thiogalactopyranoside (IPTG) was indicated by the appearance of a new band when proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) (Fig. 2B). This band had an apparent molecular size of 36 kD, the expected size of the fusion protein. The His-hSos1-cd protein was purified by metal affinity chromatography on Ni^{2+} resin (Fig. 2B); the identity of this protein was confirmed by protein immunoblot analysis of total extract and of the purified protein (Fig. 2C) with antiserum to hSos1 (anti-hSos1) (*29*). The ability of purified His-hSos1-cd to stimulate guanine nucleotide exchange on Ras was measured with recombinant human Ras proteins. The rate of dissociation of [^{3}H]GDP bound to the human H-Ras was approximately five times greater in the presence of His-hSos1-cd. The rate of [α-^{32}P]GTP binding was also approximately five times greater in the presence of His-hSos1-cd (Fig. 2E). Similar effects were observed with the human N-Ras protein (*25*). His-hSos1-cd appeared to have a specificity toward Ras because it did not affect the rate of guanine nucleotide exchange on RalA, a close relative of Ras (55% identical)

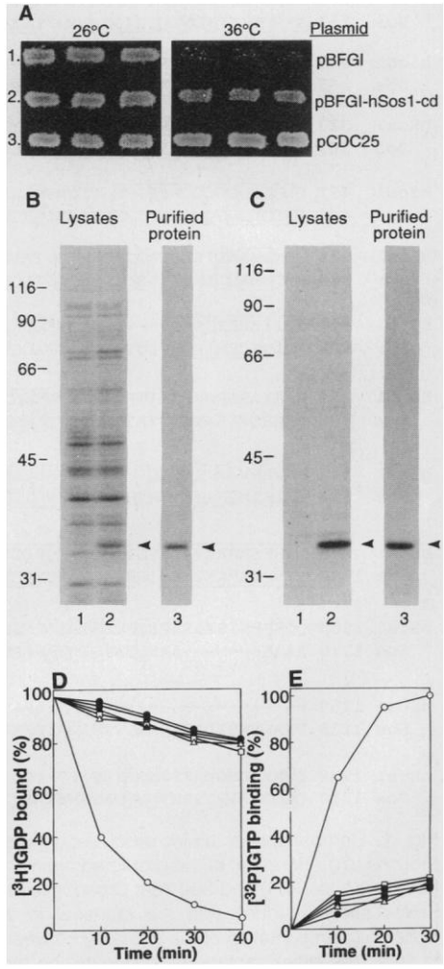

Fig. 2. Evidence that hSos1-cd functions as a nucleotide exchange factor for Ras. (**A**) Complementation of a *S. cerevisiae* $cdc25^{ts}$ mutation by a fragment of hSos1. The yeast strain OL97-1/11B which contains a mutant allele of CDC25 [cdc25-5, (*5*)] was transformed with pBFG1 as a control plasmid (row 1), with pCDC25 (row 3) or with pBFG1-hSos1-cd (row 2) (*27*). Transformants were patched, grown at 26°C for one day and then replica plated. The replicas were incubated at permissive temperature (26°C) or restrictive temperature (36°C) for 2 days. Each set shows three independent transformants. (**B**) Purification of hSos1-cd. SDS-PAGE (12.5% gel) of cell lysates prepared from *Escherichia coli* carrying plasmid pTrcHisA-hSos1-cd that were grown without (lane 1) or with (lane 2) IPTG. The hSos1-cd protein was purified (*43*) and loaded (1 μg) onto the indicated lane. Proteins were visualized by Coomassie blue staining. Molecular sizes are indicated in kD. Arrow, His-hSos1-cd. (**C**) Immunoblots of the *E. coli* cell lysates shown in (B) and of the purified hSos1-cd protein (100 ng) probed with anti-hSos1 (*44*). The immunoreactive bands were visualized with the enhanced chemiluminescence (ECL) detection system. (**D**) Effects of purified hSos1-cd on the kinetics of GDP dissociation from Ras; hSos1-cd (1 μg) (open symbols) or buffer (filled symbols) were added along with excess unlabeled GTP (500 μM) to reaction mixtures containing 10 pmol of H-Ras (○), RalA (△) or CDC42Hs (□) bound to [^{3}H]GDP. At the indicated times, portions (40 μl) of the reaction mixtures were removed and the amount of [^{3}H]GDP remaining bound to the proteins was measured by the nitrocellulose filter binding assay (*45*). (**E**) Effects of purified hSos1-cd on the kinetics of GTP binding; hSos1-cd (1 μg) (open symbols) or buffer (filled symbols) were added along with [α-^{32}P]GTP (15 pmol) to reaction mixtures containing 50 pmol of H-Ras (○), RalA (△), or CDC42Hs (□). At the indicated times, aliquots of the reaction mixtures were removed and the amount of [α-^{32}P]GTP bound to each protein was determined (*46*). Results in (D) and (E) are plotted as percentages relative to maximal binding obtained in the presence of EDTA (5 mM). All reactions were incubated at 30°C. Each data point represents the average of duplicate determination and the results shown are representative of two independent experiments.

