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The Small 11-kDa Protein from B19 Parvovirus Binds Growth Factor Receptor-Binding Protein 2 *in Vitro* in a Src Homology 3 Domain/Ligand-Dependent Manner

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The small 11-kDa proteins of B19 parvovirus contain three proline-rich regions which conform to consensus Src homology 3 (SH3) ligand sequences present in signaling molecules within the cell. We have shown that the B19 11-kDa proteins specifically interact with the growth factor receptor-binding protein 2 (Grb2) *in vitro.* Mutation of prolines within one of the three SH3 ligand-like sequences decreases the binding of B19 11-kDa proteins to Grb2, suggesting that the proline-rich region is involved in the B19 11-kDa/Grb2 interaction. Therefore, the B19 11-kDa proteins may function to alter Grb2-mediated signaling by disrupting SH3 domain/ligand interactions. These results implicate the 11-kDa proteins in B19 pathogenesis through perturbation of normal cellular signaling pathways.

Key Words: parvovirus; B19; Grb2; SH3; signal transduction; viral pathogenesis.

INTRODUCTION

B19 human parvovirus has been identified as the causative agent of erythema infectiosum causing a characteristic "slapped cheek rash." B19 infection in adults, especially women, often leads to arthritis-like joint complications. These and other symptoms associated with B19 infection are transient and self-resolve within days or weeks in healthy, immunocompetent individuals. The B19 virus targets progenitor cells of the erythroid lineage, causing a life-threatening transient aplastic crisis in individuals with an underlying hemological disorder. The virus can also establish chronic infection in those individuals with congenital or acquired immunodeficiencies, and intrauterine infection by B19 has been implicated in multiple cases of hydrops fetalis or spontaneous abortion resulting from severe anemia of the fetus (reviewed in Rogers, 1999).

A number of primary cell lines will support B19 replication when supplemented with erythropoietin, but these culture systems are not readily amenable to experimental manipulation. In the absence of a continuous permissive cell line for propagating B19, the use of transfected cell systems has aided in the elucidation of various molecular and cellular aspects of B19 replication and transcription. Recent research has focused on the functions of B19 nonstructural proteins and the roles they play in the viral life cycle and pathogenesis. B19 directs expression of all of its transcripts from a single promoter at map unit 6 (p6) (Blundell *et al.,* 1987; Doerig *et al.,* 1987; Ozawa *et al.,* 1987) and the major nonstructural protein, NS, is believed to be responsible for mediating much of B19 pathogenicity likely via induction of apoptosis (Moffatt *et al.,* 1998). The B19 parvovirus also produces a family of nonstructural 11-kDa proteins (St. Amand *et al.,* 1991; St. Amand and Astell, 1993).

Analysis of the amino acid sequence of the 11-kDa proteins (Fig. 1a) indicates an unusually high proportion of proline residues: 14 of 94 residues or 15%. Twelve of the 14 proline residues can be grouped into three regions which share similarity with sequences known to bind to Src homology 3 (SH3)[2] domains in a variety of signal-transducing molecules (Fig. 1b). These regions and domains belong to a large, diverse family of noncatalytic, folding protein modules which provide a basic building block in receptor tyrosine kinase (RTK) signaling. These domains mediate moderate- to high-affinity binding between proteins to form multicomponent complexes that enable extracellular signals to be propagated to the interior of the cell, to elicit highly specialized

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[2] Abbreviations used: DEAE, diethylaminoethyl; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; EGFP, enhanced green fluorescent protein; GST, glutathione *S*-transferase; HA, hemagglutinin; HEK, human embryonal kidney; ORF, open reading frame; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; RTK, receptor tyrosine kinase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SH3, Src homology 3; Sos, son of sevenless; wt, wild-type.

