Grb2 Is a Negative Modulator of the Intrinsic Ras-GEF Activity of hSos1[□]

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hSos1 is a Ras guanine-nucleotide exchange factor. It was suggested that the carboxyl-terminal region of hSos1 down-regulates hSos1 functionality and that the intrinsic guanine-nucleotide exchange activity of this protein may be different before and after stimulation of tyrosine kinase receptors. Using different myristoylated hSos1 full-length and carboxyl-terminal truncated mutants, we show that Grb2 function accounts not only for recruitment of hSos1 to the plasma membrane but also for modulation of hSos1 activity. Our results demonstrate that the first two canonical Grb2 binding sites, inside the carboxyl-terminal region of hSos1, are responsible for this regulation. Following different approaches, such as displacement of Grb2 from the hSos1-Grb2 complex or depletion of Grb2 levels by small interfering RNA, we found that the full-length Grb2 proteins mediate negative regulation of the intrinsic Ras guanine-nucleotide exchange activity of hSos1.

INTRODUCTION

Sos guanine-nucleotide exchange proteins mediate Ras activation induced by various receptor tyrosine kinases (RTK). Sos protein consists of several domains, each of them with a distinct function. The Sos nucleotide exchange activity on Ras is mediated by a central domain (CDC25-H domain) that is highly conserved among the various Ras guanine-nucleotide exchange factors (Boriack-Sjodin *et al.*, 1998). The amino-terminal region of Sos contains regions of homology to Dbl (DH) and pleckstrin (PH) domains involved in Rac1 activation (Nimnual *et al.*, 1998) and phospholipid binding (McCollam *et al.*, 1995), respectively. Furthermore, this amino-terminal region contains an HF motif (with homology to histone H2A; Jorge *et al.*, 2002; Sondermann *et al.*, 2003), which mediates intramolecular binding to the PH domain (Jorge *et al.*, 2002). Finally, the carboxyl-terminal region of Sos is proline-rich and contains specific sequences that bind SH3 domains of Grb2 (Simon and Schreiber, 1995).

Under steady-state growth conditions, Sos forms a complex with Grb2, as a result of which the SH3 domains of Grb2 bind to specific proline-rich sequences located in the carboxyl-terminal region of Sos (Simon and Schreiber, 1995). Previously, we identified two distinct human Sos1 isoforms (hSos1 Isf I and hSos1 Isf II) with different Grb2 binding affinity (Rojas *et al.*, 1996, 1999). These isoforms differ only by the presence in hSos1 Isf II of a 15-amino acid sequence located close to the first proline-rich motif required for Grb2 binding (Rojas *et al.*, 1996). In addition to the four proline-

rich Grb2-Binding Motifs (Grb2-BM) responsible for the interaction with Grb2 (PΨΨPPR), there are other domains containing the SH3-Minimal Binding Site (SH3-MBS) (ΨPXΨP; Zarich *et al.*, 2000). One of them, located in the specific stretch of hSos1 Isf II, is responsible for the increased Grb2 binding affinity of this isoform in comparison to isoform I (Zarich *et al.*, 2000).

Stimulation of cells with growth factors leads to the association of Sos-Grb2 complexes with activated receptors and then to the stimulation of Ras through the juxtaposition of Sos and Ras at the membrane. In this model, both the cytosolic and membrane-bound Sos forms are assumed to exhibit similar nucleotide exchange activity, and no variation of this activity is believed to occur as a consequence of relocation inside the cell. Supporting this idea, membrane targeting of these exchange factors has been shown to strengthen Ras activation in transfected cells (Aronheim *et al.*, 1994). However, other reports suggest that regardless of subcellular location, the intrinsic Ras guanine-nucleotide exchange activity of Sos (Ras-GEF activity) differs before and after stimulation of surface tyrosine kinase receptors (Li *et al.*, 1996; Rojas *et al.*, 1999). Intermolecular and intramolecular interactions involving the different modular domains of Sos proteins may help to reconcile these two apparently contradictory views. Thus, different reports propose that the carboxyl-terminal region of Sos may exert negative regulation over the activity of the whole Sos1 protein (Corbalan-García *et al.*, 1998; Zarich *et al.*, 2000). Deletion of the carboxyl-terminal region of Sos1 increases its Ras-GEF activity in vitro and in vivo, resulting in enhanced Ras-signaling and -transforming activity (Corbalan-García *et al.*, 1998; Rojas *et al.*, 1999; Zarich *et al.*, 2000). Consistent with this notion, a mutation in the *hsos1* gene creates a premature stop codon abolishing the proline-rich SH3 binding domains of the carboxyl-terminal region of hSos1 (Hart *et al.*, 2002). This mutation is associated with hereditary gingival fibromatosis, a rare, autosomal dominant form of gingival overgrowth. A

