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Functional Roles for the Pleckstrin and Dbl Homology Regions in the Ras Exchange Factor Son-of-sevenless*

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Activation of p21^{ras} by receptor tyrosine kinases is thought to result from recruitment of guanine nucleotide exchange factors such as Son-of-sevenless (Sos) to plasma membrane receptor substrates via adaptor proteins such as Grb2. This hypothesis was tested in the present studies by evaluating the ability of truncation and deletion mutants of *Drosophila* (d)Sos to enhance [^{32}P]GTP loading of p21^{ras} when expressed in ^{32}P-labeled COS or 293 cells. The dSos catalytic domain (residues 758-1125), expressed without the dSos NH$_2$-terminal (residues 1–757) or adaptor-binding, COOH-terminal (residues 1126–1596) regions, exhibits intrinsic exchange activity as evidenced by its rescue of mutant *Saccharomyces cerevisiae* deficient in endogenous GTP/GDP exchange activity. Here we show that this dSos catalytic domain fails to affect GTP·p21^{ras} levels when expressed in cultured mammalian cells unless the NH$_2$-terminal domain is also present. Surprisingly, the COOH-terminal, adaptor binding domain of dSos was not sufficient to confer p21^{ras} exchange activity to the Sos catalytic domain in these cells in the absence of the NH$_2$-terminal domain. This function of promoting catalytic domain activity could be localized by mutational analysis to the pleckstrin and Dbl homology sequences located just NH$_2$-terminal to the catalytic domain. The results demonstrate a functional role for these pleckstrin and Dbl domains within the dSos protein, and suggest the presence of unidentified cellular elements that interact with these domains and participate in the regulation of p21^{ras}.

Signal transmission by many receptor tyrosine kinases involves transient conversion of p21^{ras} proteins to the biologically active, GTP-bound state from the inactive GDP-bound form (1). When bound to GTP, p21^{ras} associates with Raf protein kinases, causing events that stimulate these and multiple other protein kinases, including the mitogen-activated protein kinases (2–4). These latter protein kinases catalyze phosphorylation of many cellular proteins, resulting in their regulation. The p21^{ras} signaling cascade modulates important cellular processes such as transcription and protein synthesis (5).

Activation of p21^{ras} proteins by receptor tyrosine kinases is thought to result from recruitment of guanine nucleotide exchange factors such as Son-of-sevenless (Sos) proteins to plasma membrane receptor substrates via adaptor proteins such as Grb2 (6–13). The mechanism of this recruitment seems to involve binding of the NH$_2$-terminal Src homology (SH)[1] 3 domain of the adaptor protein Grb2 with proline-rich motifs in the COOH terminus of Sos proteins (14, 15). Stimulation of tyrosine phosphorylation of membrane receptors or receptor substrates by growth factors provides appropriate tyrosine phosphate sites that bind the SH2 domain of Grb2. Rapid association of Grb2 (6, 11, 16) or Sos (6, 9, 17) proteins to complexes containing such tyrosine-phosphorylated species as EGF receptor, Shc, IRS-1, and Syp has been demonstrated. Furthermore, Sos protein mutants engineered to contain farnesylation or myristoylation signals exhibit plasma membrane localization and the ability to activate p21^{ras} when expressed in NIH 3T3 cells (18). Such Sos constructs in which the COOH-terminal, Grb2 binding domains were deleted displayed enhanced p21^{ras} exchange activity compared to full-length Sos (18). These data suggest that Grb2 binding to Sos proteins may have two functions: recruitment of Sos proteins to plasma membrane sites and removal of inhibition of the Sos COOH terminus on Sos catalytic activity.

More recent experiments yielded the surprising result that, in intact flies, a mutant *Drosophila* Sos protein lacking the adaptor binding COOH-terminal domain was at least as capable as full-length Sos protein in promoting R7 cell development (19). This effect is thought to result from effective modulation of the p21^{ras} signaling pathway during R cell development. Importantly, a protein encoding only the dSos catalytic domain was unable to mediate this response. The ability of the mutant dSos protein containing both NH$_2$-terminal and catalytic domains to promote R7 cell development required expression of an intact Sevenless protein (19). These data indicate that the COOH-terminal, adaptor binding domain of Sos is not sufficient to confer to p21^{ras} exchange activity to the Sos catalytic domain in intact flies. The present experiments were designed to test the role of NH$_2$-terminal and COOH-terminal regions of dSos in ^{32}P-labeled mammalian cells where p21^{ras} loading with [^{32}P]GTP can be measured directly.

