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Growth Factor Receptor-Bound Protein 2 (Grb2) Association with Hemopoietic Specific Protein 1: Linkage Between Lck and Grb2

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To analyze the growth factor receptor-bound protein 2 (Grb2) signaling pathway in lymphoid cells, we used expression cloning to isolate the genes encoding proteins that associate with Grb2. We find that the Src homology 3 domains of Grb2 directly associate, in vitro and in vivo, with murine hemopoietic specific protein 1 (HS1), a protein identical to Lck-binding protein 1. Because HS1 associates with the p56lck and p59cuk tyrosine kinases in vitro and in vivo, and becomes tyrosine phosphorylated upon various receptor stimulations, our present data suggest that HS1 mediates linkage between Lck or Lyn and Grb2 in lymphoid lineage cells. The Journal of Immunology, 1998, 161: 625–630.

The growth factor receptor-bound protein 2 (Grb2)3 is an adapter protein that consists of the Src homology 2 (SH2) domain, which mediates the Ras signal pathway (5–10). This pathway is thought to resemble the signaling pathway for photoreceptor cell differentiation in Caenorhabditis elegans (14).

In hemopoietic cells, stimulation of the TCR leads to activation of a nonreceptor type of protein tyrosine (15–20). The activation of nonreceptor-type tyrosine kinases, such as Lck and Fyn, recruits ZAP-70/Syk family tyrosine kinase (reviewed in Ref. 21), and these tyrosine kinases lead to tyrosine phosphorylation of numerous additional proteins. Some of these molecules associate with Grb2 in T cells. For example, Shc recruits Grb2 to TCR (22, 23), and 36-kDa/Lnk links Grb2 and phospholipase C (21, 24–27).

Materials and Methods

Cell lines and Ag receptor stimulation

T cell hybridoma DO-11.10 cells (43) were maintained in RPMI 1640 with 10% FBS and stimulated by plating on anti-CD3ε-coated plastic culture dishes for 10 min. Plates were prepared by incubation with 100 µg/ml of 2C11 Ab solution, followed by PBS washing. As a negative control, non-stimulated cells were used. After T cell stimulation, plates were washed with PBS, and cells were lysed directly with TNE buffer (10 mM Tris-HCl, pH 7.8, 1% Nonidet F-40, 0.15 M NaCl, 1 mM EDTA, 10 mM NaF, 10 mM NaVO4, 10 µg/ml aprotinin, and 10 µg/ml leupeptin) or digitonin buffer (10 mM triethanolamine, 10 mM iodacetamide, 1% digitonin, 0.15 M NaCl, 1 mM EDTA, 10 mM NaF, 1 mM NaVO4, 10 µg/ml aprotinin, and 10 µg/ml leupeptin, pH 7.8).

Glutathione S-transferase (GST) fusion proteins

A cDNA fragment for HS1-N was generated by PCRs (5’ primer, 5’-CCCCGGCCGCCATGTTGGAAGTCTGTAGTGGG-3’, and reverse primer, 5’-CCCGGGCCGCCCTTAGCGCAACGACCTTCTAT-3’), digested with NotI, and subcloned into a NotI site of pGEX-2T (Pharmacia, Uppsala, Sweden) expression vector. GST-HS1- #3, -HS1- #4, -HS1- #5, -HS1- #6, and P1–P4 mutants have been described (36). cDNA fragments for Grb2 N-terminal SH3 and C-terminal SH3 domains were generated by PCR (theGrb2N-terminalSH3domain,5’primer, 5’-CCGAGGGATCCATGGAAAGCATCCTGCAAAATGAC-3’, and reverse primer, 5’-GGCCGCGCCGCCATGTTGGAAGTCTGTAGTGGG-3’).
GAATTCATGTTGTTTCAATATGAGTTCTTGG-3\prime; the Grb2 C terminal SH3 domain, 5\prime; primer, 5\prime;--CGGGCGGGATCCACAGAAGC
AGCCGCTTTTGGAGC-3\prime; and reverse primer, 5\prime;--GGGAGTTACCCGTCAGTC
ACGGTCTGGTTGAC-G3\prime;). PCR fragments were digested with BamHI and EcoRI and subcloned into the BamHI and EcoRI site of pGEX-4T-1 (Pharmacia) expression vector. GST fusion proteins were expressed and purified according to published procedures (36).