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transgenic mouse construct with a comparable Sos1 chimera shows skin hypertrophy (Hart *et al.*, 2002). Furthermore, the carboxyl-terminal region of Sos contains several phosphorylation sites for p90 RSK-2 (Douville and Downward, 1997), and their phosphorylation may have a negative feedback effect on the Ras pathway.

We undertook this study to explore the mechanisms of the negative regulation of the intrinsic Ras-GEF activity of hSos1 by its carboxyl-terminal region and the possible role of Grb2 in this process.

MATERIALS AND METHODS

Cell Lines, Transfections, Antibodies, and Reagents

NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Paisley, United Kingdom) supplemented with 10% calf serum (CS, Invitrogen). Cos1 and the human HeLa, and 293T cell lines were maintained in DMEM supplemented with 10% fetal calf serum (FCS; Invitrogen). Transient transfections in HeLa, Cos1, and 293T cells were performed in p100 plates and using Jet-Pei (Polyplus-Transfection, Illkirch, France). The efficiency of transfections, as assessed using the pEGFP plasmid (Invitrogen), was between 30 and 40%. Cells for serum starvation received DMEM containing 0.5% fetal bovine serum 24 h after transfection and were then incubated for another 18 h. All assays were done 48 h after transfection. NIH3T3 and 293T cells were also transfected by calcium phosphate precipitation. Monoclonal antibodies to phospho-ERK (p-ERK) and phospho-p38 (p-p38) proteins were purchased from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal antibodies to ERK (ERK1/ERK2), p38, hSos1 and to GST came from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-hSos1, anti-pan Ras, and anti-Rac1 monoclonal antibodies were purchased from Transduction Laboratories/BD Biosciences (Heidelberg, Germany) and anti-HA monoclonal antibody from Berkeley Antibody Company (Berkeley, CA). Aplidin (Cuadrado *et al.*, 2004) was generously provided by Pharma Mar S.A. (Madrid, Spain).