MATERIALS AND METHODS

Construction of pCMV5-dSosHA Deletion Constructs—The dSos constructs engineered as described previously (19) were modified at their COOH terminus to encode a hemagglutinin (HA) epitope extension. The 9-amino acid peptide sequence Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala of the influenza HA, which is specifically recognized by the monoclonal antibody 12CA5 (20), was added to the 3′ end of the dSos constructs coding sequences. The dSos cDNA constructs encode the following amino acids: NCatCHA, 1–1596; NCatHA, 1–1125; NCatδPHHA, 1–475 and 592-1125; PHCatHA, 433-1125; CatHA 758-1125;

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[1] The abbreviations used are: SH, Src homology; HA, hemagglutinin; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; PH, pleckstrin homology; GST, glutathione *S*-transferase.

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$CatCO_2HA$, 1–53 and 681-1596; CO_2HA, 1–53, 681–690, and 1040–1596; NH_2HA, 1–690; NCatδ200HA, 1–311 and 681-1125; NCatδ300HA, 1–219 and 681-1125. The cDNA's encoding $p21^{ras}$, and the various dSos mutant constructs were subcloned into the mammalian expression vector pCMV5 (21). The $p21^{ras}$ cDNA was a gift from Dr. L. A. Feig.

Cell Lines and Transient Transfections—COS-1 cells (American Type Culture Collection RL 1650) were grown in DMEM medium containing 10% bovine calf serum, and 293 cells (American Type Culture Collection CRL 1573) were grown in DMEM medium containing 10% calf serum. Plates (6 cm) of COS-1 cells and 293 cells were transfected using the $CaPO_4$ method according to standard protocols. A total of 7 μg of plasmid DNA was used per plate.

Cell Labeling and $p21^{ras}$ Activation Assay—Two days after transfection, the cells were labeled with carrier-free [^{32}P]orthophosphate (1.0 mCi/ml) for 4 h at 37 °C in 1.5 ml of phosphate-free DMEM supplemented with 25 mM Hepes and 2 mM pyruvate. Analysis of labeled GTP and GDP bound to $p21^{ras}$ was performed as described (13, 22).

In Vitro Binding Assay—*Spodoptera frugiperda* Sf9 insect cells were cultured in Grace's complete medium and infection with high titer baculovirus stocks of either NCatCHA or NCatHA was carried out according to standard protocols. NCatCHA and NCatHA cDNA constructs were subcloned into the baculovirus transfer vector pVL1393 for expression in Sf9 cells. Frozen pellets of baculovirus-infected Sf9 cells (NCatCHA, NCatHA, and wild-type) were homogenized on ice using a 2-ml homogenizer (10 strokes) in 1 ml of cold lysis buffer (phosphate-buffered saline with 10 mM NaF, 1 mM dithiothreitol (DTT), 1 mM vanadate, 1 mM benzamidine, 1 mM phenylmethylsulfonyl chloride, and aprotinin, pepstatin, and leupeptin at 10 μg/ml each). Homogenates were spun in a microcentrifuge at 15,000 × *g* for 15 min at 4 °C. The supernatants were removed and assayed for total protein content using the Bradford method (23). Proteins from each lysate were dissolved in sample buffer, subjected to SDS-PAGE, and stained with Coomassie Brilliant Blue.

Equal amounts of protein from each lysate were incubated with 30 μl of glutathione-Sepharose, which was prebound with GST or GST-Grb2, on an end-over-end mixer at 4 °C for 2 h. GST and GST-Grb2 fusion plasmid were gifts from Dr. J. Schlessinger (New York University, New York, NY). The Sepharose was pelleted by centrifugation at 15,000 × *g* for 2 min at 4 °C. Pellets were washed twice with (20 mM Hepes, pH 7.5, 10% glycerol, 100 mM NaCl, and 1 mM DTT), twice with (20 mM Hepes, pH 7.5, 10% glycerol, 500 mM NaCl, and 1 mM DTT) and once with (20 mM Hepes, pH 7.5, 10% glycerol, 100 mM NaCl, and 1 mM DTT). Proteins remaining bound to the GST or GST-Grb2 were dissolved in SDS-PAGE sample buffer. Samples were loaded on SDS-PAGE gels (6%) and transferred to nitrocellulose filters. Filters were probed with anti-HA monoclonal antibody, and antibody-bound proteins were visualized using ECL (Amersham Corp.) according to the manufacturer's specifications.