(30), or on the more distantly related protein CDC42Hs (30% identical) (31) (Fig. 2, D and E).

To determine whether the full-length hSos1 had biochemical properties similar to hSos1-cd, we inserted a cDNA fragment that contained the entire hSos1 coding region into an expression vector under the control of the CMV promoter (RK5-hSos1). This plasmid was transfected into human kidney 293 cells. Protein immunoblot analysis of extracts from transfected cells with anti-hSos1 revealed that the hSos1 cDNA encodes a protein with an apparent molecular size of approximately 170 kD (Fig. 3A). The predicted molecular size of hSos1 is 152 kD. The slow mobility of hSos1 may result from the high proline content of the protein. Two days after transfection extracts were prepared from cells transfected with control vector (RK5) or with RK5-hSos1 and guanine nucleotide, exchange activity was measured. In the presence of extracts from cells overexpressing the hSos1 protein, the release of [^{3}H]GDP from Ras was about 2.5 times greater than that in the presence of control extracts (Fig. 3B). When unlabeled H-Ras was incubated with [^{3}H]GDP in the presence of cell extracts, the amount of [^{3}H]GDP bound to H-Ras in the presence of extracts from cells overexpressing hSos1 was about six times greater than that in the presence of extract from control cells (Fig. 3C). This effect was abolished by the addition of excess unlabeled guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γ-S), indicating that the extracts from cells overexpressing hSos1 stimulate the exchange of GDP for GTP on Ras. Taken together, our results indicate that hSos1 can function as a guanine nucleotide exchange factor for mammalian Ras proteins.

