

Interaction of Shc with Grb2 Regulates Association of Grb2 with mSOS

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Received 15 August 1994/Returned for modification 18 October 1994/Accepted 1 November 1994

The adapter protein Shc has been implicated in Ras signaling via many receptors, including the T-cell antigen receptor (TCR), B-cell antigen receptor, interleukin-2 receptor, interleukin-3 receptor, erythropoietin receptor, and insulin receptor. Moreover, transformation via polyomavirus middle T antigen is dependent on its interaction with Shc and Shc tyrosine phosphorylation. One of the mechanisms of TCR-mediated, tyrosine kinase-dependent Ras activation involves the simultaneous interaction of phosphorylated Shc with the TCR ζ chain and with a second adapter protein, Grb2. Grb2, in turn, interacts with the Ras guanine nucleotide exchange factor mSOS, thereby leading to Ras activation. Although it has been reported that in fibroblasts Grb2 and mSOS constitutively associate with each other and that growth factor stimulation does not alter the levels of Grb2:mSOS association, we show here that TCR stimulation leads to a significant increase in the levels of Grb2 associated with mSOS. This enhanced Grb2:mSOS association, which occurs through an SH3-proline-rich sequence interaction, is regulated through the SH2 domain of Grb2. The following observations support a role for Shc in regulating the Grb2:mSOS association: (i) a phosphopeptide corresponding to the sequence surrounding Tyr-317 of Shc, which displaces Shc from Grb2, abolished the enhanced association between Grb2 and mSOS; and (ii) addition of phosphorylated Shc to unactivated T cell lysates was sufficient to enhance the interaction of Grb2 with mSOS. Furthermore, using fusion proteins encoding different domains of Shc, we show that the collagen homology domain of Shc (which includes the Tyr-317 site) can mediate this effect. Thus, the Shc-mediated regulation of Grb2:mSOS association may provide a means for controlling the extent of Ras activation following receptor stimulation.

Stimulation of growth factor receptors, T-cell or B-cell antigen receptors, and many cytokine receptors activates p21^{ras} proteins (8). The conversion of Ras from its inactive GDP-bound state to its active GTP-bound state, which occurs downstream of tyrosine kinases, involves several intracellular signaling proteins (30). The adapter protein Grb2, either alone or with a second adapter protein, Shc, appears to be involved in Ras activation via many receptors. Adapter proteins have no apparent catalytic domain but contain one or more Src homology 2 (SH2) and SH3 domains (19) and mediate protein-protein interactions. It has been demonstrated that SH2 domains bind to specific phosphotyrosine-containing sequences whereas SH3 domains bind to proline-rich sequences (26, 33). Grb2 is composed of one SH2 domain and two SH3 domains (17). During epidermal growth factor (EGF) signaling, Grb2 via its SH2 domain interacts directly with the autophosphorylated EGF receptor and via its SH3 domains associates with the Ras GTP/GDP exchange factor mSOS. Thus, the simultaneous interaction of Grb2 with the receptor and mSOS helps to shuttle the exchange factor to the membrane and leads to Ras activation (2, 10, 11, 15, 27).

Several receptors (usually those which do not interact directly with Grb2) use a second adapter protein, Shc, which in turn interacts with Grb2. Shc is composed of a single SH2 domain, a glycine/proline-rich collagen homology (CH) domain, and a unique amino-terminal domain (designated N) but

no apparent catalytic domain (20). Shc exists in three isoforms of 48, 52, and 65 kDa (only the former two are expressed in hematopoietic cells). Shc was first shown to function upstream of Ras in PC12 cells; the overexpression of Shc led to neurite outgrowth, and this response was blocked by dominant negative inhibitors of Ras proteins (28). Subsequently, it has been shown that Shc is involved in Ras activation by a number of receptors which are themselves tyrosine kinases (such as insulin receptor, EGF receptor, and nerve growth factor receptor [22, 32]) as well as receptors which activate nonreceptor tyrosine kinases (such as the T-cell receptor [25] and the receptors for interleukin-2 [23], interleukin-3, granulocyte-macrophage colony-stimulating factor, and erythropoietin [6, 7]). Shc is phosphorylated on tyrosine upon stimulation of these receptors; it subsequently interacts with Grb2 (via the Grb2 SH2 domain) (28), and Grb2, in turn, interacts with mSOS. The Shc:Grb2:mSOS complex that forms upon EGF or insulin signaling has *in vitro* nucleotide exchange activity toward Ras, suggesting that a similar complex formed *in vivo* can lead to Ras activation (22). Shc is also a good substrate for Src family kinases, and in v-Src- and v-Fps-expressing cells, Shc is constitutively tyrosine phosphorylated and forms a complex with Grb2 (18, 28).

We have shown that one of the mechanisms of T-cell receptor (TCR)-mediated Ras activation (3, 9, 13, 25, 31) involves tyrosine phosphorylation of Shc. Tyrosine-phosphorylated Shc subsequently interacts with Grb2 and, in turn, mSOS. Shc also interacts via its own SH2 domain with the tyrosine-phosphorylated TCR ζ chain (25). Thus, the simultaneous interaction of Shc with the TCR, and with Grb2 and mSOS, may help to