RESULTS AND DISCUSSION

Drosophila Sos cDNA constructs were engineered to evaluate the functions of its three major domains. The catalytic domain, Cat, is defined as the region of homology to the *Saccharomyces cerevisiae* CDC25 protein, known to activate yeast Ras (24). The domain COOH-terminal to Cat containing SH3-binding motifs is denoted as C, and the region NH_2-terminal to Cat is denoted as N. Hemagglutinin epitope (HA)-tagged constructs corresponding to those depicted in Fig. 1 were prepared and ligated into the pCMV5 expression vector. COS-1 (Fig. 2) or 293 cells (not illustrated) were transiently transfected with these cDNA constructs and $p21^{ras}$ cDNA, labeled with ^{32}P for 4 h, lysed, and immunoprecipitated with anti-Ras monoclonal antibody. All of the HA-tagged Sos proteins encoded by the cDNA constructs were readily visualized by immunofluorescence microscopy when transiently expressed in either COS-1 or 293 cells (not illustrated). Furthermore, the heterologously expressed $p21^{ras}$ was visualized virtually exclusively at the cell surface by immunofluorescence microscopy (not illustrated).

Previous work showed that the catalytic domain of dSos (CatHA construct) is able to rescue *S. cerevisiae* with the $CDC25^{ts}$ mutation that inactivates endogenous Ras exchange activity (19). A similar Cat construct of human Sos produced in Sf9 cells was also shown to be fully effective in causing GTP/GDP exchange activity *in vitro* using Sf9 cell-derived K-$p21^{ras}$ protein (25). Importantly, CatHA had no significant stimulatory effect on [^{32}P]GTP binding to $p21^{ras}$ when expressed in intact COS-1 cells (Fig. 2, *lane 5*). Taken together, these previous results and the data in Fig. 2 indicate that the catalytic domain of dSos possesses intrinsic guanine nucleotide exchange activity but is unable to catalyze this reaction when this protein domain is overexpressed in mammalian cells.

The failure of CatHA to cause GTP loading of $p21^{ras}$ in intact cells suggested, as expected, that the Grb2 binding domain of dSos is necessary to carry out this function. However, a construct (CatCHA) containing both the catalytic and COOH-terminal domains of dSos also failed to cause $p21^{ras}$ activation when expressed in the COS cell system (Fig. 2, *lane 6*). Sur-

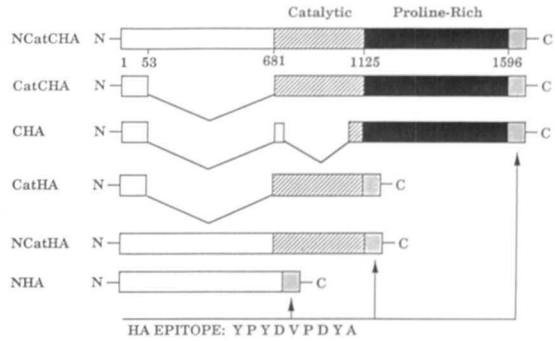

FIG. 1. **Schematic representation of the HA-tagged native and mutant Sos constructs employed in the experiment of Fig. 2.** The amino acid numbers are denoted beneath NCatCHA.