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A

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1         10         20         30         40         50
|         |          |          |          |          |
MQNNTTDMDMKSLKNCGQPKAVCTHCKHSPPCPQPGCVTKRPPVPPRLYLPPPV
PIRQPNTKDIDNVEFKYLTRYEQHVIRMLRLCNMYQNLEK
      |         |          |          |
     60        70         80         90
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B

	Position	Sequence
Consensus Grb2 SH3 domain-binding motif	N/A	xPPxPxx
B19 11 kDa	41-47	RPPVPPR
hSos1	1150-1156	PPPVPPR
hSos1	1289-1295	GPPVPPR
hSos2	1289-1295	APPVPPR

FIG. 1. (A) Amino acid sequence of the B19 11-kDa protein. Proline-rich regions corresponding to consensus SH3-binding sites are highlighted. (B) Amino acid sequence alignment of selected proline-rich SH3-binding motifs in B19 11-kDa proteins and human Sos (hSos). Conserved proline residues are highlighted.

biological responses from the cell (reviewed in Mayer and Gupta, 1998). The presence of SH3 ligand-like regions within the B19 11-kDa sequence led us to speculate that the B19 11-kDa proteins may be involved in binding to cellular proteins via their proline-rich regions. Preliminary evidence from glutathione *S*-transferase (GST) affinity chromatography experiments in our laboratory has suggested that at least two cellular proteins (of MW 85 and 26 kDa) from a human leukemic cell lysate are capable of binding the B19 11-kDa proteins (D. B. Zagrodney and C. R. Astell, unpublished observations).

In the present study, we demonstrate that the B19 11-kDa protein specifically interacts *in vitro* with the growth factor receptor-binding protein 2 (Grb2), an adaptor protein implicated in RTK-mediated signaling of mitogenic and stress stimuli. We also show that the 11-kDa protein binds Grb2 in an SH3 domain/ligand-dependent manner. Our findings implicate the 11-kDa proteins in B19 pathogenesis through perturbation of normal cellular signaling pathways by binding Grb2 or another as yet unidentified SH3 domain-containing protein. In this report, the longest (94 aa) 11-kDa protein is fused to GST or enhanced green fluorescent protein (EGFP) at the C-terminus. Within the text, we normally refer to the 11-kDa protein as a single species as the family of three 11-kDa proteins results due to use of three, in-frame, AUG codons (St. Amand and Astell, 1993), which would not occur in our fusion constructs.

RESULTS

B19 11-kDa proteins associate with Grb2 adaptor protein by far-Western blot analyses

The B19 11-kDa proteins contain 15% proline residues, including three regions conforming to the consensus xPPxPxx SH3-binding site motif (Fig. 1A). The second region, located at position 41–47, exhibits striking similarities to the SH3 ligands present in the guanine nucleotide exchange factor son of sevenless (Sos) (Fig. 1B). Because Sos uses such SH3 ligands to bind to SH3 domain-containing proteins such as the Grb2 adaptor protein, we investigated whether the B19 11-kDa proteins could also bind Grb2. We used GST, GST–11, GST–hemagglutinin (HA), and GST–HA–Grb2 fusion proteins in far-Western studies to determine whether the B19 11-kDa proteins could interact with Grb2. Affinity-purified fractions of GST and GST–11 were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to polyvinylidene difluoride (PVDF) membrane, probed with either GST–HA or GST–HA–Grb2, and detected using an anti-HA antibody (Fig. 2). Results suggest that GST–HA–Grb2 binds to membrane-bound GST–11 but not GST, confirming specificity of the interaction of the Grb2 fusion protein with the 11-kDa moiety of GST–11. Neither GST nor GST–11 binds to GST–HA, indicating that GST–11 interacts specifically with the Grb2 portion of the GST–HA–Grb2 fusion protein.

