hSos1 Contains a New Amino-terminal Regulatory Motif with Specific Binding Affinity for Its Pleckstrin Homology Domain*

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WITHDRAWN
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This article has been withdrawn by the authors (although Rocío Jorge could not be reached and Natalia Martínez passed away). The authors have become aware of errors in the preparation of Figs. 2A, 3A, 4A, and 5A, where some lanes appear to be duplicated. The authors state that although replicated experiments performed at the time of the article as well as subsequent data published by other groups (Sondermann, H., Nagar, B., Bar-Sagi, D., and 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. U.S.A. 102, 16632–16637; Yadav, K. K., and Bar-Sagi, D. (2010) Allosteric gating of Son of sevenless activity by the histone domain. Proc. Natl. Acad. Sci. U.S.A. 107, 3436–3440; and Tartaglia, M., Pennacchio, L. A., Zhao, C., Yadav, K. K., Fodale, V., Sarkozy, A., Pandit, B., Oishi, K., Martinelli, S., Schackwitz, W., Ustaszewska, A., Martin, J., Bristow, J., Carta, C., Leperi, F., Neri, C., Vasta, I., Gibson, K., Curry, C. J., Siguero, J. P., Digilo, M. C., Zampino, G., Dallapiccola, B., Bar-Sagi, D., and Gelb, B. D. (2007) Nat. Genet. 39, 75–79) support the results and conclusions presented in this published paper, they consider that the responsible course of action is to withdraw the article in the interests of maintaining the publication standards of the journal. The authors apologize for any inconvenience they may have caused. The paper with the corrected figures can be obtained by contacting the authors.

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cific association with the PH domain of hSos1 and its function in the physiological activity of hSos1.

EXPERIMENTAL PROCEDURES

Cell Lines, Transfections, and Antibodies—NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum (Invitrogen). COS1 and the human 293T (kidney keratinocyte) cell lines were maintained in DMEM supplemented with 10% fetal calf serum (fetal calf serum, Invitrogen). Transient transfections in COS1 and 293T cells were performed with the LipofectAMINE reagent (Invitrogen). COS1 cells for serum starvation received DMEM containing 0.5% fetal bovine serum 24 h after transfection and were then incubated for another 24 h. All assays were done 48 h after transfection. NIH3T3 cells were transfected (transient or stable) by the calcium phosphate precipitation technique. Morphologically transformed foci were scored after 2–3 weeks in culture (21). Transfected cells were also selected in medium supplemented, as appropriate, with Geneticin 750 μg/ml (Invitrogen). Monoclonal antibodies to phospho-MAPK protein was purchased from New England Biolabs. Rabbit polyclonal antisera to MAPK (ERK1/ERK2) and rabbit anti-PHA monoclonal antibody came from Santa Cruz Biotechnology Inc., monoclonal anti-His, from Sigma, and anti-HA and anti-AU5 monoclonal antibodies, from the Berkeley Antibody Co.

DNA Constructs—The plasmids pCEFL-KZ-HA, pCEFL-KZ-AU5, pCEFL-KZ-HA-hSos1, pCEFL-KZ-HA-NDP, pCEFL-KZ-AU5-H-Ras wt, pCEFL-KZ-AU5-H-RasV12, pGEX-4T-1, pG4D-Luc, and pCDNAIII-Ga4-Elk-I were described previously (14, 19, 21). The HF motif, DH, PH, and DH-PH domains of hSos1 (coding regions 1–640, 537–1326, 1263–1701, and 537–1835, respectively) were PCR-amplified from pCEFL-KZ-HA-hSos1 using the specific primers and providing sites BglII and NotI at the 5’ and 3’ ends, respectively. The amplified products were then subcloned into BglII and NotI sites of vectors pCEFL-KZ-HA and pCEFL-KZ-AU5 and into BamHI and NotI sites of pGEX-4T-1 (Amersham Biosciences). Likewise, the HF motif was PCR-amplified with specific primers providing sites BamHI and NotI in the 5’ and 3’ ends respectively, and subcloned into BamHI and NotI sites of pGEX30 vector (Qiagen). The myristoylated probe was obtained from pCEFL-KZ-HA-NDP by digesting the plasmid with BglII and NotI, and the small fragment BamHI-NcoI between sites BamHI and NotI of pCEFL-KZ-HA-NDP by digestion with BamHI and NcoI. The BglII and NotI sites of the wild-type hSos1 and providing restriction sites for BglII and NotI in the vector pCEFL-KZ-HA. To obtain the HF truncated mutant of hSos1, the plasmid pCEFL-KZ-HA-DH-PH was digested with HindIII and SpeI, and the HA-DH-PH fragment was subcloned into the HindIII and SpeI sites of pCEFL-KZ-HA-hSos1. K-Ras wt, K-RasV12, N-Ras wt, and N-RasV12 were PCR-amplified from pCDNA-K-Ras wt, pCDNA-K-RasV12, pCDNA-N-Ras wt, and pCDNA-N-RasV12, respectively (kind gift of J. S. Gutkind), using the specific primers providing sites BglII and NotI at the 5’ and 3’ ends. The amplified products were then subcloned into BglII and NotI sites of vector pCEFL-KZ-AU5. The sequences of the oligonucleotides utilized are available upon request.