DNA Constructs

The plasmids pCEFL-KZ-HA, pCEFL-KZ-Myr, pCEFL-AU5-H-Ras wt, pCEFL-KZ-AU5-H-Ras V12, pCEFL-KZ-HA-hSos1 Isf I, pCEFL-KZ-HA-hSos1 Isf II, pCEFL-KZ-hSos1-CMR I, pCEFL-KZ-hSos1-CMR II, pSP72-hSos1 Isf I, pGEM3-hSos1 Isf II, pGal4-Luc, and pCDNAIII-Gal4-Elk1 were as previously described (Zarich *et al.*, 2000; Jorge *et al.*, 2002). The plasmids pMexNeo-Grb3-3 (Fath *et al.*, 1994) and pEGFP-C2-SH3 A-E (Tong *et al.*, 2000) were kindly provided by B. Tocque and P. McPherson, respectively. The truncated mutants hSos1-CMRI-pr1 and hSos1-CMRII-pr1 were generated using primers for positions 3200–3465 and 3200–3510 of hSos1 Isf I and hSos1 Isf II, respectively; similarly, the truncated mutants hSos1-CMRI-pr1,2 and hSos1-CMRII-pr1,2 were generated using primers for positions 3200–3546 and 3200–3591 of hSos1 Isf I and hSos1 Isf II, respectively. These regions were PCR-amplified from pCEFL-KZ-HA-hSos1 Isf I and pCEFL-KZ-HA-hSos1 Isf II using the specific primers and providing restriction sites BamHI and Not I-SacI at the 5' and 3' ends, respectively. The amplified products were then subcloned into BamHI and SacI sites of plasmids pSP72-hSos1 Isf I and pGEM3-hSos1 Isf II, respectively. The epitope-tagged truncated mutants were obtained by inserting the coding region of the above hSos1 truncated mutants into the vectors pCEFL-KZ-HA and pCEFL-KZ-Myr. The double mutant hSos1-CMR I AA (where proline 1025 and 1028 were substituted by alanine) was obtained by PCR from pSP72-hSos1 Isf I using the specific primers and providing BglIII sites at the 5' and 3' ends and subcloned into BglIII sites of pCEFL-KZ-Myr-CMRI. All PCR-generated constructs were verified by direct sequencing. The corresponding sequences of the oligonucleotides utilized are available upon request.

RNA Interference

One pair of 21 nucleotide sense and antisense RNA oligonucleotides protected by two 3'-overhanged (2'-deoxy) thymidines (dT; small interfering RNAs [siRNAs]) were synthesized by Dharmacon Research (Lafayette, CO). These oligonucleotides are as follows: sense, 5' CAU GUU UCC CCG CAA UUA UdTdT 3'; antisense, 5' AUA AUU GCG GGG AAA CAU GdTdT 3', corresponding to human Grb2 coding nucleotides 607–627 (Jiang *et al.*, 2003). In addition, the siRNA sequence sense strand used as negative control for siRNA activity was 5' AUU GUA UGC GAU CGC AGA CdTdT. siRNA duplexes (40 nM) were transfected twice in HeLa and 293T cells using Jet-Pei at 24-h intervals. Cells were maintained in starvation medium 18 h before experiments.

Bacterial Expression of Fusion Proteins

All GST-fusion proteins were purified, following the method previously described (Zarich *et al.*, 2000; Jorge *et al.*, 2002), from *Escherichia coli* BL21

Figure 1. Structure of myristoylated-hSos1 carboxyl-truncated mutants. The mutants containing only the sites SH3-MBS of hSos1 Isf I and Isf II, without the four sites Grb2-BM, were hSos1-CMRI and hSos1-CMRII, respectively. The mutants without the last three sites Grb2-BM of both isoforms were hSos1-CMRI-pr1 and hSos1-CMRII-pr1. The mutants containing only the first and second Grb2-BM of both isoforms were hSos1-CMRI-pr1,2 and hSos1-CMRII-pr1,2. All these truncated mutants and the wt isoforms were cloned into the expression vector pCEFL-KZ-MYR. HF, HF motif; DH, Dbl-homology; PH, Pleckstrin homology; REM, Ras exchanger motif; CDC25-H, CDC25 homology; PR, Proline-rich domain; SH3-MBS, SH3 minimal binding site; Grb2-BM, Grb2 binding domain.

(DE3) harboring the plasmids pGEX-RBD, or pGEX-PAK-CRIB, containing the Raf Ras-binding domain, and the PAK Rac1-binding domain, respectively, fused to glutathione *S*-transferase (GST).

Pulldown Assays

Transfected cells were lysed in cold lysis buffer containing 25 mM HEPES, pH 7.5, 1% NP-40, 0.25% Na-deoxycholate, 10% glycerol, 1 mM EDTA, 150 mM NaCl, 10 mM MgCl₂, 1 mM sodium orthovanadate (Na₃VO₄), 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, aprotinin, pepstatin A, and trypsin inhibitor. Nucleus-free supernatants were incubated with GST-RBD, or GST-PAK-CRIB on glutathione-Sepharose beads and analyzed as previously described (Zarich *et al.*, 2000).