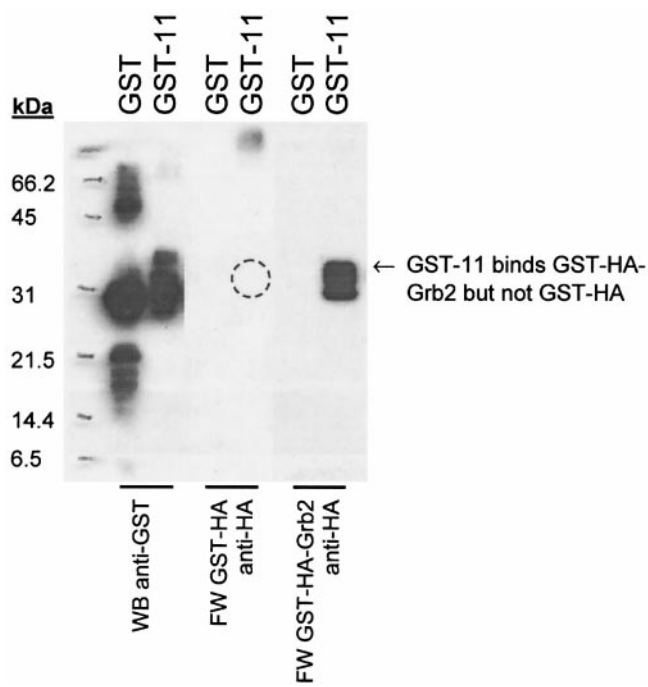

FIG. 2. B19 11-kDa proteins associate with Grb2 adaptor protein by far-Western blotting. Affinity-purified protein preparations of GST and GST–11 were resolved by 12.5% SDS–PAGE, transferred to PVDF membrane, and subjected to either Western blotting with an anti-GST antibody at 1:10,000 dilution (left) or far-Western blotting (center and right). For far-Western blotting, the membranes were first incubated with a protein probe consisting of either resin-purified GST–HA (center) or resin-purified GST–HA–Grb2 (right) diluted in blocking buffer to a final concentration of 1.0 μg/ml. Any bound GST–HA or GST–HA–Grb2 was subsequently detected using an anti-HA antibody at a 1:5000 dilution using the Western blotting protocol.

The 11-kDa/Grb2 interaction *in vitro* requires the 11-kDa proline-rich region

Because the B19 11-kDa proteins contain regions conforming to SH3 ligands similar to those employed by Sos to interact with Grb2, we postulated that the association between Grb2 and the 11-kDa protein may also be dependent on SH3 domain/ligand interactions. To examine the requirement for specific proline residues in the Grb2/11-kDa interaction, constructs encoding GST–11 with proline-to-alanine mutations within the second proline-rich region (position 41–47) were created by site-directed mutagenesis. GST–11 mutants were tested alongside wild-type (wt) GST–11, and GST in far-Western studies as described above (Figs. 3A–3C). Our findings indicate that GST–HA–Grb2 binds with reduced affinity to membrane-bound GST–11 proteins mutated in certain proline residues, as compared with wt GST–11. While the binding of Grb2 to GST–11 is not greatly affected by single mutations at proline residues 43 or 46, mutations at residues 42 and 45 do have an effect and multiple mutations in the GST–11 protein appear to significantly reduce the interaction. These results strongly suggest that this proline-rich region is involved in mediating interactions of the 11-kDa protein with Grb2 in far-Western experiments.

B19 11-kDa proteins associate with Grb2 adaptor protein in a GST pull-down assay in a SH3 domain/ligand-dependent manner

To further confirm our hypothesis, we investigated the ability of the B19 11-kDa proteins to precipitate soluble, endogenous Grb2 from a mammalian cell lysate. A GST pull-down assay was carried out to assess the interaction of soluble GST, GST–11, and GST–11 mutants with cellular Grb2. Purified GST or GST fusion proteins were incubated with human embryonic kidney (HEK) 293 cell lysate in the presence of glutathione–Sepharose resin. GST, GST–11 wt/mutants, and any associated proteins were collected with the resin by centrifugation and analyzed by SDS–PAGE (Figs. 3D and 3E). Our findings show that cellular Grb2 is effectively coprecipitated by GST–11 but not by GST, further providing support for an interaction between Grb2 and the 11-kDa proteins. GST–11 proteins mutated in certain proline residues exhibit reduced binding to Grb2, as compared with wt GST–11. Single mutations at proline positions 43 or 46 do not appear to interfere with the ability of GST–11 to coprecipitate Grb2 while mutations at positions 42 and 45 as well as multiple mutations do interfere. These results parallel those from the far-Western blotting experiments and provide strong support that the second proline-rich region of the 11-kDa proteins is involved in mediating the Grb2/11-kDa interaction.