Bacterial Expression of Fusion Proteins—S. J. Taylor (Cornell University) kindly provided the plasmid pGEX-RBD containing the Raf-Ras-binding domain (amino acids 1–149) fused to glutathione S-transferase (GST). The GST-RBD protein was purified (from Escherichia coli BL21(DE3) harboring that plasmid) following the method described previously (19). Similarly, the BL21(DE3) strain of E. coli was transformed with the vector pGEX-T4 encoding the fusion protein GST-NDP, GST-HF, GST-DH, GST-PH, GST-DH-PH (all containing domains of hSos1), or GST-PH2 (containing the PH domain 2 of Ras) kindly provided by J. S. Gutkind. This bacterial strain was also transformed with the vector pGEX-4T-1 encoding the fusion protein GST-PH (containing the PH domain of PKD) kindly provided by T. Iglesias (22). Protein purification was performed according to the method described previously (21). The M15 strain of E. coli harboring plasmid pREP4 (Qiagen) was transformed with the vector pQE30 encoding the His motif of hSos1 containing six consecutive histidine residues (the 6xHis tag) at their amino terminus. The 6xHis-His peptide was purified as described previously (23).

HF in Vitro Binding Assays—Fifty pmol of 6xHis-HF protein, purified and eluted, were incubated with 50 pmol of either GST-NDP, GST-HF, GST-DH, GST-PH, GST-DH-PH of hSos1, GST-PH2 of Ras, or GST-PH-PKD, or GST proteins coupled to glutathione-Sepharose beads and incubated for 60 min at 4 °C following the methodology described previously (21).

Regulatory Studies—COS1 cells transfected with hSos1 were stimulated with 10 μg/ml leupeptin, aprotinin, pepstatin A, and trypsin inhibitor. Nuclear-free supernatants were incubated with GST-RBD on glutathione-Sepharose beads and analyzed as previously described (19).

Reporter Gene Analysis—NIH3T3 cells were transfected with 0.6 μg of constructs encoding for either hSos1 or Ras, together with 16 ng of pCDNAIII-Ga4-Elk-I, 0.1 μg of pRL-TK (a plasmid expressing the renilla luciferase), and 0.3 μg of the reporter plasmid (pGa4Luc). Cells were kept for 24 h in DMEM supplemented with 0.5% fetal calf serum and 18 h later were stimulated for 8 h with 30% fetal calf serum. The assays were performed as described previously (19).