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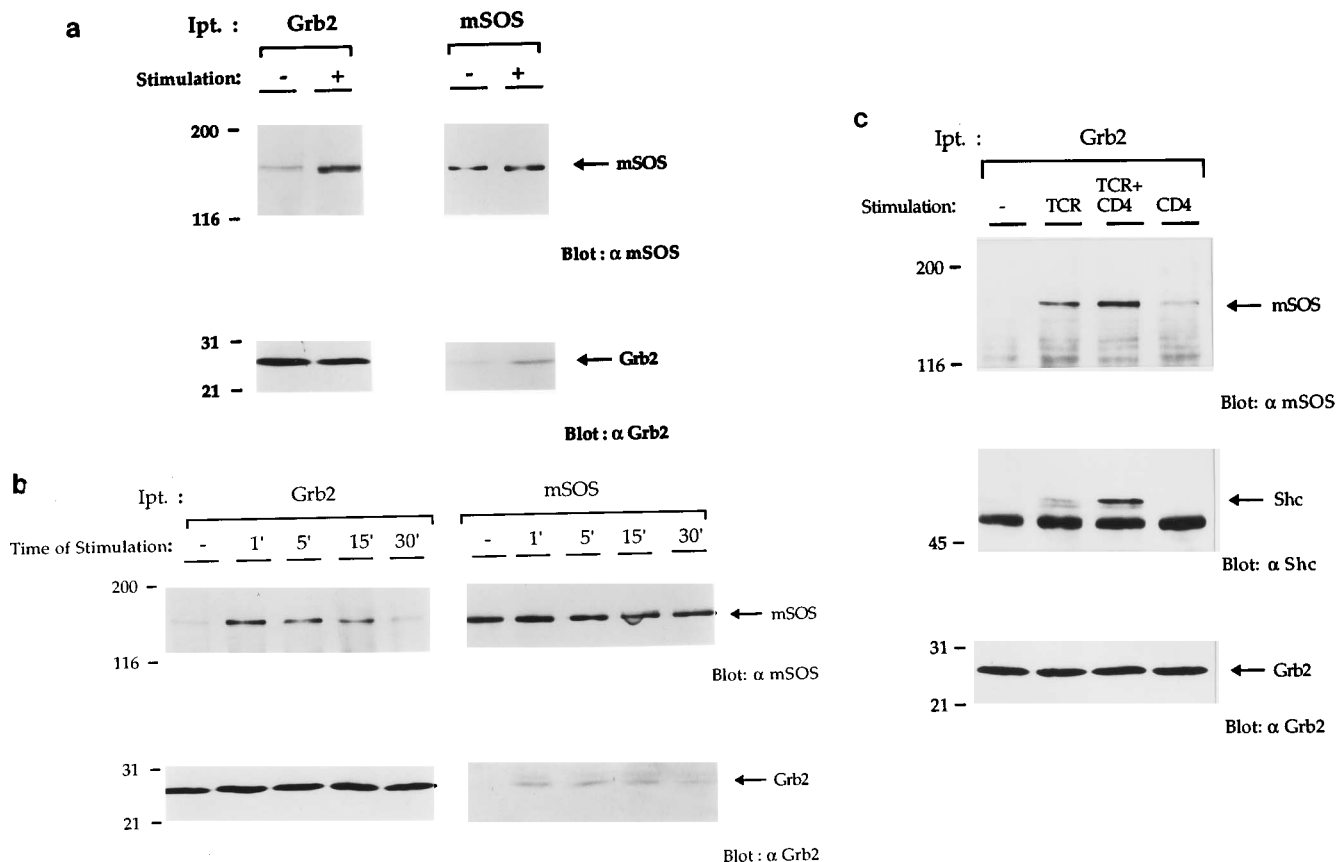


FIG. 1. Enhancement of Grb2 association with mSOS upon T-cell activation. BYDP hybridoma cells were stimulated at 37°C for 2 min (a) or the indicated times (in minutes) (b) by cross-linking with anti-Grb2 and anti-mSOS antibodies, immunoprecipitated (Ipt.) with Grb2 or mSOS, and immunoblotted for the levels of Grb2 and mSOS. (c) BYDP cells were stimulated for 2 min with either TCR and CD4, or CD4 alone, immunoprecipitated with Grb2, and immunoblotted for the levels of coprecipitating mSOS and Shc. Rough quantitation of Grb2 and mSOS levels from multiple experiments indicated a three- to fivefold increase in Grb2:mSOS association after T-cell activation. Sizes are indicated in kilodaltons.

shuttle the Ras nucleotide exchange factor to the membrane, thereby leading to Ras activation.

Unlike in fibroblasts, in which Grb2 and mSOS seem to exist constitutively in a complex at all times (10, 11, 15, 27), we report here that in T cells, there is a significant increase in the levels of Grb2:mSOS association following TCR activation. We demonstrate that this enhanced association of Grb2 with mSOS is regulated via the SH2 domain of Grb2, for the interaction of phosphorylated Shc with the Grb2 SH2 domain enhances the Grb2:mSOS association. Fusion proteins encoding different domains of Shc indicate that the CH domain of Shc, which contains Tyr-317, is sufficient to mediate this effect.

MATERIALS AND METHODS

Cells. The murine T-cell hybridoma BYDP has been described previously (24) and was grown in RPMI 1640 complete medium supplemented with 10% fetal calf serum. Human peripheral blood lymphocytes (PBLs) were isolated by using standard techniques. The mononuclear cells were isolated by Ficoll-Hypaque purification. The adherent cells were removed by incubation on plastic at 37°C for 1 h. The B cells were removed by passage over a nylon wool column. The cells recovered from the column were washed, counted, and stimulated as described below.

T-cell stimulations. BYDP cells (3×10^7 /ml) were incubated with anti-TCR antibody (F23.1; 1 μ g/ml) and/or anti-CD4 antibody (OKT4D; 1 μ g/ml) for 10 min on ice. Rabbit anti-mouse immunoglobulin G (10 μ g/ml) was added for cross-linking, and the cells were incubated for a further 10 min on ice and then incubated at 37°C for the indicated times. After a pulse spin and a quick wash with serum-free medium, cells were lysed (lysis buffer contained 1% Nonidet P-40, 50 mM Tris [pH 7.6], 150 mM NaCl, 1 mM Na_3VO_4 , 10 mM NaF, 10 μ g

each of leupeptin and aprotinin per ml, and 2 mM phenylmethylsulfonyl fluoride). Similar results have been obtained with the anti-CD3 antibody 145-2C11. Human PBLs were stimulated with anti-CD3 antibody (OKT3; 1 μ g/ml) and/or anti-CD4 antibody (OKT4D; 1 μ g/ml).