Screening of cDNA library

Screening has been described (36). Briefly, 5 \times 10^6 plaque clones from the murine pre-T cell line KKF cDNA library (36, 44) were plated at a density to produce 5 \times 10^5 plaques per 150-mm agarose plates. After incubation for 4 h at 42°C, plates were overlaid with nitrocellulose filters presoaked in 10 mM isopropl-\beta; -d-galactopyranoside (IPTG), as described (45). Incubation was continued for 4 h at 37°C. Filters were then removed, washed with TBST buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, and 0.05% Tween-20) at 4°C, and blocked in TBST containing 5% skim milk for 30 min at 4°C. After blocking, the GST-Grb2 N-terminal SH3 and C-terminal SH3 probes were added at a concentration of 1 \mu g/ml, and incubation was continued overnight. Filters were washed three times with TBST and incubated with anti-GST Ab (36) at a dilution of 1/2000 for 1 h at 4°C. After washing with TBST three times, alkaline phosphatase-conjugated anti-rabbit IgG (Dako, Glostrup, Denmark) was added at a dilution of 1/2000 for 30 min at 4°C. After washing with TBST, filters were incubated with alkaline phosphatase reaction solution (0.5 mM MgCl2 and 25 mM Na2CO3, (pH 9.8), containing 0.4 mM of nitroblue tetrazolium and 0.4 mM 5-bromo-4-chloro-3-indolylphosphate-p-toluidine salt (Wako Junyaku, Tokyo, Japan)). To obtain the specific clones that bound to each of the Grb2 SH3 domains, clones were detected with the Grb2 N-terminal, Grb2 C-terminal SH3 domains, or GST, respectively, at the third screening.

HS1 lysogen

The lysogen carrying the \alpha; 11 phage with the HS1 gene was induced with IPTG or uninduced. The IPTG-induced and uninduced proteins (+ and −, respectively) were analyzed by Western blotting, using GST fusion proteins as probe (1 \mu g/ml). Filters were washed with TBST, and protein complexes were detected by incubation with anti-GST Ab. Lysates with or without IPTG were directly incubated with anti-\beta; -gal Ab (Cappel, Oregon, PA). After washing with TBST three times, alkaline phosphatase-conjugated anti-rabbit IgG was added at a dilution of 1/2000 for 30 min at 4°C. After washing with TBST, filters were incubated with alkaline phosphatase reaction solution.

GST fusion protein-binding assay

Approximately 400 ng of GST fusion proteins were separated by SDS-PAGE and Western blotted with biotinylated GST fusion probes (1 \mu g/ml). Filters were washed with TBST-high salt buffer (10 mM Tris-HCl, pH 8, 1 M NaCl, and 0.05% Tween-20). The protein complex was detected by alkaline phosphatase-conjugated streptavidin (Life Technologies, Gaithersburg, MD).

GST fusion protein-Sepharose-binding assay

Cell lysates (1 \times 10^7 cells) were cleared by centrifugation and treatment with excess protein A-Sepharose (Pharmacia). The precleared cell lysates were incubated at 4°C overnight with glutathione-Sepharose beads (Pharmacia) bound to 50 \mu g of GST fusion proteins. Beads were washed five times with TNE buffer and lysed with SDS sample buffer, and the resulting solutions were boiled for 10 min. Proteins were separated by SDS-PAGE and transferred to nitrocellulose filters. Filters were blocked with TBST containing 10% BSA (Miles, Kankakee, IL) and incubated with anti-Grb2 (Transduction Laboratories, Lexington, KY) Ab. The anti-Grb2 Ab was detected by peroxidase-conjugated anti-mouse and anti-rabbit IgG (Dako), respectively, followed by the enhanced chemiluminescence system (ECL; Amersham, Arlington Heights, IL).