RESULTS

Overexpression or Deletion of the Amino-terminal Half of hSos1 (NDP) Impairs the Transforming Phenotype Induced by Normal Ras; the HF Motif of hSos1 Upstream DH Domain Is Critical for This Biological Effect—To ascertain the function of the amino-terminal half of hSos1, we cloned this region (NDP, residues 1–600; NDP = Nterm–Hi1) in a mammalian expression vector and also used an enzymatically truncated mutant of this region (ΔNDP-hSos1). This region includes the DH and PH domains of hSos1 (residues 1–200) and the terminal half displays very close homology to the histone (histone fold) motif (Fig. 4A). Phosphorylation pathway activation is to elicit mitogenesis in NIH3T3 cells. We cotransfected NIH3T3 cells with H-Ras wt and the truncated mutant was shown in experiments in which the different constructs were cotransfected with normal ras genes. The overexpression of H-Ras wt alone produced weak but reproducible transforming activity, which was enhanced severalfold when hSos1 wt was included in the cotransfection experiments (Fig. 1). However, over-expression of ΔNDP-hSos1 (Fig. 1A) or NDP region (Fig. 1B) together with H-Ras wt consistently resulted in a significant reduction in the number of transformed foci produced by normal H-Ras alone. These results suggest that hSos1 needs its amino-terminal half (NDP region) for the synergistic effect with H-Ras wt. This NDP region was induced in the positive control of hSos1 function, in agreement with previously published observations on the regulatory effect of the amino-terminal region of Sos (17, 18, 20).

To determine whether the ability to reduce the number of transformed foci induced by normal H-Ras can be assigned to any particular region of NDP, we over-expressed the different domains (HF, DH–PH, DH, and PH) together with H-Ras wt. Thus, ectopic expression of HF, DH–PH, and DH domains led to the same effect as the complete NDP region, inhibiting the transforming activity of H-Ras wt (Fig. 1B). In sharp contrast, overexpression of the PH domain had no effect on the number of transformed foci (Fig. 1B). Further, cotransfection of the truncated mutant ΔHF-hSos1 (hSos1 without HF motif) together with H-Ras wt more actively induced focus formation than hSos1 wt (Fig. 1A). The ectopic expression of the different hSos1 constructs did not affect the expression levels of endogenous Sos1 (data not shown). Taken together, these results suggest that the HF motif of hSos1 protein is involved in the negative control of hSos1 activity.

Overexpression or Deletion of the NDP Region (or Their Domains) of hSos1 Impairs MAP Kinase Activation Induced by Mitogenic Stimulation—We confirmed the negative effect of
FIG. 1. Focus formation assays in NIH3T3 cells cotransfected with H-Ras wt and hSos1 constructs. A. NIH3T3 cells were cotransfected with 1 μg of pCEFL-KZ-AU5-H-Ras wt and 1 μg of pCEFL-KZ-HA-hSos1 wt or the truncated mutants pCEFL-KZ-HA-ΔHF-hSos1 and pCEFL-KZ-HA-ΔNDP-hSos1 as indicated at the top of the panel. B. NIH3T3 cells were cotransfected with 1 μg of pCEFL-KZ-AU5-H-Ras wt and 1 μg of each pCEFL-KZ-HA-hSos1 construct denoted at the top of the panel. In all cases, after 14 days the dishes were Giemsa-stained to score the transformed foci. All plasmid DNAs produced similar numbers of marker-selectable colonies. The data are the averages and standard deviation of four independent assays performed in duplicate. At the top of each panel is shown the scheme of domains of hSos1 and the amino-terminal fragments of this protein used in the assays. HF, histone fold motif; DH, Dbl homology domain; PH, pleckstrin homology domain; REM, Ras exchanger motif; CDC25-H, CDC25 homology domain; PR, proline-rich region.
**Fig. 2.** The ectopic overexpression of the NDP region of hSos1 inhibits MAP kinase activation. A, 293T cells were transfected with vector (pCEFL-KZ-HA) as negative control, or pCEFL-KZ-AU5-H-RasV12 as positive control (alone or cotransfected with pCEFL-KZ-HA-NDP), or with the corresponding pCEFL-KZ-HA-hSos1 constructs described in the legend for Fig. 1. The transfected cells were serum-starved for 18 h and then treated without (+ lanes) or with epidermal growth factor (EGF; 100 ng/ml, 10 min; − lanes). Cell lysates were prepared, and equal amounts of protein were resolved on 10% SDS-PAGE and immunoblotted for activated MAPK using an antibody that recognizes phosphorylated activated MAPK. Immunoreactive bands were viewed by ECL. Activated MAPK (p-ERK1 and p-ERK2) are indicated in the upper autoradiograms (IB: α-p-ERK). Equal protein loading was confirmed by immunoblotting for MAPK protein levels (IB: α-ERK). The expression levels of the transfected HA-hSos1 constructs and AU5-H-RasV12 were detected by immunoblotting the cell extracts with the corresponding anti-tag monoclonal antibody.
the HF motif on Sos activity by evaluating its function in the signaling pathways downstream of Sos. Specifically, we investigated whether over-expression of the NDP region or its different domains (HF, DH-PH, DH, and PH) affected the Raf-MEK-MAPK pathway. Therefore, 293T cells were transiently transfected with full-length hSos1 (hSos1 wt), the truncated mutants ΔHF-hSos1 and ΔNDP-hSos1, the NDP region, or their different domains. After serum starvation, cells were stimulated with epidermal growth factor (EGF, Fig. 2A). As a positive control we used the oncogenic version of H-Ras (H-RasV12). The results show that over-expression of full-length hSos1 induces, under starved conditions, a level of ERK activation comparable with that seen for naive cells stimulated by epidermal growth factor, whereas ΔHF-hSos1 appears more potent than full-length hSos1 in eliciting this response. However, the over-expression of ΔNDP-hSos1, NDP region, HF, DH-PH and DH domains, but not the PH domain, led to a significant reduction of activated ERK1/ERK2 (Fig. 2A). This inhibitory effect on the MAP kinase pathway produced by over-expression of NDP occurs upstream of Ras because the cotransfection of H-RasV12 and NDP induced similar levels of activated ERK1/ERK2 as H-RasV12 alone (Fig. 2A). The same results were obtained with ΔNDP-hSos1 mutant and by the HF, DH-PH, or DH domains (data not shown).