Preferential interaction of the EGFP–11-kDa fusion protein expressed in COS-7 cells with cellular Grb2

To establish a fully *in vivo* system in which the 11-kDa protein might be shown to interact with cellular Grb2, COS-7 cells were transfected with a plasmid (pEGFP-11) expressing an EGFP–11-kDa fusion protein or with the control vector (pEGFP-C2) expressing EGFP. The efficiency of transfection varied with the EGFP plasmid used, yielding an ~20-fold higher transfection efficiency for pEGFP-C2 than for pEGFP-11. Hence, lysates of EGFP-expressing cells were diluted accordingly with nontransfected lysate to match levels of EGFP–11 expression in pEGFP-11-transfected cells. (The levels of EGFP–11 and EGFP were estimated by comparing the number of fluorescing cells expressing EGFP or EGFP–11. Since the cells expressing EGFP were often brighter than the EGFP–11-positive cells, this method likely underestimates the level of EGFP.) Lysates were incubated with anti-Grb2 antibody and precipitated with protein A–Sepharose resin. Proteins which bound to the antibody were analyzed by SDS–PAGE and Western blotting (Fig. 4). We find that in our hands both EGFP and EGFP–11 were immunoprecipitated under the conditions tested; however, there appears to be enhanced binding of EGFP–11, as suggested by the increased signal intensity of the band corresponding to EGFP–11 compared with EGFP in the immunoprecipitation samples after correcting for transfection efficiency differences.

DISCUSSION

This study provides *in vitro* evidence in support of our hypothesis that the small 11-kDa proteins produced by parvovirus B19 may play a role in the viral life cycle by interfering with the host environment through its interactions with cellular signaling molecules such as Grb2 or other SH3 domain-containing proteins. The binding of Grb2 to the 11-kDa protein may perturb the normal function of the Grb2 adaptor in binding the guanine nucleotide exchange factor Sos. Interaction of Grb2 with Sos involves the binding of proline-rich SH3 ligands in Sos with the cognate SH3 domains in Grb2. The B19 11-kDa proteins also contain similar proline-rich regions, which led to the hypothesis that the binding of Grb2 to the 11-kDa protein may be mediated by the same types of SH3 domain/ligand interactions as those responsible for Grb2/Sos interactions during normal host signaling.

Ten mutants of GST–11 in which one or more proline residues were selectively replaced by alanine residues were tested alongside GST and GST–11 in far-Western and GST pull-down experiments to determine the requirements of the 11-kDa/Grb2 interaction for the second proline-rich region in the 11-kDa proteins. It is known that SH3 ligand peptides can potentially bind in either orientation and the proline-rich sequences are known to exist in a left-handed polyproline type II helix (Pawson, 1995).