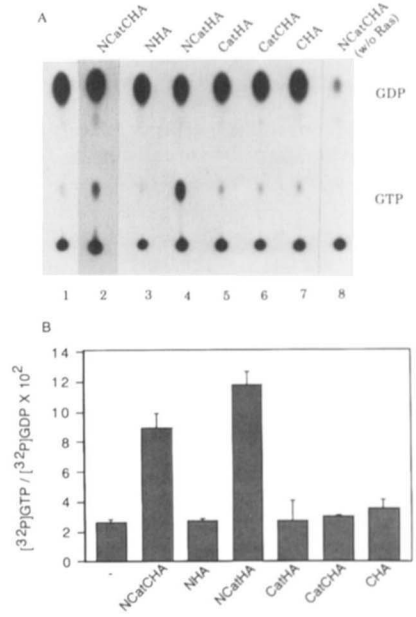

FIG. 2. **Stimulation of $p21^{ras}$ GTP/GDP exchange in intact cells by expression of recombinant dSos constructs NCatCHA or NCatHA.** *A*, COS-1 cells were transiently co-transfected with the $p21^{ras}$ (2 μg) and the HA-tagged dSos cDNAs (5 μg each) shown. [^{32}P]Orthophosphate-labeled cells were then lysed, and the cleared lysate was immunoprecipitated with anti-$p21^{ras}$ antibody as described (13, 22). The positions of the GTP and GDP standards are indicated. *Lane 1* represents cells that were transfected with $p21^{ras}$ only; *lane 8*, with NCatCHA only. *B*, graphic representation of $p21^{ras}$ activation by expression of NCatCHA or NCatHA in COS-1 cells. The GTP and GDP spots on the TLC plate were excised, and the amount of [^{32}P]orthophosphate incorporated was determined using a β counter. The ratio of GTP to GDP was calculated and expressed as a percentage, and the values shown represent the average of three individual experiments with *vertical bars* denoting standard errors. The *leftmost bar* on the graph represents cells that were transfected with $p21^{ras}$ only.

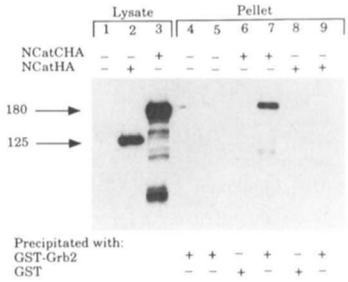

FIG. 3. **Association of recombinant NCatCHA but not NCatHA protein with GST-Grb2 fusion protein.** *Lanes 1–3* represent equal amounts of protein from total lysate of Sf9 cells infected with wild-type, NCatHA, and NCatCHA baculoviral stocks, respectively. The positions of NCatHA and NCatCHA are indicated. In *lanes 4–9*, equal amounts of sample were loaded, and proteins associating with GST or GST-Grb2 were detected by immunoblotting with anti-HA. The sample loaded in *lane 4* represents GST-Grb2 incubated with phosphate-buffered saline alone. The sample loaded in *lane 5* represents GST-Grb2 incubated with lysates from wild-type infected cells. Proteins remaining bound to the GST or GST-Grb2 were dissolved in SDS-PAGE sample buffer. Samples were loaded on SDS-PAGE gels (6%), and transferred to nitrocellulose filters. Filters were probed with anti-HA monoclonal antibody, and antibody-bound proteins were visualized using ECL (Amersham) according to the manufacturer's specifications.

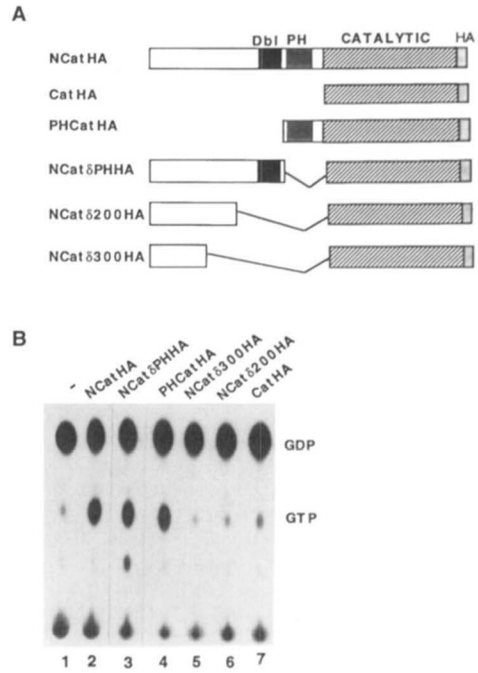

FIG. 4. **Modulation of p21^{ras} GTP/GDP exchange in intact cells by expression of truncation and deletion constructs of NCatHA.** *A*, schematic representation of the various dSos constructs which were tested for their ability to activate p21^{ras} in intact COS and 293 cells. *B*, human kidney 293 cells were transiently transfected via the $CaPO_4$-mediated method with p21^{ras} and the HA-tagged dSos cDNAs shown. Cells were labeled, and GDP and GTP associated with p21^{ras} was determined (13, 22).