To confirm the results presented above, the NDP region and its domains were studied for their ability to affect the MAPK pathway. We used a reporter assay in NIH3T3 cells cotransfected with hSos1 or H-RasV12 constructs, together with a chimerical Gal4-Elk1 transcription factor and the reporter plasmid TATA-Gal4-Luc. Fig. 2B shows the results obtained in a set of experiments in which we measured the luciferase activity under starved and serum-stimulated conditions. In accordance with the above results, we also detected inhibition of MAPK activation by the NDP region under basal and stimulated conditions. In the case of the PH domain, it was also detected with a version of the NDP region targeted to the plasma membrane (NDP-myristoylated) (Fig. 2B). This inhibition was also observed with a version of NDP constitutively targeted to the cell membrane (NDP-myristoylated) (Fig. 3, A and B). This assay was performed with the three types of mammalian Ras (K-, H-, and N-Ras). Fig. 3B summarizes the results obtained when we analyzed the AU5-Ras-GTP/AU5-Ras levels under basal and stimulated conditions. In line with the focus formation and MAP kinase results, whereas Ras activation was completely blocked by the over-expression of NDP and HF peptides, the levels of Ras-GTP upon serum stimulation were unaffected by ectopic expression of PH domain (Fig. 3B). Nevertheless, the inhibitory effect on Ras activation due to the over-expression of DH-PH and DH domains was more clearly detected with K-Ras than with H-Ras or N-Ras (Fig. 3B).

Given that the truncated mutant ΔHF-hSos1 was more potent than the full-length hSos1 inducing transforming foci (Fig. 1) and MAP kinase activation (Fig. 2), we decided to determine whether it was also more efficient in inducing Raf inhibition. Therefore, COS1 cells were cotransfected with full-length HA-hSos1 or its truncated mutants. HA-hSos1 deletion mutants were detected as described above. NIH3T3 cells transfected with AU5-H-RasV12 were analyzed in parallel. The results indicate that HA-Ras in cell lysates expresses either the truncated mutant ΔHF-hSos1 showed more Ras activation under basal conditions (Fig. 4A). The amounts of AU5-H-Ras-GTP under basal and stimulated conditions, standardized to AU5-Ras levels (Fig. 4B), in the cells containing HA-hSos1 wt were higher than in cells transfected with AU5-H-Ras wt alone but lower than in cells containing AU5-H-RasV12. Interestingly, under the same basal and stimulated conditions, HA-ΔHF-hSos1 induced Ras-GTP levels to a greater extent than full-length hSos1 (Fig. 4B), which is in agreement with the results observed in focus formation and MAP kinase activation assays.