Immunoprecipitation

Cell lysates (1 \times 10^6 cells) were prepared by lysis with digitonin buffer, cleared by centrifugation, and treated with excess protein A-Sepharose. The precleared cell lysates were incubated with 5 \mu g/ml of anti-Grb2 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) or 5 \mu g/ml of anti-rabbit IgG Ab (Sigma, St. Louis, MO). Immunocomplexes were recovered by the addition of 10 \mu l of protein A-Sepharose (Pharmacia) and Western blotted with anti-mouse HS1 Ab (Sumitomo Denko, Kanagawa, Japan) or anti-Grb2 Ab (Transduction Laboratories).

Results

**HS1 is a Grb2 N-terminal SH3-binding protein**

To better understand the molecular mechanisms of the Grb2 signaling pathway in lymphocytes, we used expression cloning to isolate and analyze genes encoding proteins that associate with Grb2 in lymphoid cells. A set of SH3 domain fusion GST proteins was bacterially synthesized and analyzed on SDS gels to confirm their predicted size (Fig. 1A). The GST fusion protein containing the N-terminal SH3 region of Grb2 (GST-Grb2NTSH3) was used to screen a \alpha; 11 cDNA expression library obtained from the murine pre-T cell line KKF (36, 44). After three rounds of

![Figure 1](https://example.com/gallery/figure1.png)

**FIGURE 1.** Probe proteins and cDNA products isolated by expression cloning. A. The following GST fusion proteins were obtained from Escherichia coli lysates, analyzed by SDS-PAGE, and stained with Coomassie blue: GST protein alone (lane 1), Grb2 N-terminal SH3 domain (amino acids 1–58, GST-Grb2NTSH3, lane 2); Grb2 C-terminal SH3 domain (amino acids 159–217, shown as a GST-CTSH3, lane 3); Lck SH3 domain (amino acids 66–126, GST-LckSH3, lane 4); and HS1 SH3 domain (amino acids 434–486, GST-HS1SH3, lane 5). Numbers on the right indicate molecular size. Amino acid sequences of each SH3 domain are shown at the bottom of the gel. Bold letters indicate potential contact sites in the SH3 domain to a proline-rich region (56, 57). Dots indicate spacers for the alignment of each amino acid sequence. **B.** Schematic representation of the mSos and HS1 structure. Open boxes indicate coding regions. Bars underneath each schematic indicate the regions of the cDNAs isolated. mSos1, vertical lines on the right indicate proline-rich regions; vertical striped box shows pleckstrin domain; and horizontal striped box shows Ras guanine exchange domain. HS1, arrowheads represent the four-tandem 37-amino-acid repeat motifs; diagonal striped box indicates proline-rich region; dotted box shows the E-P region containing the proline-glutamate repeat; and filled box indicates the SH3 domain. Amino acid sequence and location of potential Grb2 SH3 binding sites in mSos1 and HS1 are shown above each schematic. Numbers to the left of these amino acid motifs indicate position of starting amino acids.

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Thus, the C-terminal SH3 domain fusion protein used in this experiment to produce HS1 and mSos1 as fusion proteins with bacterially synthesized fusion proteins, lysogens of 15N/HS1 were isolated and probed with the various GST-SH3 fusion proteins. Proteins associating with GST fusion protein probes were detected by anti-GST Ab (lanes 1–10). The β-gal-15N/HS1 fusion protein in lysates with or without IPTG was detected by anti-β-gal Ab (lanes 11–12). Numbers on the right indicate molecular size.

Screening, five strongly positive clones (15N, 22N, 30N, 38N, and 91N) were isolated. DNA sequencing of clones 22N, 38N, and 91N revealed that these were identical to regions of the murine Sos1 gene (Fig. 1B). Clones 22N/Sos1 and 91N/Sos1 are identical, representing nucleotides 3206–4670 or amino acids 1057–1366. Clone 38N/Sos1 contains nucleotides 2486–3830 or amino acids 817–1265. Both regions in these clones cover several typical SH3-binding amino acid sequences: PPPVPPR (amino acids 1153–1159), PAIPPR (amino acids 1182–1188), and PPLPPR (amino acids 1214–1221) (Fig. 1B). These regions appear to serve as binding sites for Grb2, based on previous reports for the amino acid sequence of SH3 binding domains (6, 7). Clones 15N and 30N are 938 bp in size, and DNA sequencing revealed that they encode nucleotides 118–1055 or amino acids 40–351 of HS1 (Fig. 1B). The deduced amino acid sequence (amino acids 40–351 of HS1) (36, 46) for clone 15N contains a four-tandem 37-amino-acid repeat motif (amino acids 64–211) and a partial proline-rich region (amino acids 274–351). Figure 1B shows the structure of HS1 and the region represented by clone 15N.