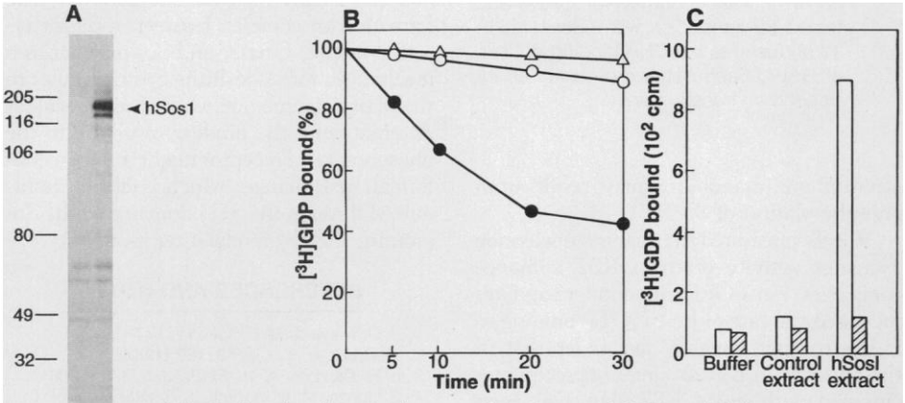

Fig. 3. Acceleration of release and exchange of GDP and GTP-γ-S by extracts prepared from cells overexpressing full-length hSos1. (**A**) Immunoblots of cell lysates prepared from human kidney 293 cells transfected with a control vector (lane 1) or with an expression vector encoding the full-length hSos1 (lane 2). Proteins resolved by SDS-PAGE were electrophoretically transferred to a nitrocellulose strip, which was then probed with anti-hSos1 (*44*). The immunoreactive bands were visualized with the ECL detection system. (**B**) Effects of cell extracts on the kinetics of GDP dissociation. H-Ras bound to [^{3}H]GDP (5 pmol) was incubated at 37°C with a portion (500 μl) of extracts (6 mg of protein per milliliter) from control cells (○) cells overexpressing the hSos1 protein (●), or with buffer (△), in the presence of 2 mM unlabeled GTP. At the indicated times, H-Ras was immunoprecipitated and the amount of [^{3}H]GDP associated with the immune complexes was determined (*47*). Results are plotted as percentages of the values obtained at 0 min. Each data point represents the average of three independent determinations. (**C**) Effects of cell extracts on exchange of GDP for GTP. Unlabeled H-Ras-GDP complex (10 pmol) was incubated for 15 min at 37°C with [^{3}H]GDP (100 pmol) in the presence of buffer alone, extract from control cells (control extract), or extract from cells overexpressing hSos1 (hSos1 extract) in the presence (hatched bars) or absence (open bars) of GTP-γ-S. The amount of [^{3}H]GDP bound to H-Ras was measured after immunoprecipitation of Ras (*47*). Results represent the averages of two independent determinations.

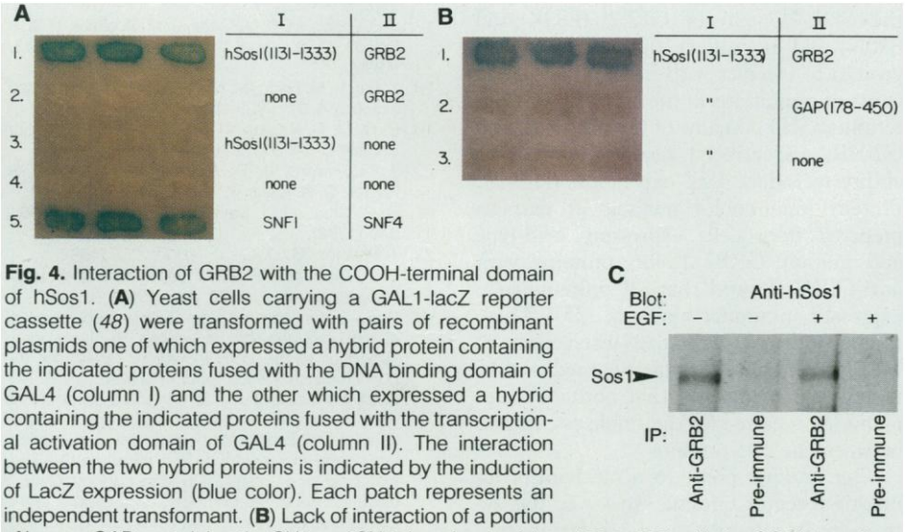

Fig. 4. Interaction of GRB2 with the COOH-terminal domain of hSos1. (**A**) Yeast cells carrying a GAL1-lacZ reporter cassette (*48*) were transformed with pairs of recombinant plasmids one of which expressed a hybrid protein containing the indicated proteins fused with the DNA binding domain of GAL4 (column I) and the other which expressed a hybrid containing the indicated proteins fused with the transcriptional activation domain of GAL4 (column II). The interaction between the two hybrid proteins is indicated by the induction of LacZ expression (blue color). Each patch represents an independent transformant. (**B**) Lack of interaction of a portion of human GAP containing the SH2 and SH3 domains (residues 178 to 450) with the COOH-terminus of hSos1 (row 2). (**C**) Interaction of GRB2 with hSos1 in vivo. Quiescent HER14 cells were stimulated with epidermal growth factor (EGF) (250 ng/ml) for 2 min. Lysates prepared from unstimulated or EGF-stimulated cells were subjected to immunoprecipitation with anti-GRB2 or with preimmune serum. The immunoprecipitates were resolved by a SDS-PAGE (10% gel) and immunoblotted with anti-Sos1 (*44*).