Preparation of Subcellular Fractions

Cells were resuspended in sucrose-hypotonic lysis buffer (20 mM HEPES, pH 7.5, 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin), swelled on ice for 1 h, and disrupted by forcing cells through a 27-gauge needle 60 times. Cytosolic and crude membrane fractions were separated by high-speed centrifugation as described (Rojas *et al.*, 1999). The P100 membrane-containing pellets were dissolved in buffer A (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). The S100 cytoplasmic fractions were adjusted to the composition of buffer A, and myristoylated hSos1 proteins were detected by immunoblotting from 150 μg of each fraction, as determined using the DC Protein Assay kit from Bio-Rad (Richmond, CA).

Reporter Gene Analysis

NIH3T3 and 293T cells (p60 plates) were transfected by calcium phosphate precipitation (Zarich *et al.*, 2000) with 0.6 μg of constructs encoding for either hSos1 or Ras, together with 16 ng of pCDNAIII-Gal4-Elk1, or pCDNAIII-Gal4-c-Jun, 0.1 μg of pRL-TK (a plasmid containing the *Renilla* luciferase gene under control of the HSV-TK promoter) and 0.3 μg of the reporter plasmid pGal4-Luc (containing the *Photinus* luciferase gene controlled by six copies of a Gal4 responsive element), adjusting the total amount of plasmid DNA with empty vector. After overnight incubation, the cells were washed and kept for 24 h in DMEM supplemented with 0.5% calf serum. Cells were then lysed with passive lysis buffer (Promega, Madison, WI). *Photinus* and *Renilla* luciferase present in the nucleus free supernatants were assayed with the Dual-Luciferase Reporter Assay System (Promega), and light emission was quantitated with a Monolight 2010 luminometer as specified by the manufacturer (Analytical Luminescence Laboratory). The *Renilla* luciferase activity present in each sample was used to normalize *Photinus* luciferase activity for transfection efficiency.

Figure 2. Relative Ras-Raf-MEK-ERK pathway activation elicited by Myr-hSos1 wt versus carboxyl-truncated mutants. (A) Cos1 cells were transfected with 1 μ g of the above Myr-hSos1 constructs or vector alone. The transfected cells were serum-starved for 18 h. Cell lysates were prepared, and the levels of p-ERK were determined using specific antibodies and normalized to that of total ERK. Moreover, Ras-GTP was recovered from cell lysates by binding to immobilized GST containing the Ras-GTP binding domain of Raf and detected by immunoblotting with anti-pan Ras antibody. Endogenous Ras was detected by immunoblotting of the cell extracts with anti-pan Ras antibody. Immunoreactive bands were viewed by ECL. Myr-hSos1 (full-length or mutant) constitutively targeted to cell membrane were detected by immunoblotting with anti-hSos1 antibody of the P100 membrane-containing pellets. Results shown are from a representative experiment. Similar results were obtained in four additional experiments. The fold increase values of p-ERK are the average of five separate assays (in each case with an SD lower than 15% of the average). (B) Quantitation of Ras-GTP standardized to Ras levels for the same type of experiments indicated in A. The histogram represents the average and SD of five separate assays. (C) NIH3T3 cells were cotransfected with the plasmids pcDNAIII-Gal4-Elk1, pGal4-Luc, and pRL-TK together with expression plasmids pCEFL-KZ-Myr containing the indicated hSos1 constructs described in Figure 1, or well H-Ras Val 12 as positive control. The transfected cells were serum-starved and assayed 24 h later for luciferase activity. The data represent *Photinus* luciferase activity standardized for the *Renilla* luciferase activity present in each cellular lysate, expressed as fold induction compared with control cells. The data are the mean and SD of four separate assays performed in triplicate.