The HF Motif Binds Specifically to the PH Domain of hSos1 in Vitro as Well as in Vivo—Taken together, the above results suggest that the HF motif of hSos1 exerts a negative regulatory effect on hSos1 activity. Because the NDP region (containing HF, DH, and PH domains) is necessary for hSos1 function, a possible mechanism to explain this negative effect could be through molecular interactions of the HF motif with the domains of the NDP region. To test this hypothesis, we analyzed the interaction between HF and NDP as well as with each one of its corresponding domains. The NDP region or its domains (HF, DH, DH-PH, PH) were expressed as GST fusion proteins, and the HF motif was also expressed as a 6xHis fusion peptide. The purified 6xHis-HF peptide was incubated with similar amounts of purified GST, GST-HF, GST-DH, GST-DH-PH, GST-PH, and GST-NDP proteins coupled to glutathione-Sepharose using a nonradioactive Ras-GTP detection assay (19). Fig. 3A shows representative results wherein we compared the levels of Ras-GTP in transient cotransfected COS1 cells expressing AU5-K-Ras wt and HA-tagged constructs of either full-length hSos1 or the NDP region (or their domains).
FIG. 3. The ectopic overexpression of the NDP region of hSos1 blocks Ras activation. A, COS1 cells were transfected with pCEFL-KZ-AU5-K-Ras wt alone or pCEFL-KZ-AU5-K-RasV12 (positive control) or cotransfected with pCEFL-KZ-AU5-K-Ras wt and the corresponding pCEFL-KZ-HA-hSos1 constructs described in the legend for Fig. 1. The transfected cells were serum-starved for 18 h and then treated without (− lanes) or with fetal calf serum (30%, 10 min; + lanes). 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-AU5 monoclonal antibody (top autoradiogram). As control, the filter was stripped and rebotted against polyclonal GST antibody (bottom autoradiogram). The expression levels of the transfected HA-hSos1 constructs and AU5-K-Ras were detected by immunoblotting of the cell extracts with corresponding anti-tag monoclonal antibody (middle autoradiograms). Results shown are from a representative experiment. Similar results were obtained in four additional and separate experiments. The histogram represents the average and standard deviation of five separate assays. The expression levels of the transfected HA-hSos1 constructs with AU5-H-Ras and AU5-N-Ras were similar to those detected with AU5-K-Ras experiments (data not shown).
one-Sepharose beads, and the proteins bound to the beads were analyzed by immunoblotting with antibodies to 6xHis (Fig. 5A). Whereas purified GST alone, GST-HF, and GST-DH (Fig. 5A) did not bind any HF, high amounts of 6xHis-HF bound to GST-PH beads (Fig. 5A). Consistent with these results, GST-DH-PH and GST-NDP proteins (both containing PH domain) also bound HF (Fig. 5A). The yeast two-hybrid approach (data not shown) gave the same results.

The specificity of the in vitro interaction between the HF motif and PH domain of hSos1 was analyzed by comparing the in vitro binding of HF to the PH domains of Ras-GRF1 (PH2 domain) (1) and PKD (22). To this end, the purified 6xHis-HF peptide was incubated with similar amounts of purified GST-PH (hSos1), GST-PH2 (Ras-GRF1), or GST-PH (PKD) proteins coupled to glutathione-Sepharose beads; and the bound proteins were detected as described in A. The autoradiogram is from a representative experiment repeated four times with similar results.

FIG. 5. In vitro binding of the 6xHis-HF peptide to GST-PH domain. B, 50 pmol of purified 6xHis-HF peptide were incubated with 50 pmol of purified GST, GST-HF, GST-DH, GST-PH, GST-DH-PH, and GST-NDP proteins coupled to glutathione-Sepharose beads. After washing, proteins bound to the beads were run in SDS and immunoblotted against monoclonal 6xHis antibody. As control, the filter was stripped and rebotted against polyclonal GST antibody. The autoradiogram shown is from a representative experiment that was repeated four more times with similar results. B, 50 pmol of purified 6xHis-HF peptide were incubated with 50 pmol of purified GST, GST-PH (hSos1), GST-PH2 (Ras-GRF1), or GST-PH (PKD) proteins coupled to glutathione-Sepharose beads. After washing, proteins bound to the beads were analyzed as described in A. The autoradiogram is from a representative experiment repeated four times with similar results.

