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Tandem SH2 binding sites mediate the RasGAP–RhoGAP interaction: a conformational mechanism for SH3 domain regulation

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Many cellular signaling proteins contain SH3 (Src homology 3) domains that mediate protein interactions via specific proline-containing peptides. Unlike SH2 domains, whose interactions with tyrosine-containing peptides are promoted by phosphorylation of the SH2 binding site, the regulatory mechanism for SH3 interactions is unclear. p120 RasGAP (GTPase-activating protein), which contains an SH3 domain flanked by two SH2 domains, forms an abundant SH2-mediated complex with p190 RhoGAP in cells expressing activated tyrosine kinases. We have identified two closely linked tyrosine-containing peptides in p190 that bind simultaneously to the RasGAP SH2 domains upon p190 phosphorylation. This interaction is expected to bring the two SH2 domains into close proximity. Consequently, RasGAP undergoes a conformational change that results in a 100-fold increase in the accessibility of the target binding surface of its SH3 domain. These results indicate that the tandem arrangement of SH2 and SH3 domains found in a variety of cellular signaling proteins can provide a conformational mechanism for regulating SH3-dependent interactions through tyrosine phosphorylation. In addition, it appears that the role of p190 in the RasGAP signaling complex is to promote additional protein interactions with RasGAP via its SH3 domain.

Keywords: p190/RasGAP/RhoGAP/SH3 domain/SH2 domain

Introduction

SH3 domains have been identified in a variety of cellular proteins, many of which function as components of signal transduction pathways (Cohen *et al.*, 1995; Pawson, 1995). These include the large array of Src family kinases, RasGAP, phospholipase C-γ (PLC-γ), p85 phosphatidylinositol-3 kinase, Stat and the adaptor proteins, Crk, Grb2 and Nck. This conserved domain, which is ~55 amino acids in length, has been found to mediate a high-affinity interaction between the SH3-containing protein and specific proline-rich peptides which have been identified in numerous proteins (Ren *et al.*, 1993). While the precise role of SH3 domains has not been elucidated, it appears that the interactions promoted by these domains can direct such aspects of protein function as subcellular localization and cytoskeletal association (Bar-Sagi *et al.*, 1993), enzyme complex assembly (Sumimoto *et al.*, 1994) and tyrosine kinase substrate specificity (Weng *et al.*, 1994). Although the structural basis for many of these interactions has now been established (Musacchio *et al.*, 1992; Kohda *et al.*, 1993; Koyama *et al.*, 1993; Yu *et al.*, 1992, 1994; Yang *et al.*, 1994; Cohen *et al.*, 1995), the mechanism by which such interactions are regulated remains unclear. Moreover, there is evidence indicating that at least some of these interactions may be unregulated and are formed constitutively (Egan *et al.*, 1993; Gale *et al.*, 1993).

In contrast to SH3 domains, the mechanism by which SH2-mediated protein–protein interactions are regulated has been established (Cohen *et al.*, 1995; Pawson, 1995). These conserved domains, which are also found in a large number of cellular signaling proteins, mediate binding to specific phosphotyrosine-containing peptides (Moran *et al.*, 1990). For these interactions, activation of tyrosine kinases which are able to phosphorylate the tyrosine in the SH2 binding site appears to serve as the regulatory step (Moran *et al.*, 1990). Several signaling proteins contain multiple tandemly arranged SH2 and SH3 domains, thereby adding an additional layer of complexity to the potential regulatory mechanisms that direct specific protein–protein interactions within the cell (Pawson, 1995). Most likely, these tandem arrangements of SH2 and SH3 domains give rise to multiprotein complexes, or allow a single protein to participate in distinct signaling complexes, thus providing a combinatorial strategy to generate a large number of diverse functions using a relatively small number of proteins. In the case of the adaptor proteins, which consist almost exclusively of SH2 and SH3 domains, their only function appears to be that of a multi-port docking station that promotes particular combinations of protein interactions (Lowenstein *et al.*, 1992; Matsuda *et al.*, 1994). The commonly observed juxtaposition of SH2 and SH3 domains within a protein structure additionally suggests that their individual interactions with various targets potentially could be either coordinated or constrained by conformational and steric effects.