RESULTS

Mapping of the Negative Regulation of hSos1

Looking for the specific zone responsible for the negative regulation of hSos1 activity, we generated a range of carboxyl-truncated mutants of the two hSos1 isoforms by consecutive deletion of their Grb2 binding sites (Figure 1). These mutants were constitutively targeted to the cell membrane using the Src myristoylation (Myr) sequence. Localization at the cell membrane was confirmed by subcellular fractionation assays followed by immunoblot detection (unpublished data).

We analyzed the Ras-Raf-MEK-ERK pathway under serum-starved cell conditions. Therefore, mutants of both isoforms lacking the four Grb2-BM (Myr-hSos1-CMRI and Myr-hSos1-CMRII) induced the highest Ras activation in Cos1 cells. Furthermore, hSos1 mutants containing only the first Grb2-BM (Myr-hSos1-CMRI pr1 and Myr-hSos1-CMRII-pr1) elicited higher levels of Ras-GTP than mutants containing the first two Grb2-BM (Myr-hSos1-CMRI pr1,2

and Myr-hSos1-CMRII pr1,2) or full-length hSos1 proteins (Figure 2, A and B). Likewise, the activation of ERK in Cos1 cells decreased proportionally to the length of the ectopic hSos1 carboxyl-terminal region, and mutants of both isoforms lacking the four Grb2-BM (Myr-hSos1-CMRI/II) induced the highest levels of ERK activation (Figure 2A). Similar results were obtained using NIH3T3 and HeLa cells (unpublished data).

The activation of the Ras pathway can induce gene expression via the transcription factor Elk1, which lies downstream of the Ras/ERK cascade. To confirm the results presented above, we used a reporter assay in NIH3T3 cells cotransfected with the myristoylated hSos1 constructs, together with a chimerical Gal4-Elk1 transcription factor and the reporter plasmid TATA-Gal4-Luc. Figure 2C shows the results obtained in a set of experiments in which we measured the induction of luciferase activity under unstimulated cell conditions, using the transfection of H-Ras V12 as positive control. Again, mutants of both isoforms lacking the

Figure 3. Negative regulation of Myr-hSos1 by Grb2. NIH3T3 cells were cotransfected and assayed as Figure 2B with plasmids pcDNAIII-Gal4-Elk1, pGal4-Luc, pRL-TK (data are the mean and SD of four separate assays performed in triplicate). (A) Together with the indicated Myr-hSos1 constructs (hSos1-Isf I, hSos1-Isf II, hSos1-CMRI, hSos1-CMRI AA) and with HA-Spry2, or GFP-SH3 A-E (SH3-ITSN), or DNA vector. Top shows the mutant Myr-hSos1-CMRI AA used in this assay, where all proline of the SH3-MBS were mutated to alanine. (B) Together with the indicated Myr-hSos1 constructs (hSos1-Isf I, hSos1-Isf II, hSos1-CMRI, hSos1-CMRI AA) and with Grb3-3 or DNA vector.

four Grb2-BM (Myr-hSos1-CMRI/II) induced the highest levels of Gal4-Elk1 activation. However, we did not detect differences between myristoylated carboxyl-truncated hSos1 mutants containing only the first two Grb2-BM (Myr-hSos1-CMRI/II pr1,2) and full-length proteins. The same results were obtained using HeLa cells (unpublished data).

These results indicate that the carboxyl-terminal region of hSos1 encompassing the first and second Grb2-BM is the main zone responsible for the negative regulation of hSos1.

Displacement of Grb2 Increases Myr-hSos1 Activity

The above results showed a proportional relationship between negative regulation of hSos1 activity and the number of Grb2 binding sites. Then, we assessed whether the intrinsic hSos1 activity was affected by the displacement of Grb2 from the hSos1-Grb2 complex. To this end, we used an Intersectin construct (containing only the five SH3 domains: SH3-ITSN; Tong *et al.*, 2000) that interacts with the carboxyl-terminal region of hSos1 in the same positions as Grb2. We also used human Sprouty 2 protein (hSpry2), which binds Grb2, thus sequestering it from the hSos1-Grb2 complex (Hanafusa *et al.*, 2002). We compared the effect of hSpry2