We used a genetic method for detecting protein-protein interactions called the two-hybrid system (*36, 37*) to test whether hSos1 interacted with GRB2. In this system, the interaction of two hybrid proteins, one containing the DNA binding domain of GAL4, a transcriptional activator from yeast, and the other containing a transcriptional activation domain of GAL4, can be detected by the activation of a GAL1-lacZ reporter gene. A plasmid expressing GRB2 fused to the GAL4 activation domain and a plasmid expressing a fusion between the GAL4 DNA binding domain and the COOH-terminal portion of the hSos1 protein [hSos1(1131–1333)] were introduced into a yeast reporter strain carrying GAL1-lacZ. The transformants were assayed for lacZ expression, which

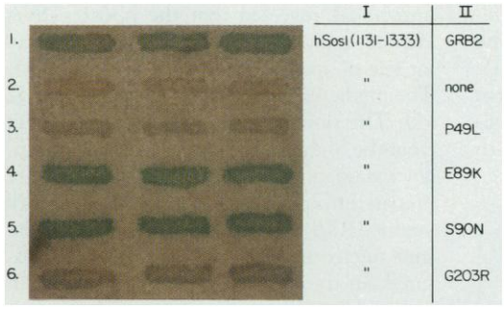

Fig. 5. Interaction of GRB2 with the COOH-terminal domain of hSos1 through its SH3 domains. Wild-type GRB2 (row 1), the SH3 domain mutants (*21*) P49L (row 3) and G203R (row 6), and the SH2 domain mutants (*21*) E89K (row 4) and S90N (row 5) were expressed as fusions with the transcriptional activation domain of GAL4 and tested for interaction with hSos1(1131–1333) fused to the DNA binding domain of GAL4. Each patch represents an independent transformant.

confers blue color to the reporter strain. The interaction between a hybrid of SNF1 and a hybrid of SNF4 (two proteins that interact with each other in the two-hybrid system) (*37*) provided the positive control (Fig. 4A). The introduction of hSos1(1131–1333) and GRB2 hybrids into the reporter strain induced lacZ expression (Fig. 4A). The expression of lacZ required the presence of hSos1 and the presence of GRB2 in their respective hybrid proteins. Expression of lacZ was not induced when hSos1 was replaced with SNF1 or when GRB2 was replaced with SNF4 in their respective hybrid proteins (*25*). Thus, the expression of lacZ probably results from the interaction between GRB2 and COOH-terminal portion of the hSos1. Expression of lacZ was not induced by hybrid proteins containing the NH_2-terminal domain (residues 1 to 579) or the catalytic domain of hSos1 (residues 603 to 1059). These results indicate that the COOH-terminal region of the hSos1 protein spanning residues 1131 to 1333 is necessary and sufficient for the interaction with GRB2.

To rule out the possibility that the interaction between GRB2 and hSos1 reflects the intrinsic binding capacity of SH2 and SH3 domains rather than a specific association between hSos1 and GRB2, we tested the ability of the COOH-terminal domain of hSos1 to interact with a region spanning the SH2 and SH3 domains of human GAP (residues 178 to 450). No interaction was detected (Fig. 4B).