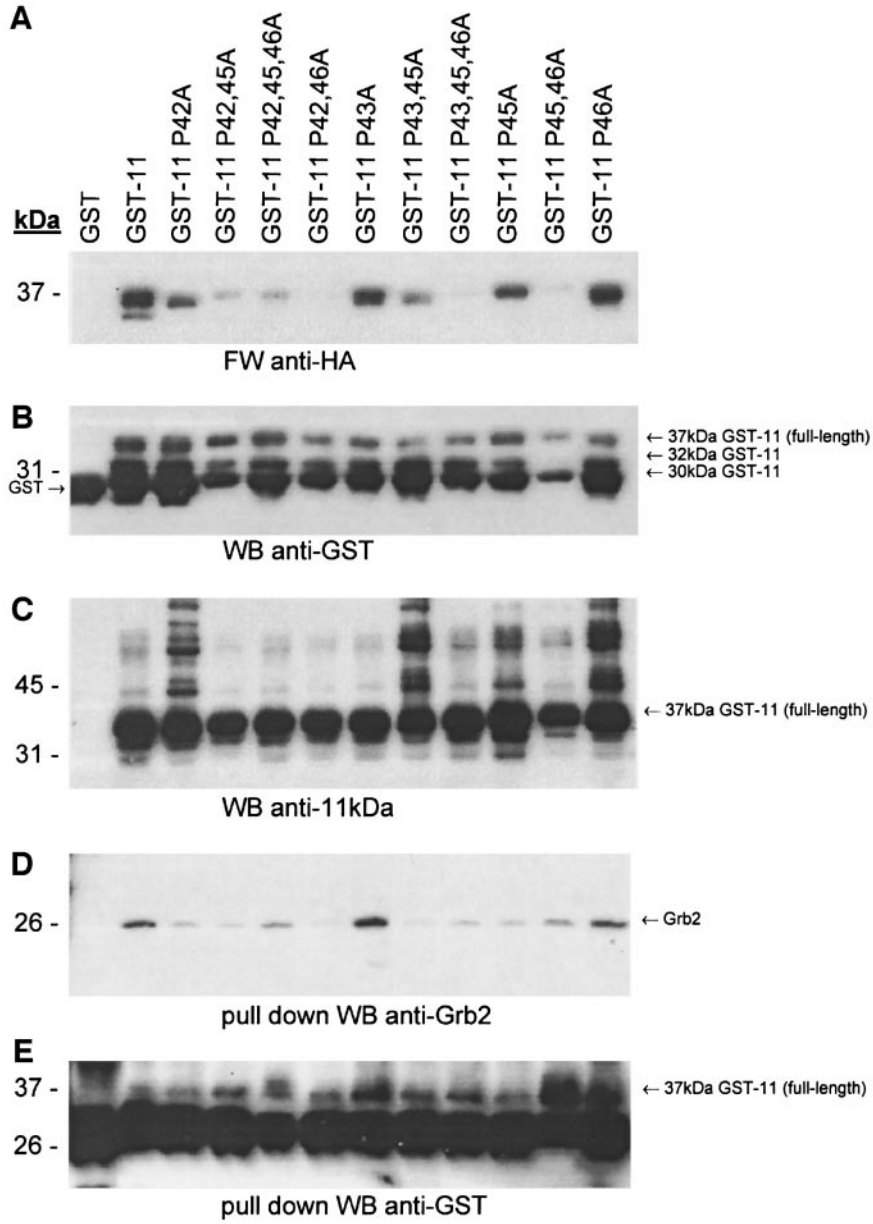

FIG. 3. The 11-kDa/Grb2 interaction *in vitro* requires the 11-kDa proline-rich region in far-Western blots and GST pull-down experiments. Affinity-purified protein preparations of GST, GST–11, and GST–11 mutants were resolved by 12.5% SDS–PAGE, transferred to PVDF membrane, and subjected to either far-Western blotting (A) or Western blotting (B and C). For far-Western blotting, the membrane was first incubated with a protein probe consisting of resin-purified GST–HA–Grb2 diluted in blocking buffer to a final concentration of 0.5 μg/ml. Any bound GST–HA–Grb2 was subsequently detected using an anti-HA antibody at a 1:5000 dilution. For Western blotting, the membranes were detected with either an anti-GST antibody (B) or an anti-11-kDa antibody (C) at 1:10,000 dilution. In B, the GST–11 affinity-purified protein included a full-length 37-kDa protein as well as two proteolytic degradation products (32 and 30 kDa). These proteins appear to have lost the 11-kDa portion as anti-11-kDa protein detects predominantly the full-length 37-kDa band. For GST pull-down experiments, HEK 293 cell lysates were incubated with resin-purified GST, GST–11, or GST–11 mutants in the presence of glutathione–Sepharose resin. Any interacting proteins were coprecipitated upon centrifugation. Proteins from pull-down samples were resolved by 12.5% SDS–PAGE, transferred to PVDF membrane, and subjected to Western blotting with either an anti-Grb2 antibody (D) or an anti-GST antibody (E) at a 1:10,000 dilution. The large band at 26 kDa is cellular GST.