One of the most extensively studied proteins that contains both SH2 and SH3 domains is the Ras regulatory protein, p120 RasGAP (Trahey *et al.*, 1988). The amino-terminal region of RasGAP contains a single SH3 domain flanked by two SH2 domains (Trahey *et al.*, 1988). The RasGAP SH2 domains have been found to associate with activated growth factor receptors and with a tyrosine-phosphorylated protein, p62, which is mitogen regulated (Ellis *et al.*, 1990; Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990; Margolis *et al.*, 1990; Reedijk *et al.*, 1990; Pronk *et al.*, 1992). These are relatively low abundance protein complexes, whereas the major cellular complex formed with RasGAP is that with p190 RhoGAP, a Rho GTPase regulatory protein (Moran *et al.*, 1991; Settleman *et al.*, 1992a). In tyrosine kinase-transformed cells, nearly all of the RasGAP is associated with p190 in a cytoplasmic

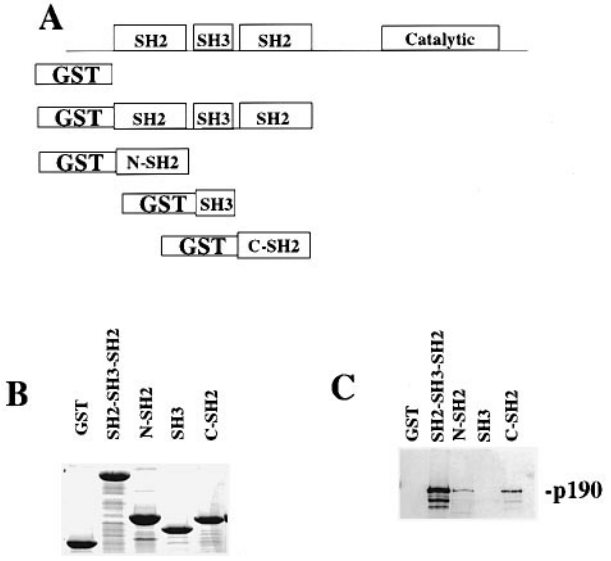

Fig. 1. Association of p190 with the amino-terminal SH2 domains of RasGAP. GST fusion proteins corresponding to various parts of the amino-terminal region of RasGAP (**A**) were expressed in bacteria and detected by SDS–PAGE followed by Coomassie Blue staining (**B**). (**C**) Lysates of Cos cells transfected with RcHA190 were incubated with the indicated GST–RasGAP fusion proteins immobilized on glutathione–agarose beads, and p190 binding was assayed by immunoblotting with the 12CA5 antibody.

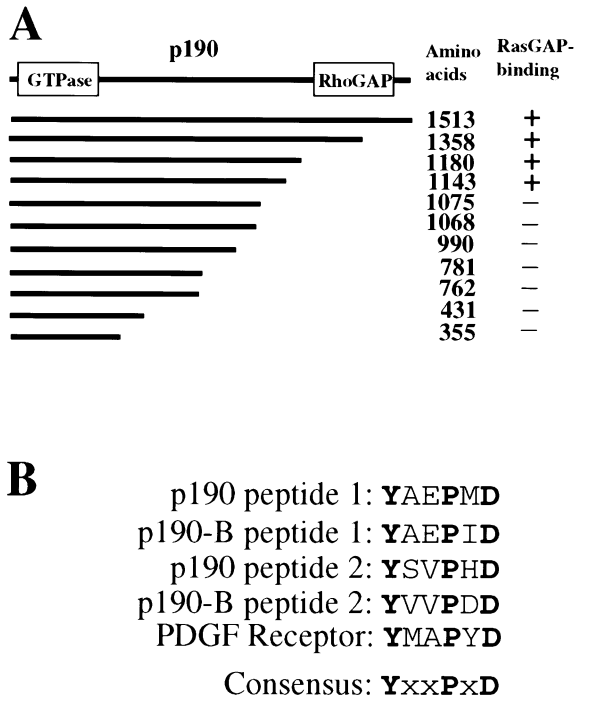

Fig. 2. Mapping the RasGAP binding domain of p190 with truncation mutants expressed in Cos cells. (**A**) Cos cells were transfected with p190 expression constructs encoding carboxy-terminal truncation mutants of the indicated sizes. At 48 h post-transfection, cell lysates were prepared and incubated with the GST–SH2–SH3–SH2 domain of RasGAP immobilized on glutathione beads. Binding was scored by immunoblotting the bound protein with p190 antibodies. Expression of each of the p190 mutants was confirmed by immunoblotting of whole cell lysates following transfection (not shown). (**B**) Alignment of two related tyrosine-containing peptide sequences found in the RasGAP binding region of rat p190 with analogous regions of a p190 homolog, p190-B (Burbelo *et al.*, 1995), and with the RasGAP binding region of the PDGF receptor (Fantl *et al.*, 1992).