Regulation of hSos1 Function by the HF Motif

Fig. 4. Deletion of the HF motif of hSos1 increases Ras activity. A, COS1 cells were transfected with pCEFL-KZ-AU5-H-Ras wt alone or pCEFL-KZ-AU5-H-Ras wt and pCEFL-KZ-AU5-HA-hSos1 wt or pCEFL-KZ-AU5-HA-hSos1 wt and pCEFL-KZ-AU5-HF-hSos1 constructs as indicated in Fig. 1. The transfected cells were serum-starved for 18 h and then treated without (− lanes) or with fetal calf serum (+ lanes). As described in the legend for Fig. 3, 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-AU5 monoclonal antibody (top autoradiogram). As control, the filter was stripped and rebotted against polyclonal GST antibody (bottom autoradiogram). The expression levels of the transfected HA-hSos1 constructs and AU5-H-Ras were detected by immunoblotting of the cell extracts with the corresponding anti-tag monoclonal antibody (middle autoradiograms). Results are from a representative experiment with similar results obtained in three additional, separate experiments. B, quantitation of AU5-H-Ras-GTP standardized (by GelWoks Analyses) to AU5-Ras levels for the experiments indicated in A. The histogram represents the average and standard deviation of four separate assays.

To extrapolate these results to an in vivo situation, we carried out transient cotransfections of COS1 cells with the plasmids pCEFL-KZ-HA-NDP (coding for the epitope-tagged (HA) NDP region of hSos1), together with either pCEFL-KZ-AU5-HF (coding for the epitope-tagged (AU5) HF motif of hSos1), or pCEFL-KZ-AU5 (as negative control). Cellular lysates and anti-AU5 immunoprecipitates obtained under starving or stimulated cellular conditions were further analyzed by immunoblotting with anti-HA antibodies. We consistently detected HA-NDP communoprecipitated with AU5-HF (Fig. 6). The immunoblot analyses with anti-HA demonstrated that HA-NDP is associated with AU5-HF, mainly under stimulated conditions (Fig. 6). Similar results were observed in 293T cells (data not shown). All of these results suggested that the NDP region of hSos1 may establish in vivo stable complexes with the HF motif, depending on mitogenic conditions.

FIG. 6. Deletion of the HF motif of hSos1 increases Ras activation. A, COS1 cells were transfected with pCEFL-KZ-AU5-H-Ras wt alone or pCEFL-KZ-AU5-H-RasV12 (positive control) or cotransfected with pCEFL-KZ-AU5-H-Ras wt and the corresponding pCEFL-KZ-HA-hSos1 wt or pCEFL-KZ-HA-H9004 HF-hSos1 constructs described in Fig. 1. The transfected cells were serum-starved for 18 h and then treated without (− lanes) or with fetal calf serum (+ lanes). As described in the legend for Fig. 3, 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-AU5 monoclonal antibody (top autoradiogram). As control, the filter was stripped and rebotted against polyclonal GST antibody (bottom autoradiogram). The expression levels of the transfected HA-hSos1 constructs and AU5-H-Ras were detected by immunoblotting of the cell extracts with the corresponding anti-tag monoclonal antibody (middle autoradiograms). Results are from a representative experiment with similar results obtained in three additional, separate experiments. B, quantitation of AU5-H-Ras-GTP standardized (by GelWoks Analyses) to AU5-Ras levels for the experiments indicated in A. The histogram represents the average and standard deviation of four separate assays.