To test the association between Grb2 and HS1 in vitro using the bacterially synthesized fusion proteins, lysogen of 15N/HS1 were used to produce HS1 and mSos1 as fusion proteins with β-gal (36), and protein lysates of lysogen with or without IPTG induction for protein synthesis were separated by SDS-PAGE, Western blotted, and probed with a series of GST-SH3 fusion proteins (Fig. 2). Several GST fusion protein probes associating with β-gal-15N/HS1 were detected by anti-GST Ab. The GST-Grb2NTSH3 probe detected a 160-kDa product in the IPTG-induced, but not the uninduced, lysate from clones 15N/HS1 (Fig. 2, lanes 3 and 4), indicating that the GST-Grb2 N-terminal SH3 protein binds to the β-gal-15N/HS1 fusion protein. The GST-LckSH3 fusion protein probe detected the 160-kDa protein in HS1 IPTG-induced, but not uninduced, lysates (Fig. 2, lanes 7 and 8), consistent with our previous report of the association between the Lck-SH3 region and HS1 (36). The other SH3 proteins, GST-Grb2CTSH3 and GST-HS1SH3, did not detect any significant bands (Fig. 2, lanes 5, 6, 9, and 10), as indicated by their detection with GST protein only (Fig. 2, lanes 1 and 2). In the expression-cloning process with the GST-Grb2 C-terminal SH3 domain probe, we isolated genes encoding SLP-76, which is known to be a Grb2-binding protein (28). Thus, the C-terminal SH3 domain fusion protein used in this experiment was capable of associating with other proteins. Anti-β-gal Ab detected 160-kDa proteins with several degraded proteins of 15N/β-gal-HS1 (Fig. 2, lane 12). Several experiments indicated that 15N/β-gal-HS1 fusion protein is most likely unstable (data not shown). Thus, the additional bands found in track 12 of Figure 2 are most likely degradation products of the 15N/β-gal-HS1 fusion protein.

Thus, the 160-kDa products detected by the GST fusion protein probes were indeed the β-gal HS1 fusion protein. These data indicate the in vitro binding of the Grb2 fusion protein to the HS1 fusion protein.

**Grb2 SH3 binding sites of HS1 are located in the proline-rich region**

To determine the binding domain of HS1 in the N-terminal SH3 region of Grb2, we constructed a series of deletion mutants of HS1 (Fig. 3A) and probed the GST fusion proteins of the mutants and the GST protein alone in Western blotting with the GST N-terminal SH3 region of Grb2 or GST proteins (Fig. 3B, middle and bottom). In this experiment, probes were biotinylated and detected by alkaline phosphatase-conjugated streptavidin. The GST-Grb2 N-terminal SH3 probe detected mutants #3 (amino acids 215–360) and #4 (amino acids 215–335), but not mutants N (amino acids 1–211), #5 (amino acids 215–280), or #6 (amino acids 372–486).
Grb2-BINDING PROTEIN