complex, and this interaction is mediated by the two RasGAP SH2 domains (Moran *et al.*, 1991; Koch *et al.*, 1992). In light of the ability of RasGAP and p190 to regulate the GTPase activity of Ras and Rho proteins, respectively, it has been suggested that this protein complex may serve to coordinate Ras- and Rho-mediated signaling pathways (Settleman *et al.*, 1992a). However, the biological consequences of RasGAP–p190 complex formation have not yet been determined. To gain insight into the regulatory mechanism and function of this protein complex, we examined the structural requirements of the SH2-mediated RasGAP–p190 interaction. Surprisingly, this analysis revealed that the likely function of p190 in the RasGAP signaling complex is to promote a substantial conformational change in RasGAP that exposes the normally inaccessible target binding surface of the SH3 domain, thereby making it available for additional protein interactions.

Results

Mapping the RasGAP binding sites in p190 RhoGAP

To establish the structural requirements of the RasGAP–p190 complex, we first developed an assay for a RasGAP SH2-dependent interaction with p190 in transfected Cos cells. GST fusion proteins containing all or part of the amino-terminal SH2–SH3–SH2 domain of RasGAP (Figure 1A and B) were immobilized on glutathione–agarose beads and used as affinity reagents to capture p190 from lysates of Cos cells that had been transfected with an amino-terminally epitope-tagged (HA) form of p190. Associated p190 was then detected by immunoblotting with anti-HA antibodies. As shown (Figure 1C), p190 readily associates with the RasGAP SH2–SH3–SH2 domain, but only weakly with the individual SH2 domains. The isolated SH3 domain does not bind p190. Similar results with baculovirus-produced p190 have been reported recently (Bryant *et al.*, 1995). To map the binding site(s) on p190 that mediates the RasGAP interaction, we prepared carboxy-terminal truncation mutants of p190 in the Cos expression vector and tested their ability to bind the RasGAP SH2–SH3–SH2 domain following transfection. This analysis localized the RasGAP binding region to amino acids 1075–1142 of p190, which are located just upstream of the carboxy-terminal RhoGAP catalytic domain (Figure 2A).

Inspection of this sequence revealed that it contains two closely spaced tyrosines (Y1087 and Y1105) within peptide sequences that have the same amino acids at positions +3 (proline) and +5 (aspartic acid) relative to the tyrosine (Figure 2B). Notably, this sequence motif (YxxPxD) is also conserved in the RasGAP SH2 binding site in the platelet-derived growth factor (PDGF) receptor (Fantl *et al.*, 1992) and at analogous positions in a recently reported p190-related protein (p190-B), which also binds RasGAP (Burbelo *et al.*, 1995). To test the possibility that these sites mediate RasGAP binding, we prepared synthetic peptides (10mers) corresponding to the two putative RasGAP binding sites in p190 (amino acids 1083–1092 and amino acids 1102–1111). Each peptide was synthesized either with or without phosphorylated tyrosine at the

relevant position and then tested for the ability to block an interaction between the GST–SH2–SH3–SH2 fusion protein and p190 expressed in baculovirus-infected insect cells. The phosphorylated peptides (100 μM) are each able to block nearly all of the complex formation with p190, whereas the unphosphorylated peptides, even when added together at high concentration, are unable to affect the interaction (Figure 3A). Fifty percent inhibition of binding could be achieved with ~10 μM phosphopeptides (not shown).

To confirm that a similar binding mechanism is used in the context of the full-length RasGAP, we performed the same peptide competition experiment using insect cell lysates expressing full-length RasGAP and p190. For this analysis, we took advantage of a previous observation that the RasGAP–p190 complex can be formed efficiently *in vitro* by mixing lysates of RasGAP and p190 baculovirus-infected insect cells (Foster *et al.*, 1994). In such cells, p190 contains readily detectable levels of tyrosine phosphorylation as revealed by anti-phosphotyrosine immunoblotting (not shown). As with the isolated SH2-containing region of RasGAP, the interaction of full-length RasGAP with p190 is completely blocked by the p190 phosphopeptides (Figure 3B). Notably, either of the two individual phosphopeptides can completely block complex formation, suggesting that both of the RasGAP SH2 domains are able to bind either of these two p190 regions. This was in fact confirmed by performing a similar peptide competition experiment with the isolated individual GST–SH2 fusion proteins (data not shown). It is possible, however, that only one of the two possible orientations of these proteins relative to each other is normally found in the cellular complex between full-length RasGAP and p190 due to additional conformational constraints.