Immunoprecipitations and immunoblotting. The lysates were immunoprecipitated with polyclonal anti-Grb2 (Santa Cruz Biotechnology, Santa Cruz, Calif.; used at 300 ng/15 $\times 10^6$ cell lysates) or anti-mSOS (Upstate Biotechnology Inc., Lake Placid, N.Y., or Transduction Laboratories, Lexington, Ky.; used at 3 μ g/20 $\times 10^6$ cell lysates) and 40 μ l of 50% protein A-Sepharose solution (Pharmacia) for 2 h at 4°C. The beads were washed four times in a mixture of 0.1% Nonidet P-40, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.4), 150 mM NaCl, 1 mM Na_3VO_4 , 5 mM NaF, and 10 μ g each of leupeptin and aprotinin per ml, analyzed on sodium dodecyl sulfate (SDS)-6 to 12% polyacrylamide gels, transferred to nitrocellulose, immunoblotted with the anti-Grb2 monoclonal antibody (Transduction Laboratories) or anti-mSOS antibody (specific for mSOS1; Upstate Biotechnology), and developed by enhanced chemiluminescence (Amersham). Polyclonal anti-Shc antibody (Transduction Laboratories) was used in immunoprecipitations, and monoclonal anti-Shc antibody (Transduction Laboratories) was used in immunoblotting.

Peptides and inhibitions. Tyrosine-phosphorylated 9-mer Shc peptide (PSPYVNVQNL) and a control peptide (PSPYVAVQNL) were synthesized as described previously (21). The sequence of the 16-mer Shc peptide was ELFD-DPSpYVNVQNLDK. The Shc peptide bound with the same affinity to the isolated Grb2 SH2 domain as did the full-length protein ($K_D \approx 1 \times 10^{-7}$ to 5×10^{-7} M), while the control peptide had no detectable binding ($K_D > 10^{-4}$ M) (34). Unstimulated or stimulated BYDP cell lysates (15×10^6) were immunoprecipitated with anti-Shc, anti-Grb2, or anti-mSOS in the presence of 30 μ M Shc peptide or the control peptide and immunoblotted for the levels of Shc, Grb2, or mSOS. Testing a range of peptide concentrations showed that inhibitory effects could be observed from 5 to 10 μ M (25a).

The sequence of the proline-rich mSOS peptide used was GTDEVPPVPPVPPRRRPEA, and the sequence of the control peptide was PVPPRRRPEA. BYDP lysates (15×10^6 cells) were immunoprecipitated with anti-Grb2, washed,