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The amino-terminal region of hSos1 (NDP region) contains DH and PH domains involved in Rac1 activation (5) and phospholipid binding, respectively. In addition, it also contains a small region (residues 1–200) upstream of DH displaying high sequence similarity with histone H2A (24). We added here as the HF motif. Ectopic over-expression of the hSos1 region and its domains (HF, DH-PH, and DH) in many mammalian cells blocks Ras, MAPK activation, and cellular transformation induced by normal Ras. In addition, using a NPD deletion mutant of hSos1, we have shown here that absolutely necessary for MAPK activation and cellular transformation. This suggests, on the basis of the published observations (17, 18, 20), that this region contains growth arrest site of the DH domain, identified on the basis of sequence conservation and structural features, lies near the interface of the DH and PH domains (9), and mutations in DH or PH could affect function in the other domain. For this reason, the inhibition of Sos1 activity observed in DH or PH mutants (16, 18, 20) could be due to a conformational distortion of its PH or DH partner, respectively. Because of this possibility, we used ectopic over-expression of isolated DH, PH or DH-PH domains. We found to our surprise that the inhibitory effect on Ras activation upon ectopic over-expression of DH-PH or DH domains was more evident with K-Ras than with H-Ras and N-Ras. Although we still have not explained these differences, the explanation could lie in the fact that the inhibitory effects were related to differences in how Ras proteins are routed to the plasma membrane and where they localize once they get there (25).

To date, the HF motif is a region that is absolutely unknown in the context of Sos1 activity. We found that deletion of the HF motif increases the hSos1 functionality measured by MAPK activation, Ras-GTP levels, or assessing its cooperative effects on the transforming activity of Ras wt. Taken together, these results and those presented above indicate inhibitory behavior of the isolated HF motif, suggesting that this domain is involved in negative regulation of Sos1 activity. No crystallographic data on the HF motif of Sos1 exist yet, but this small region seems to be independent of the DH-PH domain (9). The negative effect of the HF motif could explain the results of Corbalan-Garcia et al. (15), who found that the amino-terminal portion of Sos1 was involved in the negative regulation of its catalytic activity (15). Our results indicate that the HF motif exhibits significant and specific binding affinity for the PH

**Fig. 6. Analysis of HF-NDP complexes in vivo.** COS1 cells were transiently cotransfected with pCEFL-KZ-HA-NDP (coding the NDP region of hSos1) together with either pCEFL-KZ-AU5-HF (coding the HF motif of hSos1) or pCEFL-KZ-AU5 (as negative control). The transfected cells were serum-starved for 18 h and then treated without (−lanes) or with fetal calf serum (30%, 10 min; +lanes). Cell extracts were incubated with anti-AU5 monoclonal antibodies. The anti-AU5 immunoprecipitates or whole cell extracts were then analyzed by immunoblotting using anti-HA monoclonal and anti-AU5 antibodies as described under “Experimental Procedures.” The autoradiogram is shown from a representative experiment repeated three times with similar results.

**DISCUSSION**

The amino-terminal region of hSos1 (NDP region) contains DH and PH domains involved in Rac1 activation (5) and phospholipid binding, respectively. In addition, it also contains a small region (residues 1–200) upstream of DH displaying high sequence similarity with histone H2A (24), which we denoted here as the HF motif. Ectopic over-expression of the hSos1 region and its domains (HF, DH-PH, and DH) in mammalian cells blocks Ras, MAPK activation, and cellular transformation induced by normal Ras. In addition, using a NPD deletion mutant of hSos1, we have shown here that absolutely necessary for MAPK activation and cellular transformation. This suggests, on the basis of the published observations (17, 18, 20), that this region contains growth arrest site of the DH domain, identified on the basis of sequence conservation and structural features, lies near the interface of the DH and PH domains (9), and mutations in DH or PH could affect function in the other domain. For this reason, the inhibition of Sos1 activity observed in DH or PH mutants (16, 18, 20) could be due to a conformational distortion of its PH or DH partner, respectively. Because of this possibility, we used ectopic over-expression of isolated DH, PH or DH-PH domains. We found to our surprise that the inhibitory effect on Ras activation upon ectopic over-expression of DH-PH or DH domains was more evident with K-Ras than with H-Ras and N-Ras. Although we still have not explained these differences, the explanation could lie in the fact that the inhibitory effects were related to differences in how Ras proteins are routed to the plasma membrane and where they localize once they get there (25).