To confirm the requirement for these p190 domains in the RasGAP–p190 complex, we constructed and expressed mutated forms of p190 in which small in-frame deletions were generated that correspond to the putative binding sites. Three p190 mutants were generated: M1, a 10 amino acid deletion (1083–1092); M2, a 10 amino acid deletion (1102–1111); or M3, a 29 amino acid deletion (1083–1111) that removes both of the putative RasGAP binding peptides in p190. Each mutant was tested for RasGAP interaction in the Cos transfection assay. As shown (Figure 3C), deletion of either of the two binding sites in p190 greatly reduces the ability of p190 to interact with RasGAP, and deletion of both binding sites completely abolishes binding. Together, these results confirm the requirement for these two binding sites in p190 for the RasGAP interaction, and are consistent with a binding mechanism that involves both of the RasGAP SH2 domains.

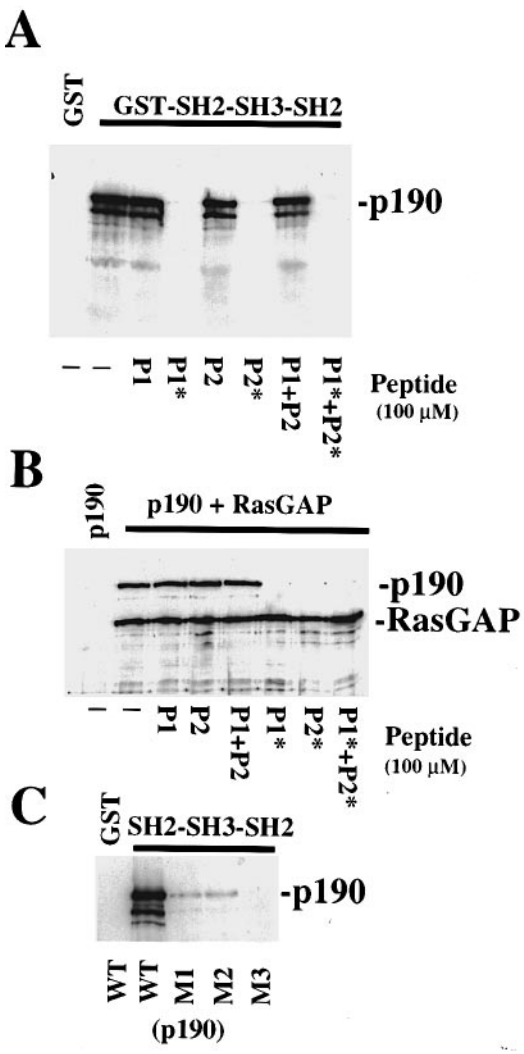

Fig. 3. Inhibition of RasGAP–p190 complex formation by specific p190 phosphopeptides. (**A**) Glutathione beads bound to the GST–SH2–SH3–SH2 domain of RasGAP were pre-incubated with or without 100 μM of specific p190 peptides (or combinations of peptides), as indicated. GST alone (left lane) was included as a negative control. Peptides are described in Materials and methods. An asterisk indicates phosphotyrosine in the peptide. After pre-incubation, the beads were incubated with insect cell lysate containing baculovirus-produced p190, then washed, subjected to SDS–PAGE and immunoblotted with p190-specific antibodies. The band just below p190 corresponds to a commonly observed p190 degradation product. (**B**) Insect cell lysates containing either p190 alone (left lane) or RasGAP plus p190 (all other lanes) were incubated in the presence of the indicated p190 peptides (100 μM) and then subjected to immunoprecipitation with anti-RasGAP antibodies followed by immunoblotting of RasGAP and p190 to detect complex formation. (**C**) Glutathione beads bound to GST alone (left lane) or GST–SH2–SH3–SH2 were incubated with lysate from Cos cells that had been transfected with epitope-tagged (HA) wild-type p190 (WT) or p190 mutants containing small in-frame deletions in the putative RasGAP binding sites. M1 lacks amino acids 1083–1092, M2 lacks amino acids 1102–1111 and M3 lacks amino acids 1083–1111. Bound protein was then analyzed by SDS–PAGE and immunoblotting with anti-HA antibody to detect p190. M1, M2 and M3 p190 mutants are detected at levels equivalent to wild-type p190 in immunoprecipitations (not shown).