Singly mutated GST–11 proteins 43 and 46 mostly retain the ability to interact with GST–HA–Grb2, while reduced interactions are observed with the P42A and P45A mutants and markedly reduced interactions are observed for several of the double and triple mutants. These results demonstrate that, while no single proline residue is critical for SH3 binding, multiple prolines together contribute to the interaction. Our findings provide strong support for the involvement of the second proline-rich SH3 ligand-like region of the B19 11-kDa proteins as a conformationally defined unit in mediating interactions with Grb2.

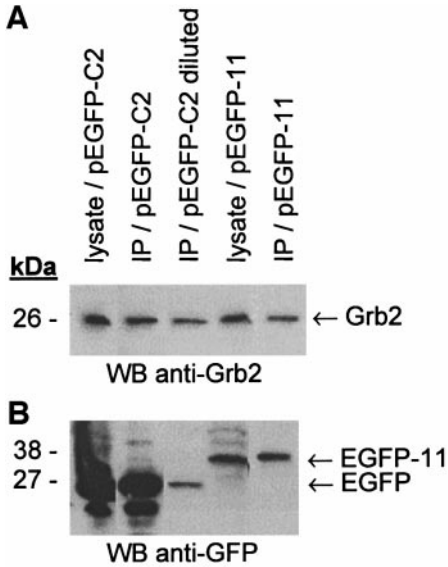

FIG. 4. An EGFP–11-kDa fusion protein expressed in COS-7 cells interacts preferentially with cellular Grb2. Undiluted (EGFP- and EGFP–11-expressing) or 25-fold diluted (EGFP-expressing) cell lysates from transfected COS-7 cells were incubated with an anti-Grb2 antibody in the presence of protein A–Sepharose. Any interacting proteins were coimmunoprecipitated upon centrifugation. Proteins from lysates and immunoprecipitation samples were resolved by 12.5% SDS–PAGE, transferred to PVDF membrane, and subjected to Western blotting with either an anti-Grb2 antibody (A) or an anti-GFP antibody (B).

The ability of the 11-kDa protein to interact with cellular Grb2 *in vitro* suggests a possible mechanism of B19 parvovirus to manipulate the host cell environment by modulating signaling pathways, thereby affecting downstream signaling events including those stemming from mitogenic stimuli. Examples of virally encoded nonstructural proteins which interact with SH3 domain proteins and/or disrupt mitogenic signaling include the hepatitis C virus NS5A protein (Tan *et al.,* 1999) and the human immunodeficiency virus type 1 Nef protein (Saksela *et al.,* 1995; Manninen *et al.,* 1998), both of which are implicated in modulation of host signaling cascades involved in virulence and pathogenesis. The B19 11-kDa proteins may function in a similar way to enhance viral replication and propagation.

Having established the involvement of at least one of the three proline-rich sequences of the 11-kDa proteins in binding to Grb2, *in vitro,* in far-Western and GST pull-down experiments, it will be instructive to mutate the remaining two regions individually or in combination to examine the requirement for and possible synergistic or compensatory effects of these proline-rich regions in the Grb2/11-kDa interaction.

Although we have demonstrated preferential interaction of EGFP–11 with cellular Grb2, the *in vivo* relevance of this interaction remains uncertain. Previously, we have shown that the 11-kDa proteins are predominantly, although not exclusively, nuclear (W. Luo and C. R. Astell, unpublished data). Grb2 is predominantly cytoplasmic, although importantly up to 20% can be located in the nucleus (Romero *et al.,* 1998). However, many proteins have SH3 domains and one or more of these proteins may be important cellular interactors. We have tested a second adaptor protein, Crk II, in GST pull-down experiments and observed nonspecific interactions of Crk II with both GST and GST–11 with no evidence of preferential interaction with GST–11, indicating that not every SH3 domain protein is able to interact with the 11-kDa proteins (data not shown). Crk II has both SH2 and SH3 domains. It is phosphorylated in response to Epidermal Growth Factor (EGF) stimulation and the phosphorylated form appears to dissociate from the EGF receptor (Hashimoto *et al.,* 1998). Other SH3 domain-containing proteins also need to be tested for their ability to interact specifically with the 11-kDa proteins. We may be able to scale up the GST–11 affinity purification of cellular interacting proteins (D. B. Zagrodney and C. R. Astell, unpublished data) in order to identify these proteins using mass spectrometry (Link *et al.,* 1999). An alternate approach may be to use a method of panning of domain cDNA libraries displayed on the surface of phage to identify other potential interacting proteins (Zucconi *et al.,* 2001).