and SH3-ITSN on the capacity of Myr-hSos1 Isf I, Myr-hSos1 Isf II, and Myr-hSos1-CMRI to activate the ERK pathway, using the Gal4-Elk1 transcriptional activation assay in NIH3T3 fibroblasts. Neither hSpry2 nor SH3-ITSN increased basal or serum-stimulated Gal4-Elk1 activity (Supplementary Figure 1). However, both proteins increased the Gal4-Elk1 activity elicited by the Myr-hSos1 constructs (Figure 3A). Induced-Gal4-Elk1 activity also occurred with the mutant Myr-hSos1-CMRI, which contains only one SH3-MBS but forms stable complex with Grb2 protein (Zarich *et al.*, 2000). When we used the mutant Myr-hSos1-CMRI AA, which does not bind Grb2 (Zarich *et al.*, 2000), the addition of hSpry2 or SH3-ITSN did not increase the Gal4-Elk1 activity (Figure 3A). Furthermore, Myr-hSos1-CMRI AA alone induced higher transcriptional activity than Myr-hSos1-CMRI. We also used the Grb2 truncated mutant Grb3-3 (Fath *et al.*, 1994), which contains only the two SH3 domains without the SH2 domain. The presence of Grb3-3 increased the Gal4-Elk1 activation induced by Myr-hSos1 Isf I, Myr-hSos1 Isf II, and Myr-hSos1-CMRI (Figure 3B). However, this effect was not observed using the mutant Myr-hSos1-CMRI AA, which does not bind SH3 domains. This result suggests that, in

Figure 4. Grb2 depletion increases the intrinsic Ras-GEF activity of Myr-hSos1. (A) HeLa cells were cotransfected with 1 μ g of plasmid vector (CONTROL), or with 1 μ g of different Myr-hSos1 constructs (hSos1-CMR I or hSos1 Isf I), and with 40 nM either control siRNA (siRNA Grb2: -), or specific Grb2 siRNA (siRNA Grb2: +). The transfected cells were serum-starved for 18 h. The Ras-GTP and p-ERK levels were detected following the same approach of Figure 1B and 2A, respectively. Endogenous Ras, ERK, and Grb2 levels were detected by immunoblotting of the cell extracts with specific antibodies. The detection Myr-hSos1 constitutively targeted to cell membrane was as Figure 1B. Results shown are from a representative experiment, and similar results were obtained in three additional experiments. The fold increase values of p-ERK and the % Grb2 levels are the average and SD of four separate assays. (B) Quantitation of Ras-GTP standardized to Ras levels. The histogram represents the average and SD of four separate assays.

addition to the SH3 domains, Grb2 also needs the SH2 domain to down-regulate the hSos1 activity in basal state. The same results were obtained using HeLa cells (unpublished data).

In all cases, the differences in Gal-Elk1 activity were detected independently of Rac activation, because the Myr-hSos1 constructs had similar influence on the Rac-GTP levels and downstream effectors (Supplementary Figure 2).

Depletion of Grb2 Increases Myr-hSos1 Activity

To assess whether Grb2 had a regulatory effect on the intrinsic Ras-GEF activity of hSos1, we knocked-down Grb2 expression in HeLa cells using siRNAs. HeLa cells were cotransfected with plasmid vector, or Myr-hSos1-CMR I, or Myr-hSos1 Isf I, and control siRNA or a specific siRNA covering a portion of the human Grb2 coding sequence. Endogenous Grb2 was decreased by 80% using this siRNA duplex (Figure 4A). Under these conditions neither endogenous levels of ERK and Ras, nor expression of the corresponding Myr-hSos1 constructs were affected. Decreased Grb2 expression in Myr-hSos1 cotransfectants correlated with an increase in ERK activation under serum-starved conditions (Figure 4A). Both Myr-hSos1-CMR I and Myr-hSos1 Isf I, in the presence of Grb2 siRNA, induced similar levels of ERK activation, whereas the depletion of Grb2 in the starved cells transfected with plasmid vector had no effect on the p-ERK levels (Figure 4A). Consistently with the p-ERK levels, the decrease of Grb2 levels in Myr-hSos1 cotransfectants correlated with an increase of Ras activation (Figure 4A). Again, both Myr-hSos1-CMR I and Myr-hSos1 Isf I, cotransfected with Grb2 siRNA, induced similar levels of Ras activation, whereas the depletion of Grb2 in the starved cells transfected with plasmid vector had no effect on the Ras-GTP levels (Figure 4, A and B). Taken together, these experiments confirmed that Grb2 negatively modulate