To test whether GRB2 and Sos1 interacted in vivo, NIH 3T3 cells overexpressing the human EGF receptor (*38*) were immunoprecipitated with antibodies to GRB2 (anti-GRB2) (*39*) and the immunoprecipitated proteins were immunoblotted with anti-hSos1. Sos1 protein was clearly present in the GRB2 immunoprecipitates obtained from quiescent and epidermal growth factor (EGF)–stimulated cells (Fig. 4C). These results suggest that GRB2 can constitutively interact with Sos1. EGF stimulation did not appear to increase the amount of Sos1 associated with GRB2. However, a shift in the position of the Sos1 band toward a slower mobility was detected in GRB2 immunoprecipitates obtained from EGF-stimulated cells. This shift in electrophoretic mobility may result from phosphorylation of Sos1.

We also measured the guanine nucleotide exchange activity of the GRB2 immunocomplexes. Anti-GRB2 immunoprecipitates increased the rate of [α-^{32}P]GTP binding to H-Ras to approximately five times that in the presence of control immunoprecipitates obtained with anti-GRB2 that had been blocked by incubation with an excess of purified GST-GRB2 fusion protein (*25*).

GRB2 contains a single SH2 domain flanked by two SH3 domains. We examined the ability of GRB2 proteins containing point mutations in the SH2 and SH3 domains to interact with hSos1 in the two-hybrid system. These mutations corresponded to changes in *sem*-5 loss-of-function alleles (*21*). GRB2 proteins containing these mutations cannot rescue *sem*-5 mutants whereas wild-type GRB2 can (*40*). Yeast cells were transformed with plasmids expressing wild-type or mutant GRB2 fused to the GAL4 activating domain and a plasmid expressing a fusion between the COOH-terminal fragment of hSos1 and the GAL4 DNA binding domain. Mutations in the SH2 domain of GRB2 (E89K and S90N) did not affect the ability of the protein to interact with hSos1 (Fig. 5). In contrast, mutations in the NH_2- or COOH-terminal SH3 domains of GRB2 (P49L and G203R, respectively) nearly abolished its ability to induce lacZ expression (Fig. 5). Protein immunoblot analysis of extracts prepared from cells expressing wild-type and mutant GRB2 fusion proteins with anti-GRB2 showed that all proteins were expressed in similar amounts (*25*). These results indicate that the interaction of GRB2 with hSos1 is primarily mediated by the SH3 domains and that both SH3 domains are required for the stable association between the two proteins.

Our findings point to a mechanism for linking receptor tyrosine kinases to Ras activation: In response to growth factor stimulation, the SH2 domain of GRB2 may bind to the autophosphorylated receptor tyrosine kinase and the SH3 domain may mediate the interaction with an Sos protein which in turn may stimulate Ras by promoting the conversion of Ras from the GDP-bound form to the GTP-bound form. Consistent with such a mechanism are recent studies demonstrating that growth factors can activate Ras by increasing guanine nucleotide exchange rates (*19, 41, 42*). It is not yet clear how the interaction between GRB2 and Sos might regulate Sos activity. The formation of complex between receptor tyrosine kinase, GRB2, and Sos may act as a mechanism for relocalizing Sos molecules to the plasma membrane where Ras is located. Alternatively, the binding of GRB2 to the phosphorylated receptor might induce a conformational change, which could be transmitted through the SH3 domains to the Sos protein, thereby modulating its activity.

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24. Screening of the rat brain library: Two mixes of oligonucleotides corresponding to the coding strand or to the complementary strand for the conserved sequence PCVPFFG were used. Nucleotides incorporated at degenerated positions were chosen on the basis of two criteria: (i) codon usage in mice and rat (the most frequent nucleotides were incorporated) and (ii) some G-T mismatches were accepted for rare codons. Oligo 1 corresponds to the coding strand, oligo 2 to the complementary strand. Oligo 1: 5′(G/T)CC(C/T/A)-TG(C/T)GT(G/C/T) CC(C/T/A)TT(C/T)TT(C/T)G-G3′; oligo 2: 5′(G/T)CC(A/G)AA(AG)AA(A/T/G)G-G(C/G/A)AC(A/G)CA(A/T/G)GG 3′. Oligonucleotides were synthesized on an Applied Biosystems DNA Synthesizer. The oligo(dT)-primed and random-primed rat brain cDNA library in λGT10, the two human fetal brain cDNA libraries, and the hu-