MATERIALS AND METHODS

Plasmids and mutagenesis

pGEX2T (Pharmacia) directs expression in *Escherichia coli* of GST, a 26-kDa protein from *Schistosoma japonicum,* under the control of the inducible *tac* promoter. GST and GST fusion proteins can be purified by affinity chromatography on glutathione–Sepharose resin. pGEX-11 (constructed by J. St. Amand) contains the B19 11-kDa open reading frame (ORF) (Fig. 1a) cloned in-frame into the *Bam*HI/*Eco*RI sites of pGEX2T. This vector directs the expression of a GST–11-kDa fusion protein, GST–11. pGEX-11 plasmids containing proline-to-alanine mutations in the 11-kDa ORF (pGEX-11 P42,45A; P42,45,46A; P42,46A; P43A; P43,45A; P43,45,46A; P45A; and P45,46A) were constructed by PCR-based site-directed mutagenesis using complementary degenerate oligonucleotide primers, 11kDPro2 (5′-cag gta cag tct gg(**c/g**) tg(**c/g**) tac tg(**c/g**) tg(**c/g**) gcg ttt agt tac-3′) and 11COMPL (5′-gta act aaa cgc (**c/g**)ca (**c/g**)ca gta (**c/g**)ca (**c/g**)cc aga ctg tac ctg-3′). These primers are partially degenerate at the first nucleotide position of each triplet corresponding to the four proline residues targeted for mutation (Pro-42,43,45, and 46). pGEX-11 P42A and pGEX-11 P46A were similarly constructed from pGEX-11 by PCR-based site-directed mutagenesis using complementary oligonucleotide primer pairs, P42A1 (5′-cgt aac taa acg **cgc** acc agt acc acc-3′)/P42A2 (5′-ggt ggt act ggt **gcg** cgt tta gtt acg-3′) and P46A1 (5′-gcc cac cag tac cag cca gac tgt acc tg-3′)/P46A2 (5′-cag gta cag tct ggc tgg tac tgg tgg gc-3′), respectively. Nucleotide mutations corresponding to the

specified amino acid changes are set in bold. Mutated pGEX-11 vectors direct expression in bacteria of mutant GST–11 fusion proteins. pGEX2T-tag (gift from K. Harder) was constructed by insertion of a HA epitope tag and polylinker into the *Bam*HI/*Eco*RI sites of pGEX2T. This plasmid expresses a GST–HA fusion protein. pGEX2T-tag-Grb2 (gift from K. Leslie and M. Welham) was constructed by cloning the cDNA encoding human Grb2 into the *Nhe*I/*Eco*RI sites of pGEX2T-tag. This vector directs the expression of a GST–HA–Grb2 fusion protein. pEGFP-C2 (Clontech) directs expression in mammalian cells of EGFP, under the control of a human cytomegalovirus immediate-early promoter. pEGFP-11 (constructed by H. Chen) contains the B19 11-kDa ORF cloned in-frame into the *Bgl*II/*Eco*RI sites of pEGFP-C2. This vector directs the expression of an EGFP–11-kDa fusion protein, EGFP–11.

Protein expression and purification

GST and GST fusion proteins were expressed in DH10b *E. coli* and purified according to the manufacturer's protocol.