the intrinsic Ras-GEF activity of hSos1 observed under serum-starvation conditions.

DISCUSSION

In this study we have explored the mechanisms of the negative regulation of hSos1 activity by its carboxyl-terminal region. We mapped the specific zone by consecutive deletions. The carboxyl-terminal region of hSos1 contains four proline-rich Grb2-BMs responsible for the interaction with Grb2 (P Ψ P Ψ PPR), and two SH3-MBSs (Ψ PX Ψ PP) that also interact with the SH3 domains of Grb2 (Zarich *et al.*, 2000). The Grb2-BMs and SH3-MBSs were considered in the generation of the myristoylated hSos1 deletion mutants (Figure 1). The myristoylation targeted the hSos1 constructs to the cell membrane and precluded the bias effect of any differences in the translocation capacities of hSos1 mutants to the cell membrane attributable to differences in their Grb2-binding affinities. Moreover, in all assays we analyzed the activation of Ras pathway under serum-starved cell conditions. Therefore, the Ras-GTP levels elicited by overexpression of Myr-hSos1 full-length or carboxyl-deletion mutants, corresponded to the basal Ras-GEF activities of these hSos1 constructs and could be considered an *in vivo* indication of their intrinsic Ras-GEF activities.

The results of this study suggest an alternative function of Grb2 as negative modulator of hSos1 activity in the basal state, which seems to be mediated by its capacity to bind the first and second Grb2-BM of hSos1 (Supplementary Figure 3A) and to depend on both SH3 and SH2 domains. Indeed, displacement of Grb2 from the hSos1-Grb2 complex, or knockdown of Grb2 levels by siRNA, increased the activity of myristoylated hSos1. Our data demonstrate that Grb2 not only recruits hSos1 to the plasma membrane upon RTK stimulation, but also controls the activity of hSos1. The first and second Grb2-BM, involved in the negative control of

hSos1 activity, are the domains of the carboxyl-terminal region with highest binding affinity for Grb2. The stoichiometry of the Grb2-hSos1 complex is not clearly defined but the presence of four Grb2-BMs and four independent SH3-MBSs in the carboxyl-terminal region of hSos1 suggests that several molecules of Grb2 may be associated with one molecule of hSos1.

These results support some previously published effects. It has been described that overexpression of the SH3 domain of PLC- γ (with binding affinity for the carboxyl-terminal region of hSos1) induces activation of the Ras-ERK pathway (Kim *et al.*, 2000). In addition, microinjection with peptide fragments of the first and second Grb2-BM of hSos1 Isf II induces activation of Ras pathway in *Xenopus* oocytes (Rojas *et al.*, 1996). Furthermore, our results rule out the possibility that Grb2 offsets the negative regulation of hSos1 activity by its carboxyl-terminal region (McCollam *et al.*, 1995; Wang *et al.*, 1995). They also indicate that the highest activity of the hSos1 carboxyl-truncated mutants is attributable to factors other than the loss of the p90 RSK-2 phosphorylation sites (Douville and Downward, 1997), such as the absence of Grb2 binding.