prisingly, transfection of a cDNA construct (NCatHA) encoding the complete NH_2-terminal and catalytic domains of dSos caused [^{32}P]GTP loading of p21^{ras} that was even greater than that elicited by full-length dSos (Fig. 2, *lanes 2* and *4*). It should be noted that NCatHA may not actually be more active than full-length dSosHA because Western blotting with anti-HA antibody showed somewhat higher expression of the former (not illustrated). Expression of either the NH_2-terminal (NHA) or COOH-terminal (CHA) domains of dSos alone had no effect on cellular p21^{ras}·[^{32}P]GTP concentrations in COS-1 cells. No p21^{ras}-associated [^{32}P]GTP could be detected when p21^{ras} cDNA was omitted from the transfection (Fig. 2, *lane 8*). Similar data were obtained with this series of dSos constructs using 293 cells, although the magnitude of p21^{ras} activations by NCatCHA and NCatHA were greater than in COS-1 cells (not illustrated).

One explanation to account for the data in Fig. 2 is that Grb2 can also bind regions in the dSos protein that reside in the NH_2-terminal or catalytic domains. In order to test this possibility, HA-tagged NCat and HA-tagged full-length dSos proteins were expressed in the baculovirus-insect cell system, and assayed for their ability to associate with a GST-Grb2 fusion protein. As shown in Fig. 3 (*lanes 2* and *3*), lysates of Sf9 cells expressing these dSos proteins displayed immunoreactive bands at the expected mobilities when electrophoresed on SDS-PAGE and blotted with anti-HA monoclonal antibody. Wild-type Sf9 cell lysates failed to display any such immunoreactivity under similar conditions (Fig. 3, *lane 1*). Incubation of Sf9 cell lysates containing full-length dSos protein with GST-Grb2 fusion protein but not GST alone resulted in the adsorption of full-length dSos (Fig. 3, *lanes 6* and *7*), whereas NCatHA did not detectably associate with the GST-Grb2 fusion protein under these same conditions (Fig. 3, *lane 9*). These data indicate that the ability of NCatHA to cause p21^{ras} activation when expressed in intact COS-1 or 293 cells is not dependent upon its binding Grb2.

Taken together, the results in Figs. 2 and 3 suggest that structural elements within the NH_2-terminal domain of dSos are required in order for the exchange activity of Cat to act on p21^{ras} in intact cells. Truncation and deletion mutants of the HA-tagged NCat cDNA were therefore engineered as depicted schematically in Fig. 4*A*, and transfected into COS-1 and 293 cells with p21^{ras} cDNA. The design of these constructs focused on two regions in dSos previously identified to contain sequence similarities to domains found in pleckstrin (26) (amino acids 475–592 in dSos) and the Rho guanine nucleotide exchange factor Dbl (27) (amino acids 380–427 in dSos), respectively. As found using COS-1 cells (Fig. 2), CatHA expression in 293 cells (Fig. 4) failed to significantly enhance [^{32}P]GTP loading of p21^{ras} compared to transfection of p21^{ras} alone. Remarkably, PHCatHA, containing the pleckstrin homology (PH) domain NH_2-terminal to Cat, greatly stimulated cellular [^{32}P]GTP·p21^{ras} concentrations to levels approaching those observed in response to NCatHA (Fig. 4*B*, compare *lanes 2* and *4*). Immunofluorescence microscopy of the transfected 293 cells revealed similar levels of expression of all constructs depicted in Fig. 4, except for somewhat higher expression of NCatHA (not illustrated). Thus, PHCatHA may actually be as active as NCatHA in this system, when normalized for protein expression. In any case, these results document for the first time a functional role of the PH domain in a p21^{ras} exchange protein. Interestingly, an NCatHA construct with the PH domain deleted also effectively stimulated [^{32}P]GTP loading of p21^{ras} (Fig. 4*B*, *lane 3*). Ablation of exchange activity was observed, however, when both the PH and Dbl homology regions of NCat were deleted (Fig. 4*B*, *lanes 5* and *6*).