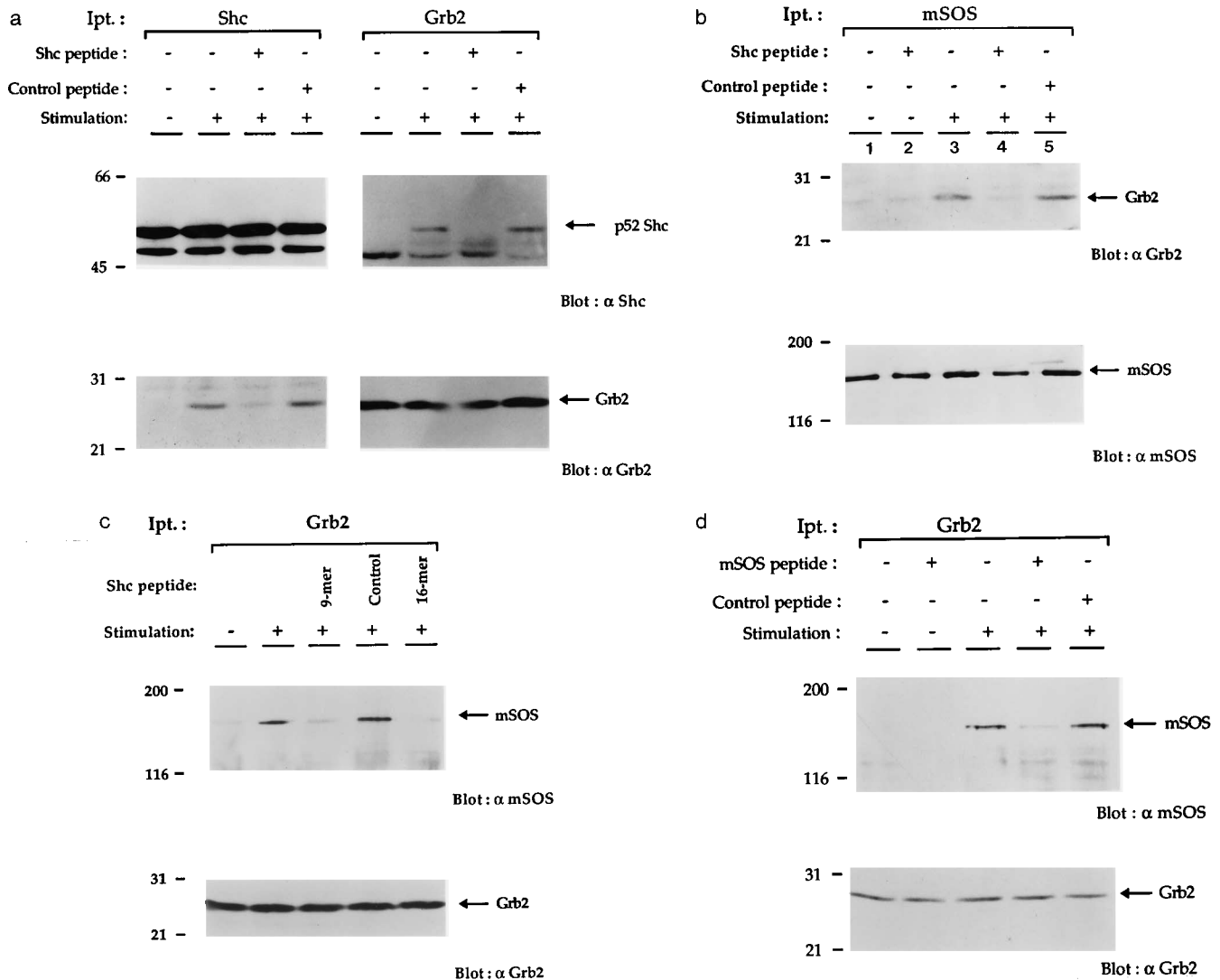


FIG. 2. Influence of Shc peptide on Grb2:mSOS association. (a) BYDP cells (15×10^6) were stimulated as for Fig. 1a, immunoprecipitated (Ipt.) with anti-Shc or anti-Grb2 in the presence of $30 \mu\text{M}$ Shc peptide or the control peptide, and immunoblotted for the levels of coprecipitating Shc or Grb2. The peptides had no effect on Shc phosphorylation as determined by antiphosphotyrosine blotting of Shc immunoprecipitates (25a). (b) Stimulated or unstimulated lysates (20×10^6 cell equivalents) were immunoprecipitated with anti-mSOS in the presence of Shc peptide or control peptide ($30 \mu\text{M}$), and the levels of coprecipitating Grb2 were assessed by immunoblotting. (c) BYDP cells (15×10^6) were stimulated and after lysis immunoprecipitated with anti-Grb2 in the presence of 9-mer or 16-mer Shc peptide or control peptide ($30 \mu\text{M}$). The levels of mSOS in these precipitates were assessed by immunoblotting. (d) Influence of proline-rich mSOS peptide on enhanced Grb2:mSOS association. BYDP cell lysates (15×10^6) were immunoprecipitated with anti-Grb2 and protein A-Sepharose beads. After washing, the beads were incubated with a proline-rich peptide from mSOS or control peptide (1 mM) and washed again, and the levels of mSOS still bound to the beads were assessed by immunoblotting. Sizes are indicated in kilodaltons.

incubated with the mSOS peptide or the control peptide (1 mM) for 1 h on ice, washed once, and analyzed for the level of mSOS still bound to Grb2.

GST-Shc fusion proteins and in vitro phosphorylation. Glutathione *S*-transferase (GST)-Shc fusion proteins were generated by PCR using primers 5' and 3' of the desired regions on Shc cDNA. The DNA fragments were cloned into pGEX2T (Pharmacia) and expressed in *Escherichia coli*. The fusion proteins encoded the Shc sequence as follows: CH, amino acids (aa) 228 to 386; N+CH, aa 1 to 386; CH+SH2, aa 200 to 471; and SH2, aa 375 to 471. The construct containing mutated N and CH domains (mtN+CH; aa 1 to 386) was generated by PCR using a Shc cDNA carrying a Tyr-317-to-Phe point mutation (kindly provided by P. G. Pelicci, Perugia, Italy). The fusion proteins were expressed in *E. coli* and induced with 0.2 mM isopropylthiogalactopyranoside (IPTG) for 4 to 6 h. After cell lysis (with a buffer containing 50 mM Tris, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mg of lysozyme per ml, and 10 μg each of pepstatin, leupeptin, and aprotinin per ml), the fusion proteins were recovered by using glutathione-agarose beads.

GST-Shc fusion proteins (10 μg) bound to glutathione beads were phosphor-

ylated with purified p56^{lck} (Upstate Biotechnology; used at 5 $\mu\text{l}/100 \mu\text{l}$ of reaction buffer) in a buffer containing 10 mM MnCl₂, 5 mM HEPES (pH 7.4), 100 μM ATP, and 10 μg each of leupeptin and aprotinin per ml for 4 h at 30°C. After washing, the beads were incubated with unactivated T-cell lysates (15×10^6 cells) for 2 h at 4°C, washed, resolved by SDS-6 to 12% polyacrylamide gel electrophoresis and immunoblotted with anti-Grb2, anti-mSOS, or antiphosphotyrosine (RC20H; Transduction Laboratories). Unphosphorylated or phosphorylated GST-CH domain fusion proteins (GST-CH) were eluted from the glutathione beads (elution buffer, pH 7.4, containing 75 mM HEPES, 150 mM NaCl, 50 mM reduced glutathione, 5 mM dithiothreitol, 1 mM EDTA, 0.5% Nonidet P-40, 0.02% SDS, and 10 μg each of leupeptin and aprotinin per ml; eluted twice for 15 min each time on ice) and incubated with unactivated T-cell lysates (15×10^6 cells) in the presence of the Shc peptide or control peptide ($30 \mu\text{M}$). When more than one condition was tested in an experiment, the phosphorylation and elution were performed in bulk and the samples were split just before use.

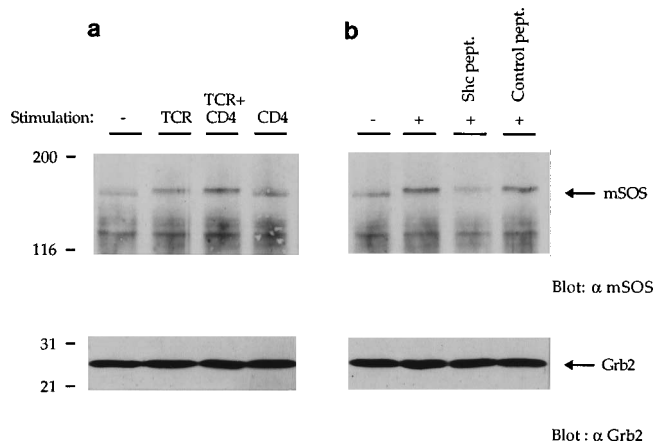


FIG. 3. Enhanced Grb2:mSOS association upon T-cell activation in human PBLs. PBLs were stimulated with anti-TCR and/or anti-CD4 antibody, immunoprecipitated with Grb2, and immunoblotted with anti-mSOS (a). Unactivated (-) or TCR-plus-CD4-activated (+) T-cell lysates were incubated with Shc peptide or control peptide (30 μ M) and assessed for the level of mSOS precipitated with Grb2 (b). Sizes are indicated in kilodaltons.