A small region of p190 is necessary and sufficient for RasGAP binding

To determine whether the identified RasGAP binding region of p190 is sufficient for the interaction, we constructed and expressed a GST fusion protein, GST–GBD (GAP binding domain), that contains an 89 amino acid region of p190 comprising the two RasGAP binding sites, and examined its ability to bind RasGAP (Figure 4A). The fusion protein was immobilized on beads and used to capture RasGAP from Src-transformed fibroblast lysates. In parallel, RasGAP and associated p190 were co-immunoprecipitated from cell lysates with anti-RasGAP antibodies. Bound proteins were detected by immunoblotting with RasGAP- and p190-specific antibodies. As shown in Figure 4B, GST–GBD (but not GST alone) efficiently binds RasGAP in whole cell lysates. Notably,

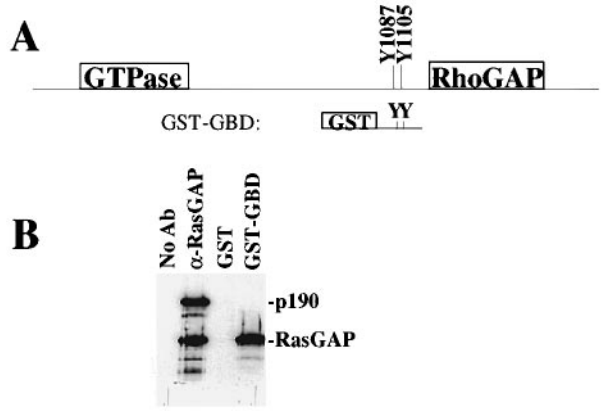

Fig. 4. A small region of p190 is sufficient for RasGAP binding. (**A**) The location of the GAP binding domain (GBD) in the p190 protein that was expressed as a GST fusion protein. (**B**) Lysate from Src-transformed Rat-1 cells was incubated with beads bound to either GST, GST–GBD or RasGAP antibody, as indicated. Bound protein was analyzed by SDS–PAGE followed by immunoblotting with anti-RasGAP and anti-p190 antibodies.

whereas anti-RasGAP immunoprecipitates contain both RasGAP and p190, the GST–GBD complex contains only the monomeric, non-p190-complexed form of RasGAP. These results demonstrate that this small region of p190 is both necessary and sufficient for RasGAP interaction. In addition, the fact that p190 is not present in the GST–GBD complex indicates that all of the cellular interactions of p190 with RasGAP are mediated by this domain in p190.

Bacterially produced GST–GBD is not tyrosine phosphorylated, indicating that complex formation can be achieved *in vitro* in the absence of phosphorylation. A similar observation was made previously in the baculovirus system (Bryant *et al.*, 1995). Phosphorylation-independent binding is only seen at high protein concentrations, whereas at lower, more physiological protein concentrations, there appears to be a strict requirement for tyrosine phosphorylation, similar to that seen with most other SH2 interactions (see below). The finding that tyrosine phosphorylation is dispensable for binding at high protein concentrations, whereas small peptide deletions that include the tyrosines disrupt binding, also indicates an important role for amino acids near the tyrosine residues in the SH2 binding sites. An important role for the +3 amino acid (relative to tyrosine) has been demonstrated for several SH2-mediated interactions (Songyang *et al.*, 1993), and a role for amino acids on the amino-terminal side of the tyrosine has also been reported (Bibbins *et al.*, 1993). In addition, the fact that SH2 binding by the synthetic p190 peptides requires tyrosine phosphorylation suggests that the tandem SH2 binding sites in p190 form a structure that is highly conducive to RasGAP binding, but which cannot be mimicked even by the combination of the two synthetic p190 peptides.

The RasGAP binding domain of p190 is the major site of tyrosine phosphorylation in vivo

p190 has been reported to undergo tyrosine phosphorylation and association with RasGAP in cells expressing activated tyrosine kinases (Ellis *et al.*, 1990; Moran *et al.*, 1991). To determine whether the identified RasGAP