man skeletal muscle cDNA library in λGT10 were purchased from Clontech Laboratories, Inc. All these libraries were plated on C600 Hfl. Screening of the rat brain library: Oligonucleotide mixes 1 and 2 were labeled on the 5′ end with T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and separated from unincorporated [γ-^{32}P] on a Sephadex G-25 column. About 300,000 phage were plated on C 600 Hfl, transferred to nitrocellulose, and screened with oligo 1. Hybridization conditions were: 5× SSPE [0.9 M NaCl, 50 mM NaH_2PO_4 (pH 7.4), 5 mM EDTA], 5× Denhart's solution, 0.1% SDS, at 53°C for 20 min. After autoradiography, nitrocellulose filters were washed in the hybridization solution at 68°C for 3 hours and re-hybridized with oligo 2, under the same conditions. Plaques (*24*) that save a positive signal with both probes were replated twice, until cloned. Only three clones gave a strong signal with both oligos and were studied further. The screening of the human cDNA libraries with rat and human Sos probes was done at 64°C for 5 hours; washings were done twice in 1× SSC, 0.1% SDS, at 64°C for 5 hours, and then twice in 1× SSC, 0.1% SDS, at 68°C for 20 min. The λ DNA was prepared from confluent lysis agarose plates by the miniprep technique and electro-eluted Eco RI inserts were subcloned in the Eco RI site of the pUC19 plasmid. Restriction sites were mapped and suitable restriction fragments were subcloned in M13, mp18, or mp19. Single-stranded DNA templates were prepared and sequenced by the di-deoxynucleotides chain termination methods, with the Klenow fragment of DNA polymerase (Amersham) as described [J. Sambrook, T. Fritsch, T. Maniatus, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), or according to manufacturers instruction, with minor modifications. We screened about 0.5 × 10^6 plaques of a human fetal brain cDNA library with the rat Sos1 probe and isolated 7 clones that encoded most of human Sos1 but lacked the 5′ end encoding the NH_2-terminus. We used the most 5′ Eco RI fragment from these isolates to screen about 2 × 10^6 plaques of a human pheochromocytoma cDNA library [Grima *et al.*, *Nature* **326**, 707 (1987). Twelve clones were isolated, one including the starting ATG.