RESULTS

Enhanced association between Grb2 and mSOS upon T-cell activation. A murine T-cell hybridoma was activated by antibody-mediated cross-linking of TCR and CD4, and the level of Grb2 associated with mSOS was examined. There was a significant increase in the amount of mSOS precipitated by anti-Grb2 antibody from activated T-cell lysates compared with unactivated lysates (Fig. 1a). Similarly, increased amounts of Grb2 were coprecipitated with mSOS after T-cell activation. Rough quantitation of the bands indicated a three- to fivefold increase in the Grb2:mSOS association compared with the basal levels seen in unstimulated cells. This finding suggested that unlike in fibroblasts, in which growth factor stimulation does not alter the levels of the Grb2:mSOS complex (10, 11, 15, 27), T-cell stimulation leads to a significant increase in the levels of Grb2:mSOS association. It was evident from a time course of activation that the enhanced association was a regulated phenomenon. The increased Grb2:mSOS interaction was observed as early as 1 min and returned to nearly basal levels by 30 min (Fig. 1b). Similar enhanced Grb2:mSOS association was also observed in the human Jurkat T-cell line upon TCR stimulation (25a).

A Shc phosphopeptide inhibits the enhanced Grb2:mSOS association. The level of mSOS associated with Grb2 was greater when TCR and CD4 were cross-linked together compared with cross-linking of the TCR alone (Fig. 1c). We have previously shown that compared with TCR cross-linking alone, cross-linking of the TCR and CD4 together leads to greater levels of overall tyrosine phosphorylation (including enhanced Shc tyrosine phosphorylation) as well as increased levels of the Shc:Grb2 complex (as seen by anti-Shc immunoblotting in Fig. 1c) (24, 25). This finding suggested that the enhanced Grb2:mSOS association may be regulated via the interaction of tyrosine-phosphorylated proteins with the SH2 domain of Grb2. Moreover, when the kinetics of activation were slowed by stimulating cells at 30°C, we observed in Shc immunoprecipitates that maximal levels of Grb2 were already found in a complex with Shc by 5 min, while the peak level of mSOS in the complex was observed only at 15 min after stimulation (25a). This finding indicated that the Shc-Grb2 complex was formed prior to the Shc:Grb2:mSOS complex and that the interaction of Shc

with Grb2 may regulate the affinity of Grb2 for mSOS. Since the SH2 domain of Grb2 interacts with tyrosine-phosphorylated Shc (28), we synthesized a 9-aa phosphopeptide corresponding to the site on Shc (aa 315 to 323 with the sequence PSpYVNVQNL, termed Shc peptide) that has been shown to bind the Grb2 SH2 domain (29, 32, 33). A control peptide in which the critical asparagine was changed to alanine (PSPYVAVQNL), which does not bind to the Grb2 SH2 domain (34), was also synthesized.

The Shc peptide, when added during immunoprecipitation, was capable of dissociating the interaction between Grb2 and phosphorylated Shc (which occurs upon T-cell activation), while the control peptide had no effect (Fig. 2a). The addition of the peptide had no effect on the level of Shc phosphorylation, and besides the loss of Grb2, it also resulted in the loss of mSOS from Shc immunoprecipitates, suggesting that the mSOS interaction with Shc occurs through Grb2 (25a). We then determined whether the Shc peptide could influence the association between Grb2 and mSOS. mSOS was immunoprecipitated from activated or unactivated T-cell lysates in the presence of the Shc or control peptide, and the amount of coprecipitating Grb2 was assessed by immunoblotting (Fig. 2b). The addition of the Shc peptide reduced the amount of Grb2 associated with mSOS in activated T-cell lysates to the levels seen in unactivated lysates, while the control peptide had no effect. It is noteworthy that the Shc peptide alone, when added to unstimulated lysates, had no effect on Grb2:mSOS association (Fig. 2b, lane 2). Since this peptide can interact with the Grb2 SH2 domain, the simple occupancy of the Grb2 SH2 domain is insufficient to regulate its association with mSOS.

Similar results were obtained when Grb2 was immunoprecipitated from activated lysates in the presence of the Shc peptide and assessed for coprecipitating mSOS (Fig. 2c). A longer Shc peptide of 16 aa gave results similar to those for the 9-mer Shc peptide. Thus, upon T-cell activation, interactions of the Grb2 SH2 domain regulate the Grb2:mSOS association. Although regulated via the Grb2 SH2 domain, the enhanced association between Grb2 and mSOS still occurred via the SH3 domains of Grb2 (Fig. 2d). A proline-rich peptide, corresponding to the mSOS sequence (which binds to the Grb2 SH3 domain) (10, 15, 27), was able to inhibit the enhanced association between Grb2 and mSOS, while a control peptide lacking the critical prolines had no effect.

To rule out the possibility that the enhanced Grb2:mSOS association is a phenomenon seen only in transformed T cells, we stimulated freshly isolated human PBLs with anti-TCR and/or anti-CD4 antibodies and examined the levels of mSOS association with Grb2 (Fig. 3). Again, there was increased binding of Grb2 and mSOS upon TCR stimulation which was further enhanced by cross-linking of CD4 with the TCR. Similar to the results obtained with the T-cell hybridoma, the enhanced association seen in PBLs was also inhibited by the Shc peptide.