#6(amino acids 361–486) (Fig. 3B, middle). Coomassie blue staining confirmed the proper molecular size of each fusion protein (Fig. 3B, top). Biotinylated GST alone did not detect any significant bands (Fig. 3B, bottom). Thus, the N-terminal SH3 domain of Grb2 binds to the proline-rich region in HS1 in vitro. To precisely locate the binding regions in the proline-rich region of HS1, we constructed several small proteins covering the proline-rich region, P1, P2, P3, and P4 (Fig. 4A). GST fusion proteins P1, P2, P3, and P4 were separated by SDS-PAGE, Western blotted, and probed with biotinylated GST alone or with biotinylated GST fusion protein probes. The Lck SH3 probe was used as a positive control, since it binds to P2 and P3 (36). Both Grb2 (N-terminal SH3) and Lck (SH3 region) probes bound to P2 and P3, but not to P1 or P4 (Fig. 4B, GST-Grb2NTSH3 and GST-LckSH3, lanes P2 and P3). Note the very faint signals in the P2 and P3 lanes detected with the Grb2 C-terminal SH3 domain probe (Fig. 4B, GST-Grb2CTSH3). The other protein probes, HS1-SH3 and GST alone, did not detect any of the four proteins. Thus, binding of the Grb2 N-terminal SH3 domain to HS1 is highly specific, despite the strong similarity in amino acid sequence of several other SH3 regions examined (Fig. 1A).

Grb2-binding pattern of HS1 in T cells

The association of Grb2 and HS1 in cell lysates was further confirmed by immunoprecipitation with the GST fusion protein. T cell hybridoma DO-11.10 cells, with or without TCR stimulation, were lysed and incubated with GST fusion proteins noncovalently coupled to glutathione-Sepharose beads. Bound proteins were separated by SDS-PAGE, Western blotted, and probed with anti-Grb2 Ab. An Ab, commercially available anti-Grb2 Ab, detected a 26-kDa protein (Fig. 5A, lane 1), but not other proteins, suggesting that this 26-kDa band represents the Grb2 molecule. As expected, the middle region in HS1 (HS1-#3), which covers a proline-rich region, associated with Grb2, whereas the HS1 N-terminal region (HS1-N) did not bind to Grb2 (Fig. 5A). The precipitation of Grb2 by the HS1 C-terminal (HS1-#6) and the HS1 SH3 domain (HS1SH3) (Fig. 5A, upper panel) was unexpected, because the isolated DNA clone (15N/HS1) for the Grb2-binding protein does not cover this region (see Fig. 3A). These data indicate that Grb2 can associate with HS1 at two distinct sites. The Grb2 binding region in the HS1 proline-rich region was further examined using the P1-P4 mutants. Immunoprecipitation experiments with GST fusion proteins indicated that P2 and P3 bound to Grb2 regardless of TCR stimulation, whereas P1, P4, and GST proteins did not bind to Grb2 (Fig. 5B). These data confirm that the two proline-rich regions of HS1 bind to Grb2.

Grb2 association with HS1 in vivo

To examine the in vivo association of Grb2 with HS1, cell lysates obtained from T and B cell lines were immunoprecipitated with anti-Grb2 Ab or rabbit IgG as a control, and immunoblotted with anti-mouse HS1 Ab (Fig. 6, top). Anti-mouse HS1 Ab detected an 85-kDa protein in the Grb2 immunoprecipitates from T and B cell lines (Fig. 6, top lanes 5, 6, and 8), but rabbit IgG did not (Fig. 6, lanes 3, 4, and 7), showing that Grb2 associates with HS1 in vivo not only in T lineage cells, but also in B lineage cells. Note that the
Figure 6. Analysis of in vivo association between HS1 and Grb2. Cell lysates from the murine T cell hybridoma cell line DO-11.10 or murine B cell line Ig6.3 were immunoprecipitated with Ab specific for Grb2 or unrelated IgG Ab as a control. Cell lysates were obtained from stimulated (+) or unstimulated (−) T cell hybridoma. Bound proteins were separated by SDS-PAGE, blotted, and probed with anti-HS1 Ab (top) or with anti-Grb2 Ab (bottom). Signals were detected by the horseradish-conjugated anti-rat IgG for HS1 or anti-mouse IgG for Grb2 using the ECL detection system.

Discussion

We previously demonstrated the association of HS1 with Lck in vivo (36) and tyrosine phosphorylation of HS1 upon TCR stimulation (36, 40), indicating that HS1 is involved in the TCR signaling pathway through Lck. Similarly, HS1 associates with Lyn in vivo and in vitro and is tyrosine phosphorylated upon BCR stimulation in B cells (37, 41, 42). Several recent observations have implicated Lck signaling in the activation of the Ras pathway (47), and Lck has been reported to control mitogen-activated protein kinase activity in murine T cell lines (48). In the present study, we show that HS1 associates with Grb2 in vivo and in vitro. Since HS1 associates with Lck (36), it is likely that HS1 links signals from Lck to Grb2.