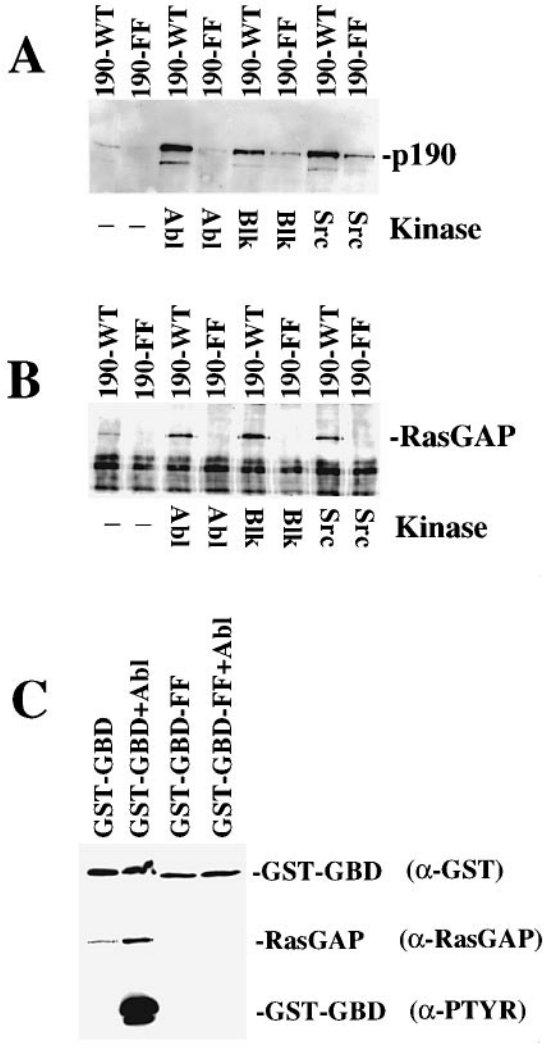

Fig. 5. The RasGAP binding domain of p190 undergoes tyrosine phosphorylation *in vivo*. (**A**) Cos cells were transfected with the wild-type RcHA190 expression vector or the double tyrosine (1087F, 1105F) mutant p190 (190-FF) in the presence or absence of the indicated tyrosine kinases. After 48 h, cell lysates were assayed by immunoprecipitation of transfected p190 with the 12CA5 antibody followed by immunoblotting with anti-phosphotyrosine antibody. (**B**) The same blot was stripped and re-probed with anti-RasGAP antibody to detect RasGAP–p190 complex in transfected cells. (**C**) Cos cells were transfected with a vector expressing the GST–GBD fusion protein (or the double tyrosine mutant, GST–GBD-FF) plus or minus an Abl kinase vector and then assayed by immunoblotting for either expression of GST–GBD (top panel), or for RasGAP association with GST–GBD (middle panel) or tyrosine phosphorylation of GST–GBD (lower panel) after binding of GST–GBD in whole cell lysates to glutathione beads.

binding sites in p190 are the major sites of phosphorylation by tyrosine kinases *in vivo*, we examined the ability of several tyrosine kinases to promote phosphorylation of p190 tyrosines 1087 and 1105. p190 expression constructs were transfected into Cos cells together with activated forms of either Src, Blk or Abl tyrosine kinases, and tyrosine phosphorylation of p190 was examined by immunoprecipitation of transfected p190 followed by immunoblotting with anti-phosphotyrosine antibodies. As shown (Figure 5A), co-transfection of each of these kinases with p190 results in substantial tyrosine phosphorylation of p190. However, co-transfection of these kinases with

a p190 mutant in which tyrosines 1087 and 1105 have been substituted with phenylalanine (190-FF) did not give rise to a significant increase in p190 tyrosine phosphorylation, suggesting that most of the tyrosine phosphorylation on p190 *in vivo* occurs on the two tyrosines in the RasGAP binding domain.

The fact that the basal level of p190 tyrosine phosphorylation seen in the absence of co-transfected kinase is reduced in the double tyrosine mutant (190-FF) also indicates that these are the major sites of p190 tyrosine phosphorylation by endogenous tyrosine kinases. For the Src and Blk kinases, it appears that there is at least one additional site of p190 tyrosine phosphorylation, which is not seen with Abl. By re-probing this same immunoblot with anti-RasGAP antibodies, we were able to examine the relationship between phosphorylation of tyrosines 1087 and 1105 of p190 and the ability to associate with RasGAP. As shown in Figure 5B, phosphorylation of tyrosines 1087 and 1105 is necessary for p190 to co-precipitate endogenous RasGAP. Thus, these two tyrosines in the RasGAP binding domain of p190 appear to be the major sites of phosphorylation by several tyrosine kinases *in vivo*. Moreover, it seems likely that an important role for tyrosine phosphorylation of p190 by several different kinases is to regulate its association with RasGAP.