Phosphorylated CH domain of Shc is sufficient to mediate the enhanced Grb2:mSOS association. To directly address a role for Shc in regulating Grb2:mSOS association and to determine if a specific domain of Shc mediates this effect, we generated GST fusion proteins encoding different domains of Shc (Fig. 4a). Shc is composed of a unique N domain, a glycine/proline-rich CH domain, and a single SH2 domain (20). The Tyr-317 of Shc is located in the CH domain of Shc. Fusion proteins encoding the N and CH domains, the CH domain alone, the CH and SH2 domains, or the SH2 domain alone were generated. These fusion proteins, bound to glutathione beads, were first tyrosine phosphorylated *in vitro*, using the

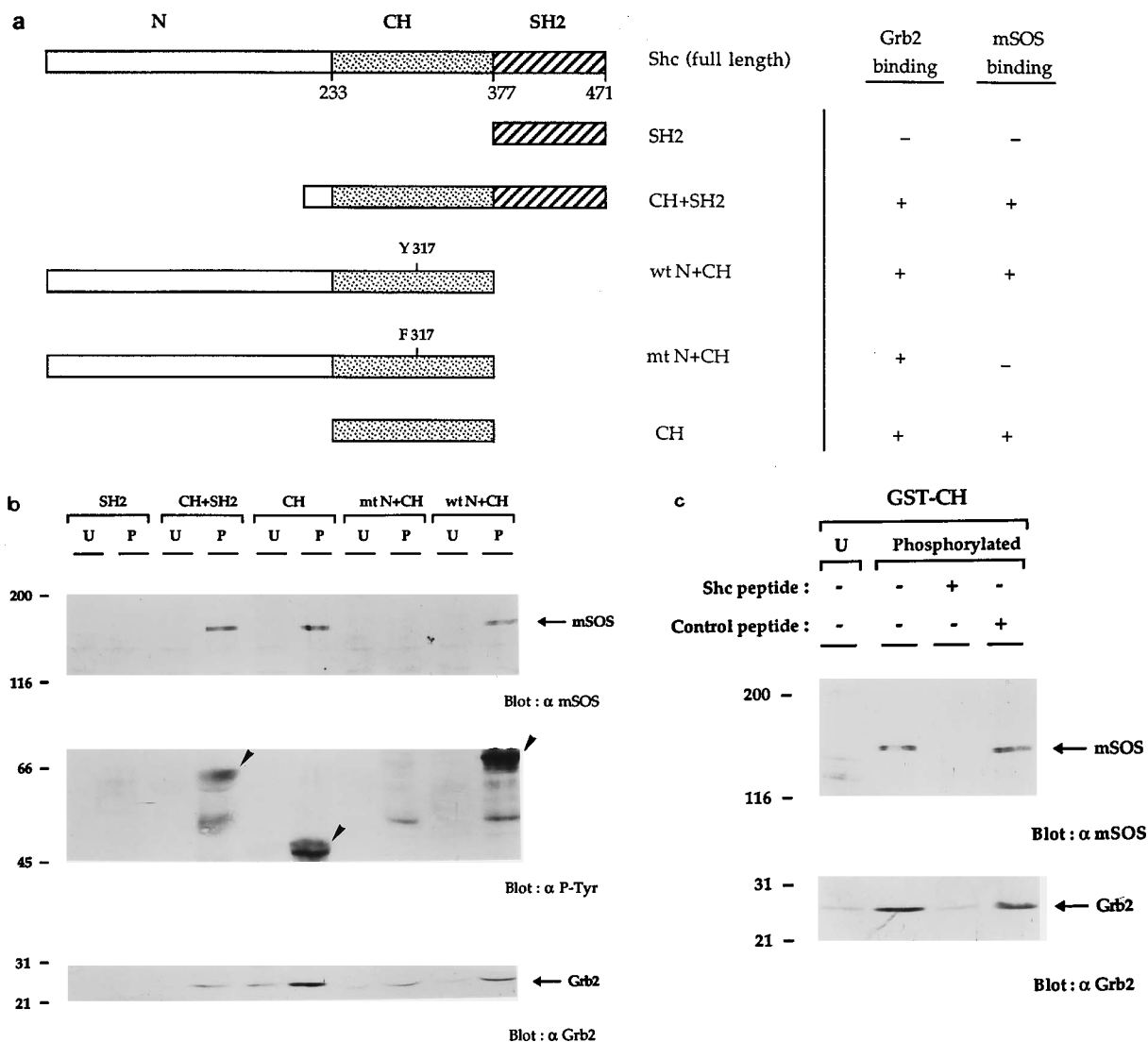


FIG. 4. Interaction of in vitro-phosphorylated GST-Shc fusion proteins with Grb2 and mSOS. (a) Schematic representation of different GST-Shc fusion proteins. wt, wild type; mt, mutant. (b) Unphosphorylated (lanes U) and in vitro Lck-phosphorylated (lanes P) GST-Shc fusion proteins bound to glutathione-agarose beads were incubated with unactivated T-cell lysates, washed, and assessed for the binding of Grb2 and mSOS. Arrowheads in panel b indicate the tyrosine-phosphorylated fusion proteins. P-Tyr, phosphotyrosine. (c) GST-CH fusion proteins bound to glutathione beads were incubated with unactivated T-cell lysates in the presence of Shc peptide or control peptide and assessed for their interactions with Grb2 and mSOS. Sizes are indicated in kilodaltons.

T-cell-specific Src family tyrosine kinase p56^{lck}. Subsequently the beads were incubated with lysates from unactivated T cells and assessed for their interaction with Grb2 and, in turn, mSOS (Fig. 4b). The CH domain alone (containing Tyr-317), when phosphorylated, was sufficient to bind Grb2 and, in turn, mSOS. Phosphorylated N+CH and CH+SH2 fusion proteins also gave similar results, while fusion proteins that did not encode the CH domain (such as the Shc-SH2 fusion) did not bind Grb2. The addition of Shc peptide inhibited the interaction of Grb2 with phosphorylated CH domain, confirming that the association occurred via the Grb2 SH2 domain (Fig. 4c). The phosphorylated N+CH fusion protein carrying a Tyr-317-to-Phe mutation weakly interacted with Grb2 (possibly through Tyr-240, which carries a less than optimal Grb2 SH2 domain binding site [33]) but did not precipitate any detectable mSOS, suggesting that binding of Grb2 to Shc Tyr-317 plays an important role in regulating the Grb2:mSOS association.