25. P. Chardin *et al.*, unpublished data.
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27. The yeast expression vector pBFG1 is an *E. coli*–yeast shuttle vector, with a yeast 2μ origin of replication, a LEU2 gene, the strong yeast phosphoglycerate kinase (PGK) promoter, the PGK terminator, and a multicloning site allowing expression of genes fused 3′ to three repeats of the hemagglutinin epitope (HA epitope) [M. Tyers, G. Tokiwa, R. Nash, B. Futcher, *EMBO J.* **11**, 1773 (1992)]. The region of hSos1 encoding amino acids 707 to 1059 was amplified by the polymerase chain reaction (PCR) with Pfu DNA polymerase and adequate primers. The amplified fragment was cloned into plasmid pBFG1. The yeast strains used in this part of the study (with their relevant genotype in between brackets) are OL97-1/11B (cdc25-5) (*5*) STS1 (ras1::URA3, ras2ts) (M. Wigler, unpublished data), and TT1A1 [cdc25::URA3 (pTRP1-CDC25)] (*2*). Work with *S. cerevisiae* was performed according to standard procedures [M. D. Rose, F. Winston, P. Hieter, *Methods in Yeast Genetics. A Laboratory Course Manual.* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989)].
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29. PCR was used to insert an initiating ATG codon 5′ to the exchange factor domain of hSos1, residues 601 to 1019, and a stop codon at its 3′ end with convenient restriction sites. This fragment was then inserted in a T7 promoter–based vector derived from pET3c (P. Chardin, unpublished data). Expression of a 50-kD protein corresponding to this exchange factor domain was observed in *E. coli* harboring this plasmid after induction with IPTG. The identity of this protein was confirmed by microsequencing of the NH_2-terminus. Bacterial lysates were fractionated by SDS-PAGE (12% gels). The band corresponding to the hSos1 catalytic domain was excised and used to generate rabbit polyclonal antibodies (anti-hSos1).
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39. Anti-GRB2 has been described (*23*). For immunoprecipitations, HER14 cells were lysed in buffer containing 10 mM tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF), leupeptin (10 μg/ml), 1 mM sodium orthovanadate, 100 mM sodium fluoride, and 10 mM sodium pyrophosphate. Lysates were clarified by centrifugation and immunoprecipitations were done for 90 min at 4°C with anti-GRB2 and Protein A–Sepharose beads.
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43. Sedimented bacteria were solubilized in sonication buffer [10 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, 1 μM fluoride PMSF, 20 μM leupeptin, 2 μg/ml aprotinin (2 μg/ml), and 2 μM pepstatin] and clarified by centrifugation. The lysate was incubated with Pro-Bond resin (Invitrogen) for 2 hours at 4°C and proteins were eluted with increasing imidazole concentrations. Fractions containing the histidine-tagged hSos1 catalytic domain (His-hSos1-cd) were identified by immunoblotting with anti-hSos1, pooled, dialyzed, concentrated against buffer A [20 mM tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM dithiotreitol (DTT), and 100 mM NaCl], and used immediately in guanine nucleotide exchange assays.
44. For immunoblotting, proteins resolved by SDS-PAGE were electrophoretically transferred to nitrocellulose filters at a constant voltage of 90 V for 1.5 hours in tris-glycine (25 and 192 mM, respectively) buffer containing 20% methanol. Filters were blocked with 5% bovine serum albumin (BSA) overnight and then incubated with anti-hSos1 (1:200 dilution) for 1 hour. Blots were incubated with goat antibodies to rabbit immunoglobulin conjugated to horseradish peroxidase (Organon Teknika) and the immunoreactive bands were visualized using the ECL detection system (Amersham).
45. Purified H-Ras, N-Ras, RalA, or CDC42Hs (10 pmol) were bound to [^{3}H]GDP by incubation for 30 min at 30°C in 200 μl of buffer consisting of 20 mM tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM DTT, 100 mM NaCl, BSA (100 μg/ml), and [^{3}H]GDP (100 pmol, 10 Ci/mmol. Then, nonradioactive GTP (500 μM) and purified His-hSos1-cd (1 μg in 25 μl of buffer A) were added.
46. Purified H-Ras, N-Ras, RalA, or CDC42Hs (50 pmol) were incubated in buffer A (100 μl) at 30°C. The reaction was initiated by the addition of [α-^{32}P]GTP (15 pmol, 3000 Ci/mmol) and purified His-hSos1-cd (1 μg in 25 μl of buffer A). At various intervals, samples (20 μl) were withdrawn and the amount of [α-^{32}P]GTP bound was measured.