To date, the HF motif is a region that is absolutely unknown in the context of Sos1 activity. We found that deletion of the HF motif increases the hSos1 functionality measured by MAPK activation, Ras-GTP levels, or assessing its cooperative effects on the transforming activity of Ras wt. Taken together, these results and those presented above indicate inhibitory behavior of the isolated HF motif, suggesting that this domain is involved in negative regulation of Sos1 activity. No crystallographic data on the HF motif of Sos1 exist yet, but this small region seems to be independent of the DH-PH domain (9). The negative effect of the HF motif could explain the results of Corbalan-Garcia et al. (15), who found that the amino-terminal portion of Sos1 was involved in the negative regulation of its catalytic activity (15). Our results indicate that the HF motif exhibits significant and specific binding affinity for the PH
domain of hSos1. However, the HF motif does not bind to the DH domain, and neither does it bind to itself. The homology shown by the HF motif with histone H2A (24) (involving residues 90–173 of hSos1) suggests that HF and histone H2A have a similar structure. Histone H2A is an α protein with a relatively simple structure (Fig. 7A). In this structure a long helix occupies an axial position, with one short helix at each end. As a result of this organization, a central cleft, which can be used for interactions with other molecules, remains. In fact, visual inspection of different protein complexes involving the H2A fold shows that the central cleft is probably a good binding site. This is also confirmed in the case of the nucleosome by accessibility computations (Fig. 7, A and B), which is an indicative that central helix residues are involved mainly in protein-protein contacts. In particular, helices from other proteins tend to lie against the long H2A helix through this cleft. This suggests that the observed interaction between the hSos1 HF and PH domains could be caused by binding of the PH to the cleft in the HF motif. In vivo assays showed that the isolated HF motif originated a stable complex with NDP upon mitogenic stimulation. Interestingly, this HF-NDP complex was not detected under starvation conditions. We propose a hypothetical model, based on our data, that may help to envisage the role of the HF motif in hSos1 activity regulation. In a basal state the HF motif could inhibit Sos activity by intramolecular binding to the PH domain. This would prevent the association of the DH region with an unknown molecule(s) required for the efficient activation of Sos (18, 20). Upon stimulation of the cells by engagement of mitogenic receptors, Sos1 function is unregulated by events that weaken the contacts between the HF motif and the PH domain. This in turn would allow the interaction of the DH-PH region with specific targets necessary for activation. Oligomerization of Ras-GEFs in multiple subdomains was also reported (26); although oligomerization of Sos1 has not been described, this possibility cannot be ruled out.

Similar autoinhibitory regulations are observed in other GEF proteins. Indeed, there is evidence that the activation of proto-Dbl involves an autoinhibitory interaction between its amino-terminal sequence and the PH domain (27). The amino-terminal region of proto-Dbl is an α-helix that binds directly to the DH domain, restricting the access to Rho GTPases (28). Furthermore, mammalian p115RhoGEF becomes activated upon Go_{13} binding to its amino-terminal domain, suggesting that this interaction may relieve the intrinsic constraint of the DH domain (29).

There are several possibilities as to how the HF motif-PH domain interaction could be affected. Phosphorylation events that modify either the HF motif or the PH domain may relieve the structural constraint of the HF motif-PH domain interaction. However, serine/threonine rather than tyrosine phosphorylation would be more likely to play a role in this effect, because no tyrosine phosphorylation was detected in the NDP region of hSos1. Likewise, another potential mechanism may involve the interaction of either the HF motif or the PH domain with some docking/scaffold complex proteins upon mitogenic cell stimulation. This occurs with Cdc24 (GEF in budding yeast), which needs to be recruited to the targeting site via interaction with Far1, a scaffolding protein that recognizes a conserved motif found in the amino-terminal region of Cdc24 (30). Finally, the phosphoinositol lipids may affect the HF motif-PH domain interaction. The SoS1 PH domain shows an ~5-fold higher affinity for phosphatidylinositol 3,4,5-trisphosphate than for phosphatidylinositol 4,5-diphosphate (31), and the specific phosphoinositol composition in a local environment could trigger conformational changes of the PH domain that may eventually reduce the HF-PH binding affinity. Some reports suggest that, irrespective of the subcellular location, the intrinsic Ras-GEF activity of Sos may be different before and after stimulation of surface tyrosine kinase receptors (13, 14). Our proposed model for HF-PH interactions may help to reconcile these two apparently contradictory views. Whether this model is applicable to other Ras exchange factors is currently being studied, as is the identification of the putative proteins that interact with the HF motif upon mitogenic stimulation.

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