We then assessed whether soluble phosphorylated GST-CH (eluted from beads with glutathione) when added to unactivated T-cell lysates could enhance the association between Grb2 and mSOS. The addition of phosphorylated GST-CH led to a significant increase in the amount of Grb2 precipitated with mSOS, while the addition of unphosphorylated GST-CH had no effect (Fig. 5a). That phosphorylated Shc mediates this enhancement was demonstrated by two observations: (i) the coprecipitation of the 45-kDa phosphorylated GST-CH with mSOS (assessed by antiphosphotyrosine immunoblotting; Fig. 5a, middle panel) and (ii) the loss of Grb2 (correlating with the loss of GST-CH) in mSOS immunoprecipitates after addition of the Shc peptide, but not the control peptide (Fig. 5a). Taken together, these data indicate that the interaction of the phosphorylated CH domain with the Grb2 SH2 domain is sufficient to regulate the association between Grb2 and mSOS.

We and others have observed an unidentified 140-kDa ty-

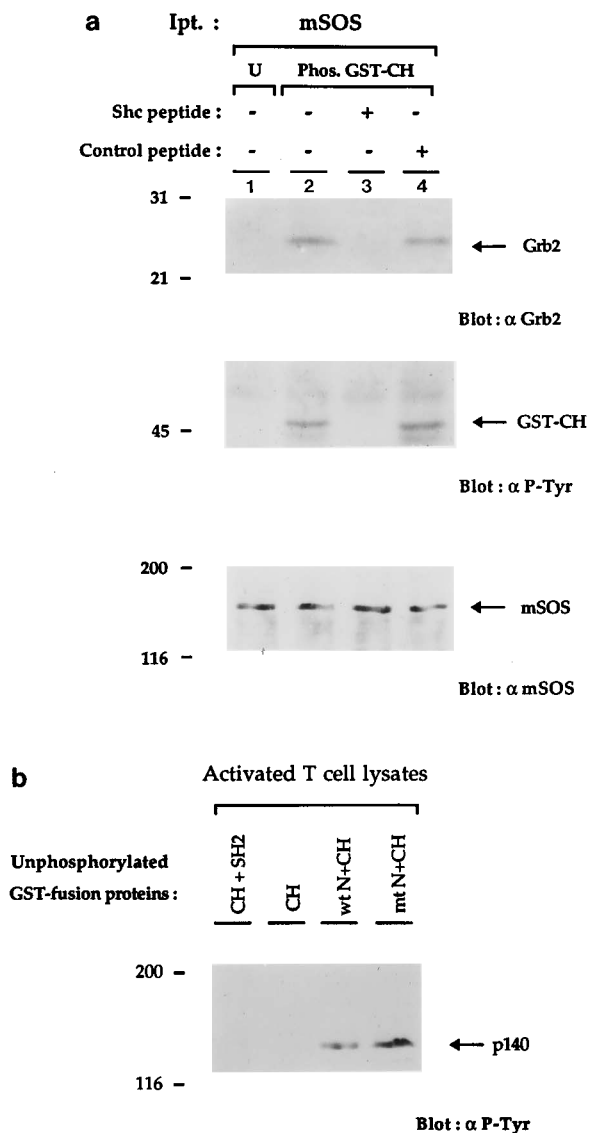


FIG. 5. Addition of phosphorylated GST-CH is sufficient to enhance the Grb2:mSOS association. (a) Unphosphorylated or in vitro-phosphorylated GST-CH fusion proteins (eluted from the glutathione beads by using glutathione) were added to unactivated T-cell lysates. mSOS was immunoprecipitated (Ipt.) in the presence of Shc peptide or control peptide, and the level of coprecipitating Grb2 was assessed by immunoblotting. Antiphosphotyrosine (α P-Tyr) blotting revealed the presence of GST-CH (45 kDa) only in lanes 2 and 4 (middle panel). The bottom panel shows the level of mSOS precipitated in each lane. (b) Activated T-cell lysates (1.5×10^7 cells) were incubated with different unphosphorylated GST-Shc fusion proteins, and the presence of the 140-kDa protein was analyzed by antiphosphotyrosine immunoblotting. The slightly higher level of p140 seen in mtN+CH is not a reproducible result. wt, wild type; mt, mutant.

rosine-phosphorylated protein (p140) in Shc immunoprecipitates (7, 25). To examine if p140 plays a role in regulation of Grb2:mSOS association, we assessed the interaction of p140 with different domains of Shc. Incubation of lysates from activated T cells with unphosphorylated fusion proteins showed that the N and CH domains (both the wild-type and the mutant with Phe-317) associated with p140, whereas the CH domain alone did not bind p140 (Fig. 5b). Since the CH domain is capable of enhancing Grb2:mSOS association, a direct role for this 140-kDa protein seems unlikely.

DISCUSSION

We present evidence that T-cell stimulation via the TCR leads to a significant enhancement of Grb2 association with mSOS. We have characterized several aspects of this phenomenon. First, the induced Grb2:mSOS association correlates with the state of activation of the cells. The enhanced association is observed within 1 min after T-cell activation but returns to basal levels by 30 min after stimulation. Second, the enhanced binding of Grb2 and mSOS is regulated via the Grb2 SH2 domain. A phosphopeptide derived from the Shc sequence, which binds to the Grb2 SH2 domain, was able to inhibit the enhanced association between Grb2 and mSOS. Since the enhanced binding still occurred via the Grb2 SH3 domains, these data suggested that interaction of tyrosine-phosphorylated proteins with the Grb2 SH2 domain modulates the affinity of Grb2 SH3 domains for mSOS. Third, the interaction of tyrosine-phosphorylated Shc with Grb2 is sufficient to enhance the Grb2:mSOS association. Using fusion proteins encoding different regions of Shc, we have been able to narrow the region of Shc necessary for this regulation to the 145-aa CH domain of Shc. Our data also suggest that the Tyr-317 of Shc is essential for this enhancement. Fourth, cross-linking the coreceptor CD4 with the TCR, which leads to greater phosphorylation of Shc, results in greater association between Grb2 and mSOS compared with cross-linking of the TCR alone. This finding suggests that engagement of the coreceptor CD4 can modify the extent of Ras activation by the TCR through the regulation of Grb2:mSOS association.