Far-Western and Western blotting

Whole-cell lysates, precipitated proteins, or purified GST and GST fusion proteins were subjected to SDS–PAGE and transferred to PVDF membranes (Bio-Rad or Pall Corp.). Membranes were probed with specific primary antibodies and the appropriate horseradish peroxidase-conjugated secondary antibodies. Proteins of interest were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech). For far-Western blotting, 2 μg of GST, GST–11, and GST–11 mutant proteins was resolved by SDS–PAGE and transferred to PVDF membranes. The procedure is essentially identical to that of Western blotting except for an additional incubation with a protein probe solution containing 0.5–1.0 μg/ml GST–HA or GST–HA–Grb2 dissolved in blocking buffer, prior to detection with an antibody directed against the HA epitope (Boehringer Mannheim).

Cell lines

COS-7 and HEK 293 cells were cultured as monolayers in complete medium consisting of low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 10 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] at pH 7.4, 37°C, and 5% CO_2. Cells were routinely trypsinized and passaged in a 1:10 dilution in fresh medium when they reached about 90% confluency, usually every 3 to 4 days.

Transfection

COS-7 cells were transfected by a modified diethylaminoethyl (DEAE)–dextran procedure. Briefly, nearly confluent cells in 100-mm culture dishes were passaged at a 1:6 dilution 1 day prior to transfection. Five micrograms of plasmid DNA purified by ethanol precipitation was dissolved in 100 μl of sterilized distilled H_2O, mixed with 200 μl of 2 mg/ml DEAE–dextran, and diluted to a 3-ml final volume with serum-free DMEM. Cells were washed twice with additive-free DMEM and then overlaid with the transfection solution. After 8 h of incubation at 37°C and 5% CO_2, the transfection solution was removed and replaced with 3 ml of phosphate-buffered saline (PBS) containing 10% dimethylsulfoxide (DMSO). Cells were subjected to the DMSO shock for exactly 5 min and then washed twice with PBS before the addition of 10 ml of complete medium. Transfected cells were incubated at 37°C, 5% CO_2 for 2 days before being harvested for immunoprecipitation experiments.

Lysate preparation

For the GST fusion protein pull-down assays and immunoprecipitation experiments, nearly confluent cells were washed once in PBS and resuspended at a concentration of $\sim 3 \times 10^6$ cells per milliliter of 20 mM Tris–Cl, pH 7.4, supplemented with 2 μg/ml antipain, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 4 μg/ml aprotinin. Cells were lysed by Dounce homogenization after a 15-min incubation on ice. The nuclei were pelleted and the supernatant was stored on ice and used within 2 h.

GST protein pull-down assay

GST protein pull-down assays were performed according to a protocol similar to that described in Byrne *et al.* (1996). Briefly, 500 μl of Dounce homogenized HEK 293 cell lysate (~0.5–1.0 mg protein) was incubated with end-to-end rotation for 2 h at 4°C with a 10-μl bed volume of PBS-equilibrated glutathione–Sepharose 4B resin (Amersham Pharmacia Biotech), in the presence of 10 μg of affinity-purified GST or GST fusion protein. The resin and associated proteins were washed three times with ice-cold PBS and analyzed by Western blot as described above.

Immunoprecipitation

The protocol for immunoprecipitation studies was derived from the method described by Bonifacino *et al.* (1999). Briefly, 500 μl of Dounce homogenized lysate (~0.5–1.0 mg protein) from COS-7 cells transfected with pEGFP-11 or with pEGFP-C2 (diluted 25-fold to match EGFP–11 levels) was incubated with end-to-end rotation for 2–3 h at 4°C with a 15-μl bed volume of equilibrated protein A–Sepharose CL-4B resin (Amersham Pharmacia Biotech) which was previously bound to 0.5–1.0 μg rabbit anti-Grb2 polyclonal antibody (StressGen Biotechnologies) in the presence of 0.1 mg bovine serum albumin in 20 mM Tris–Cl, pH 7.4, for 1 h at 4°C. The resin and

associated proteins were washed twice with 20 mM Tris–Cl, pH 7.4, and analyzed by Western blotting using a mouse anti-green fluorescent protein monoclonal antibody (StressGen Biotechnologies).

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