HS1 has a four-tandem 37-amino-acid repeat motif, two proline-rich regions, and an SH3 domain (36, 49). Previously, we found that the Lck SH3 domain binds to the two proline-rich regions (P2 and P3) of HS1 (36). In the present study, we showed that the Grb2 N-terminal SH3 domain also binds to the proline-rich regions (P2 and P3) in HS1 by filter-binding assay (Figs. 3 and 4), and that Grb2 binds to the proline-rich regions in HS1 by Sepharose bead-binding assay (Fig. 5, A and B). These data suggest that one HS1 molecule is potentially able to associate with two SH3 domains, implying that two SH3 domains, such as the Lck and Grb2 SH3 domains, are able to physically interact with the HS1 proline-rich regions. Surprisingly, we also found that Grb2 binds to the HS1-SH3 region by Sepharose bead-binding assay (Fig. 5A), indicating either a direct or an indirect intracellular association of the cellular Grb2 and the GST-HS1 fusion proteins. The PXXP motif (amino acids 155–158) at the C-terminal end of the Grb2 SH2 domain might bind to the HS1-SH3 region. Alternatively, the Grb2 might associate with HS1 at the HS1-SH3 domain indirectly, with another molecule(s) providing the link in the association of these molecules. Thus, these in vitro associations of Grb2 and HS1 suggest that multiple sites of HS1 appear to be involved in Grb2-HS1 interaction. These in vitro results suggest that Grb2 might recruit HS1 and HS1 association molecules such as Lck to the receptor complex using the Grb2-SH2 domain.

We did not detect a stable multiple complex, such as Lck/HS1/Grb2 in T cells in vivo, possibly because formation of such complexes occurs very rapidly under certain conditions such as activation or deactivation, or because the Ab used for immunoprecipitation affects formation of further complexes. Alternatively, association of the proteins might not be sufficiently strong to maintain complexes during immunoprecipitation experiments. We previously found a constitutive interaction between Lck and HS1 (36), and demonstrated significant changes in the binding pattern of Lck and HS1 upon cell activation, which induces additional binding of HS1 to the Lck-SH2 region (42). Conformational changes or changes in the affinity of the protein interaction upon cell activation might be required for complex formation with multiple molecules, and lead to further signal transduction of Grb2. Thus, such complexes might form in vivo under certain conditions that induce conformational changes in the component molecules.

We observed a strong association between Grb2 and the HS1 in the absence of TCR stimulation, but a weakened association upon TCR stimulation. A similar binding pattern has been described with respect to insulin stimulation of 3T3-L1 adipocytes, which results in hyperphosphorylation of Sos and the dissociation of Grb2 and Sos (50–53). Those authors suggested that the dissociation of the Sos-Grb2 complex might deactivate and desensitize the Ras signaling pathway. Similarly, the present data indicating reduced HS1 binding to Grb2 in vivo upon TCR stimulation might reflect the activation or inactivation of the Grb2 signaling.

Recently, several molecules have been described as linkers between Src family tyrosine kinases and Grb2, such as p120 c-Cbl (32, 33), Sam68 (34), and RAFTK (35) in T cells. These molecules are tyrosine phosphorylated upon TCR stimulation and are thought to mediate Src family tyrosine kinase signaling. HS1 is also tyrosine phosphorylated upon various stimulations such as TCR activation. Currently, it is not clear whether these molecules link signals between Src family tyrosine kinase and Grb2 simultaneously or only on a particular occasion. HS1 might play a specific role in linkage between Src family tyrosine kinase and Grb2 for apoptotic signals, because HS1 has been shown to be involved in apoptotic signals (40, 54, 55).

In this study, we demonstrated the clear association of HS1 with Grb2, in vitro and in vivo, indicating that HS1 links signaling between Lck and Grb2. The potential physiologic function of HS1 as a linker between Grb2 and Src family tyrosine kinase is currently under investigation.

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References