hSos1 may also be involved in Rac activation (Nimnual *et al.*, 1998; Scita *et al.*, 1999, 2001). The pathway Sos-Rac1 is mediated by a complex of Sos proteins with the molecular adaptors Eps8 and E3b1-Abi-1 (Scita *et al.*, 1999, 2001). Therefore, it appears that Sos proteins can be engaged in dual interactions, each leading to the activation of a different biological response. Thus, the Sos-Grb2 complex is disrupted upon RTK activation (Cherniack *et al.*, 1994; Douville and Downward, 1997; Innocenti *et al.*, 2002), whereas the Sos-E3b1-Eps8 complex is not (Innocenti *et al.*, 2002). Furthermore, the activation of Ras by growth factors is short-lived, whereas the activation of Rac is sustained (Innocenti *et al.*, 2002). Although the Rac pathway can induce Elk1 activation, the differences detected among the myristoylated hSos1 constructs, such as Gal-Elk1 activity, were independent of Rac, because all hSos1 mutants had a similar effect on Rac and downstream effectors. Moreover, the Rac-GEF activity of hSos1 is only elicited upon RTK stimulation and it depends on Abl tyrosine phosphorylation of hSos1 (Sini *et al.*, 2004), whereas all assays with the myristoylated hSos1 mutants were done without RTK activation.

The dual role of Grb2, recruiting hSos1 to the plasma membrane upon RTK stimulation and down-regulation of the Ras-GEF activity of hSos1 under basal conditions, has relevant physiological implications. Although Grb2 limits the access of hSos1 to its substrate (Ras proteins tagged to plasma membrane) only after cell-mitogenic activations, the negative regulation of hSos1 activity by Grb2 would reduce the stimulus-independent Ras activation (in different endomembrane cell-compartments) by unspecific hSos1 location due to its overexpression. In normal conditions, the cytoplasmic concentration of Grb2 is much higher than that of Sos1 (unpublished data), which can explain the absence of tumors and transforming activity elicited by Sos1 overexpression (McCollam *et al.*, 1995; Wang *et al.*, 1995; Rojas *et al.*, 1996). However, both in mice (Wang *et al.*, 1995) and in humans (Hart *et al.*, 2002), mutations have been described that abolish the proline-rich SH3-binding domains in the carboxyl-terminal region of Sos1 and induce cellular transformation. In our siRNA assays the expression of Grb2 was still higher than that of endogenous hSos1, even after an 80% reduction (Figure 4A and unpublished data). This explains why that down-regulation of Grb2 did not increase Ras or ERK activation in the absence of hSos1 overexpression. Finally, the negative regulation of the Sos1 activity by Grb2

may account for the fact that the intrinsic Ras guanine-nucleotide exchange activity of Sos (Ras-GEF activity) differs before and after stimulation of surface tyrosine kinase receptors (Li *et al.*, 1996; Rojas *et al.*, 1999), including NGF, EGF, platelet-derived growth factor, and macrophage-stimulating protein receptor.

Although it is still speculative, a putative model of negative regulation of hSos1 could be analogous to the regulation of Src (Supplementary Figure 3B). Indeed, Src activity in basal state is low because of the intramolecular interaction between its phosphorylated tyrosine 527 and its SH2 domain (Sun *et al.*, 1998). Similarly, Grb2 bound to the carboxyl-terminal region of hSos1 could also bind some tyrosine phosphate residue(s) of an unknown hSos1-binding protein via its SH2 domain, which would reduce the hSos1 activity. Stimulation of RTK activity accounts for tyrosine phosphate residues anchoring the hSos1-Grb2 complex to the membrane through the SH2 domain of Grb2. This interaction would relieve hSos1 inhibition, and some tyrosine phosphatase could dephosphorylate the unknown hSos1-binding protein and thus provide stable stimulation of the Ras-GEF activity. Identifying phospho-tyrosine protein(s) that mediate the functional role of Grb2 as a negative regulator of the hSos1 activity, is expected to help define the physiological mechanism(s) whereby the carboxyl-terminal region of hSos1 down-regulates its Ras-GEF activity. It may also be possible to reconstruct this mechanism in vitro by protein purification of all components.

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