Upon T-cell stimulation, Grb2 interacts with phosphorylated Shc as well as an unidentified 36-kDa phosphoprotein (p36) (3, 31). It should be noted that the Shc peptide displaces both Shc and p36 from the Grb2 SH2 domain (25a). While our data clearly demonstrate a role for Shc in regulating the Grb2:mSOS association, whether p36 also regulates this association remains to be determined. It is also noteworthy that we do not observe increased binding of GST-Grb2 fusion proteins to mSOS when incubated with activated T-cell lysates. Different concentrations of fusion proteins and various amounts lysates gave similar results, suggesting that bacterially expressed Grb2 proteins behave differently from those expressed in T cells.

One mechanism by which Shc mediates the enhanced Grb2:mSOS association may be that the interaction of phosphorylated Shc with the Grb2 SH2 domain regulates the affinity of the Grb2 SH3 domains for mSOS. Thus, it was surprising to discover that the Shc peptide alone, although capable of interacting with Grb2 SH2 domain, was unable to enhance the Grb2:mSOS association by itself. Both a 9-mer and a 16-mer Shc peptide as well as various peptides derived from Grb2 binding motifs in other signaling proteins (such as the EGF receptor, SH-PTP2, Bcr-Abl, and insulin receptor substrate 1) did not, by themselves, enhance the Grb2:mSOS association in unactivated T-cell lysates (25a). Recently, Cussac et al. (5) reported that the binding of phosphopeptides to GST-Grb2 fusion proteins did not influence the affinity of the Grb2 SH3 domains for proline-rich mSOS peptides. We have also observed that in vitro, when GST-Grb2 fusion proteins were used, SH2 domain occupancy by a phosphopeptide did not alter SH3 domain affinity for a proline-rich peptide, and conversely, SH3 domain occupancy did not influence SH2 domain affinity or specificity for phosphopeptides (34). This may be because of the use of proline-rich peptides instead of the native mSOS protein, the use of bacterially expressed Grb2 proteins, or both.

However, phosphorylated GST-Shc fusion protein when added to unactivated lysates was capable of enhancing this

association. This finding indicated that the simple occupancy of the Grb2 SH2 domain is not sufficient and that some component of the Shc structure (not present in the peptide) is required for regulating binding of Grb2 to mSOS. The observation that the GST-Shc fusion protein carrying a Tyr-317→Phe point mutation, although capable of interacting weakly with Grb2 (perhaps via Tyr-240), is unable to enhance the Grb2:mSOS association suggests that sequences surrounding Tyr-317 of Shc may play a role in this regulation. In contrast to the data obtained with Grb2, addition of appropriate phosphopeptides which interact with the SH2 domain(s) of either the tyrosine phosphatase SH-PTP2 or the p85 regulatory subunit of phosphoinositide-3 kinase increases the catalytic activities of these proteins (1, 4, 14).

Another means by which Shc may regulate the Grb2 binding to mSOS is through the recruitment of other proteins, which can directly or indirectly influence the affinity of Grb2 SH3 domains for mSOS. In this regard, our data would appear to rule out a direct role for the Shc-associated 140-kDa phosphoprotein, since it does not interact with the CH domain of Shc, which is sufficient for regulating the Grb2:mSOS association.

The currently available data, except in one case in which Grb2 was overexpressed (2), indicate that Grb2 and mSOS exist in a constitutive complex and that growth factor stimulation does not alter the levels of Grb2 complexed with mSOS (10, 11, 15, 16, 27). Since no change in catalytic activity of mSOS has been observed before or after receptor triggering, the translocation of mSOS (via Grb2) from the cytoplasm to the membrane has been proposed as the major rate-limiting step in Ras activation following receptor stimulation (30). The Shc-mediated regulation of Grb2:mSOS association following TCR activation may provide a mechanism for controlling the extent of Ras activation. Quantitative differences in Ras activation and downstream signaling through Ras are proposed as the means by which EGF and nerve growth factor mediate proliferative and differentiation response, respectively, of PC12 cells (12). Since different levels of Grb2:mSOS complex can be attained through different stimuli (such as the engagement of the CD4 coreceptor with TCR), this may provide a mechanism for achieving quantitative differences in Ras activation. The ability of Shc to regulate the Grb2:mSOS interaction may be a reason for the wide use of Shc in signaling via many receptors. In support of this notion, during insulin receptor signaling, no detectable mSOS was found associated with Grb2:insulin receptor substrate 1 complex, while a significant amount of mSOS was observed in the Shc:Grb2 complex, suggesting a similar role for Shc in insulin-mediated Ras activation (22). The data presented here also strongly suggest that the so-called adapter proteins, such as Grb2 and Shc, are more than simply connector proteins and that their interactions are also subject to regulation.

ACKNOWLEDGMENTS

This work was supported by a NIH grant (to S.J.B.) and a grant from F. Hoffmann-LaRoche. K.S.R. is supported by a fellowship from The Medical Foundation, Boston, Mass. U.L. is supported by a fellowship from the American Cancer Society, Massachusetts Division.

We thank P. G. Pelicci for the Shc cDNA constructs and J. McGlade and T. Pawson for the GST-Shc CH+SH2 fusion protein. We thank Z. Songyang for helpful discussions and S. Sawadkosal and V. Igras for help with the human PBL experiments. We thank B. Neel, R. Kapeller, J. Pratt, and D. Barber for critical reading of the manuscript.

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