Since we had already observed that the RasGAP–p190-GBD interaction could be formed *in vitro* in the absence of p190 tyrosine phosphorylation when using high concentrations of GST–GBD fusion protein, we wanted to confirm that this interaction mimics the normal *in vivo* interaction of RasGAP and p190 that takes place when the proteins are present at more physiologic concentrations. For this analysis, we prepared mammalian expression constructs from which the GST–GBD fusion protein and a double tyrosine mutant (GST–GBD-FF) could be expressed in transfected cells. Cos cells were transfected with these plasmids in the presence or absence of a co-transfected Abl expression vector, and cell lysates were incubated with glutathione–agarose to capture expressed GST–GBD and associated RasGAP. As shown (Figure 5C), both GST–GBD and the GST–GBD-FF mutant are expressed efficiently in transfected cells, and GST–GBD (but not GST–GBD-FF) is tyrosine phosphorylated in the presence of Abl. Significantly, the presence of Abl increases the binding of GST–GBD to RasGAP, whereas GST–GBD-FF binding to RasGAP is not detectable even in the presence of Abl. Thus, these results demonstrate that despite the fact that at high protein concentration the phosphorylation requirement for binding can be bypassed *in vitro*, interaction of this binding region of p190 with RasGAP *in vivo* is regulated by phosphorylation of these two tyrosines in p190.

p190 promotes a conformational change in RasGAP that exposes the SH3 domain

The juxtaposition of the two SH2 binding sites in p190 (13 amino acid separation) suggests that binding of RasGAP to p190 via both of the SH2 domains potentially could promote a substantial conformational change in RasGAP that might affect the SH3 domain that is situated between the two SH2 domains. Therefore, we examined the possibility that the role of p190 in the RasGAP complex is to regulate the accessibility of the RasGAP SH3 domain. For these studies, we made use of a monoclonal antibody (B4F8) that we had previously generated that specifically recognizes the RasGAP SH3 domain. To confirm the antibody specificity, GST fusion proteins containing various regions of the amino-terminal portion of RasGAP were analyzed by immunoblotting with monoclonal antibody B4F8. As shown in Figure 6A, this antibody specifically recognizes an isolated 58 amino acid region of RasGAP that comprises only the SH3 domain. We hypothesized that by titrating the amount of this antibody in an immunoprecipitation of recombinant RasGAP, we could compare its ability to bind the RasGAP SH3 domain in the presence or absence of associated p190, and thereby measure accessibility of the SH3 domain.

To test this possibility, RasGAP-containing insect cell lysate was mixed with either a 20-fold excess of p190-containing lysate or an equivalent amount of lysate prepared from uninfected cells, and the mixed lysates were subjected to immunoprecipitation with a range of B4F8 antibody concentrations. Immunoprecipitated proteins were analyzed by immunoblotting with anti-RasGAP antibody to assess the efficiency of immunoprecipitation. As shown (Figure 6B), in the presence of excess p190, RasGAP can be immunoprecipitated by the SH3-specific antibody with much greater efficiency than RasGAP in the absence of p190. Based on the antibody titration results, we estimate that in the presence of p190 the accessibility of the RasGAP SH3 domain is increased by ~100-fold. As a control, the same experiment was performed with an antibody that recognizes a carboxy-terminal region of RasGAP and, as expected, no difference in immunoprecipitation efficiency was observed in the presence or absence of p190 (Figure 6C). This result indicates that the conformational effects of p190 on RasGAP are confined to the SH3 domain.

We next examined the ability of the isolated RasGAP binding region of p190 to promote the same conformational change in RasGAP. For this experiment, insect cell lysate containing RasGAP was incubated with either the purified GST–GBD fusion protein (RasGAP binding domain of p190) or GST alone as a control. The samples were subjected to B4F8 immunoprecipitation as before. As shown (Figure 6D), GST–GBD is able to promote a conformational change in RasGAP as efficiently as the full-length p190 protein (100-fold). This result indicates that the only region of p190 required to promote access to the RasGAP SH3 domain is the region containing the tandem SH2 binding sites. We have also found that high concentrations of the RasGAP binding p190 phosphopeptides, even when mixed together, have no effect on the accessibility of the SH3 domain when incubated with RasGAP (data not shown). This suggests that simple occupancy of the RasGAP SH2 domains is not sufficient to expose the SH3 domain, but rather, the two SH2 domains must be brought into a specific conformation—a function that p190 apparently is able to serve due to its closely spaced SH2 binding domains. This hypothesis was confirmed using the antibody titration strategy with mutated forms of GST–GBD (GST–GBD-M1 and GST–GBD-M2) that contain 10 amino acid deletions that remove the individual RasGAP binding sites. As shown in Figure 6D, neither of these fusion proteins is able to affect the accessibility of the SH3 domain when incubated with