A simple hypothesis that explains these data is that either PH or Dbl homology regions of dSos are sufficient to allow the catalytic domain of dSos to interact with cellular p21^{ras} proteins. Thus, the absence of both of these regions in NCatHA abolishes activity, while the presence of either domain positioned NH_2-terminal to the Cat domain confers high activity. The results presented here lead us to postulate that interactions of the PH and Dbl homology regions with other elements in the cellular complexes that regulate p21^{ras} proteins are necessary for localizing the catalytic domain appropriately to operate in intact cells. The identities of the hypothetical elements that bind PH or Dbl regions of dSos are unknown but

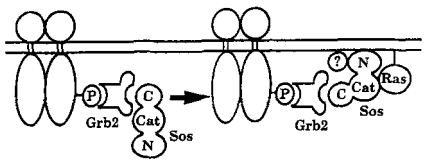

FIG. 5. **Model for the role of NH_2-terminal domains of dSos in facilitating GTP/GDP exchange on $p21^{ras}$ in intact cells.** Subsequent to the binding of Grb2·Sos complexes to tyrosine phosphate sites on receptor or receptor substrate proteins (*left*), association of PH or Dbl regions in the Sos NH_2 terminus to unknown components (?) hypothetically position Sos to activate $p21^{ras}$. Alternatively, Sos·Grb2 complexes may be anchored to the membrane through binding of NH_2-terminal sequences prior to receptor activation and binding of Grb2 to tyrosine phosphates (not shown).

could be known proteins in $p21^{ras}$-activating complexes or as yet unidentified cellular components. It should be noted that our results were derived from complex intact cell systems transiently overexpressing $p21^{ras}$ and the Sos constructs. Much larger increases in GTP loading of $p21^{ras}$ are achieved under these conditions compared to growth factor stimulation. Thus, it is not possible to draw conclusions related to possible regulation of these hypothetical PH- or Dbl-binding components in growth factor-mediated $p21^{ras}$ activation.

At last count, 71 proteins have been reported to contain sequences with similarity to the PH domains of pleckstrin (28), but the presumed cellular partner or partners that associate with these domains have remained elusive. Suggestions that $\beta\gamma$ subunits of trimeric G proteins (29) and phosphatidylinositol derivatives (30) bind PH domains have not yet been confirmed in intact cells. That the PH domain of Bruton tyrosine kinase binds protein kinase C has been reported recently (31), but not yet extended to other PH domains. A three-dimensional structure for the PH domain of the B-spectrin protein has been recently reported, indicating some similarity to retinol binding motifs (32), but retinol does not bind this structure.

The Dbl homology region of dSos represents only a small portion of the regions in CDC24 (27, 33), Vav (27), and Dbl (27) that appear to be required to catalyze GTP loading of Rho-like GTP-binding proteins. The degree of sequence similarity between the Dbl homology region in dSos and the corresponding regions of these Rho exchangers is somewhat low, but compelling. Thus, Sos proteins do not catalyze Rho exchange activity, but might bind such GTP-binding proteins. This hypothesis deserves testing in future experiments.

The key result reported here, that the Grb2 binding region of dSos is not sufficient to allow Cat function in COS-1 or 293 cells, is not necessarily inconsistent with the recent data and interpretation of Aronheim *et al.* (18) that implicates a critical role for Grb2 in recruiting Sos proteins to receptor complexes. However, our data reveal an additional requirement for the NH_2-terminal domain of dSos in $p21^{ras}$ regulation, which may function in conjunction with or subsequent to the association of dSos·Grb2 complexes to tyrosine phosphates within membrane complexes (Fig. 5). Consistent with Aronheim *et al.* (18), we find evidence for an inhibitory effect of the dSos COOH terminus on catalytic activity in that CatCHA is unable to activate $p21^{ras}$ (Fig. 2, *lane 6*). Perhaps the function of the PH or Dbl regions of dSos is to bind membrane components that position dSos to better interact with membrane-bound $p21^{ras}$, while Grb2 plays a role in relieving an inhibitory influence of the Sos COOH terminus (Fig. 5). Strong support of these concepts are provided by Karlovich *et al.* (19) using genetic complementation, showing a dominant inhibitory function of CatC and physiological activity of NCat in intact flies. Wang *et al.* (34) have also recently found enhanced transforming activity of mutant mSos proteins with COOH-terminal truncations. The striking similarity of results obtained by these independent methodologies strongly reinforce the concept that regions within the NH_2-terminal region of Sos proteins are necessary for their physiological function.

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