47. Human kidney 293 cells were transfected with the indicated expression plasmids. Transfected cells were sonicated in buffer consisting of 20 mM MOPS (pH 7.5), 1 mM $MgCl_2$, 200 mM sucrose, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF, leupeptin (100 μg/ml), pepstatin (100 μg/ml), 0.1% aprotinin, 10 mM benzamidine, trypsin inhibitor (10 μg/ml), and 1 mM sodium orthovanadate. The homogenates were centrifuged at 100,000*g* for 20 min at 4°C, and the supernatants were used immediately. Purified H-Ras (5 pmol) was incubated with [^{3}H]GDP (50 pmol, 10 Ci/mmol) in 50 μl of buffer [25 mM tris-HCl (pH 7.5), 1 mM DTT, BSA (100 mg/ml), and 5 mM EDTA] for 30 min at 37°C. The bound [^{3}H]GDP was stabilized by the addition of $MgCl_2$ (final concentration, 5 mM). Reactions were initiated by the addition of cytosol (500 μl, 6 mg of protein per milliliter) supplemented with $MgCl_2$ (5 mM) and nonradioactive GTP (2 mM). At various intervals, samples (100 μl) were diluted to 1 ml with immunoprecipitation buffer [25 mM tris-HCl (pH 7.5), 0.5 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 0.5% deoxycholate, 0.05% SDS, leupeptin (100 μg/ml), pepstatin (100 μg/ml), 0.1% aprotinin, 10 mM benzamidine, trypsin inhibitor (10 μg/ml), and 1 mM sodium orthovanadate]. Ras was immunoprecipitated with the Y13-259 antibody as described (*49*) and the immune complexes were washed five times with the immunoprecipitation buffer. Nucleotides were eluted from the immune complexes by incubation with 1% SDS and 20 mM EDTA for 5 min at 65°C and quantified by scintillation counting.
48. The two-hybrid system (*36*) uses a yeast strain in which an integrated lacZ gene is under control of a GAL1 promoter, a plasmid expressing the GAL4 DNA binding domain and a plasmid expressing the GAL4 activation domain. We used the yeast strain YPB2 (MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3,112 can^R gal4-542 gal80-538, LYS2::$GAL1_{UAS}$-$LEU2_{TATA}$-HIS3, URA3::$GAL1_{17mers(3x)}$-$CYC1_{TATA}$-lacZ). Replicative plasmid pGBT10 is a pGBT9 plasmid in which the frame of the cloning site has been modified. It has a TRP1 gene for selection in yeast and an alcohol dehydrogenase promoter that drives expression of the GAL4–DNA binding domain (amino acid 1 to 147). Fusion at the COOH-terminus of this domain can be done by cloning in the site GAA TTC GGA TCC CAT TTA AAT GTC GAC. Replicative plasmid pGAD10GHU is a derivative of pGAD1 [C. Chien, P. L. Bartel, R. Sternglanz, S. Fields, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 9578 (1991)] provided by G. Hannon. This replicative plasmid with a LEU2 marker and a URA3 marker allows expression of the GAL4-activation domain (amino acid 768 to 881) from an ADH promoter. At the 3′ end of the DNA encoding this domain, the multicloning site of plasmid pGAD was replaced by part of the multicloning site of plasmid Bluescript SK$^-$. Some of the inserts cloned in these plasmids were cloned by PCR with adequate primers, using PfU Polymerase (Stratagene). The sequence of all PCR-generated products was verified. Otherwise, classical DNA cloning techniques were used [J. Sambrook, T. Fritsch, T. Maniatus, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989)]. LacZ expression assays were done with a paper filter assay with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, Boehringer Mannheim) as described [S. Dalton, R. Treisman, *Cell* **68**, 597 (1992)], except that Whatman 50 paper was used instead of nitrocellulose paper.
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50. We thank A. Lamouroux and J. Mallet for the pheochromocytoma cDNA library; S. Fields, P. Bartel, and G. Hannon for the strain and the plasmids used in the two-hybrid system; R. Waldmann and C. Nicolette for their help with the sequence alignments, L. Feig for the RalA protein; R. A. Cerione the CDC42Hs protein; L. Rogers and M. Riggs for their help in sequencing, D. Moinier for performing the microsequencing of the *E. coli*–expressed hSos protein; and F. Aguila, P. Renna, J. Duffy, and M. Ockler for photographic work. Supported by INSERM and the Association pour la Recherche sur le Cancer (ARC 6103) to P.C., by CNRS and NATO to J.H.C , by The Collen Foundation and National Fund for Scientific Research (Belgium) to L.V.A., by NIH grants CA55360 and CA46370 to D.B., and by The American Cancer Society and The National Cancer Institute (M.H.W.). M.H.W. is an American Cancer Society professor.

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