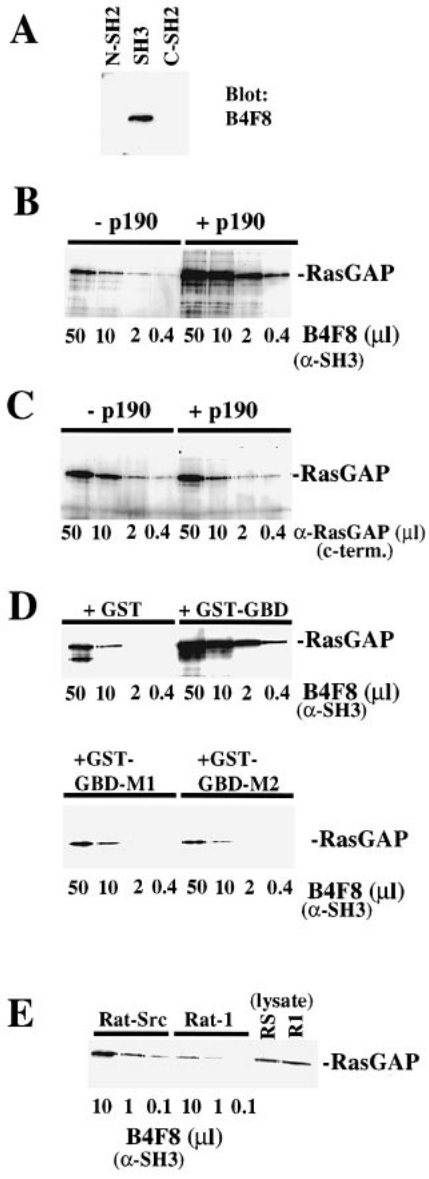

Fig. 6. p190 promotes a conformational change in RasGAP that exposes the target binding surface of the SH3 domain. (**A**) Anti-RasGAP antibody B4F8, which was previously found to recognize the amino-terminal region of RasGAP, was tested by immunoblotting for specificity for the SH3 domain. GST fusion proteins (10 ng) containing each of the RasGAP SH2 domains or the isolated 58 amino acid SH3 domain were analyzed by SDS–PAGE followed by immunoblotting with the B4F8 monoclonal antibody. (**B**) RasGAP-containing insect cell lysate was incubated (~10 ng of RasGAP per lane) with a 10-fold excess of either p190-containing insect cell lysate (+p190) or an equivalent amount of lysate from uninfected cells (–p190), and then subjected to immunoprecipitation with the indicated amounts of B4F8 antibody (~25 μg/ml). Samples were then analyzed by SDS–PAGE followed by immunoblotting with anti-RasGAP antibody. (**C**) A similar antibody titration experiment was performed using a RasGAP antibody that recognizes a carboxy-terminal region of RasGAP. (**D**) A B4F8 antibody titration experiment was performed [as in (B)] by incubating the RasGAP insect cell lysate in the presence of 2 μg of either purified GST protein, the GST–GBD fusion protein or GST–GBD mutants M1 and M2 prior to immunoprecipitation. (**E**) A B4F8 antibody titration experiment was performed to compare the accessibility of the RasGAP SH3 domain in Src-transformed Rat-1 fibroblasts and the parental normal Rat-1 line. In (B) and (D), the estimation of a 100-fold conformation effect of p190 binding is based on the observation that similar amounts of RasGAP are immunoprecipitated in the presence or absence of p190 with 0.4 and 50 μl of B4F8 antibody, respectively.

RasGAP. This result indicates that it is the coordinated action of both RasGAP binding sites in p190 that results in exposure of the SH3 domain.

To demonstrate that regulation of the RasGAP SH3 domain by tyrosine-phosphorylated p190 occurs *in vivo*, we took advantage of the fact that in Src-transformed Rat-1 fibroblasts, there is abundant p190 tyrosine phosphorylation and RasGAP–p190 complex formation relative to the untransformed parental Rat-1 cells (Moran *et al.*, 1991; Foster *et al.*, 1994). Titration of the SH3-specific antibody in RasGAP immunoprecipitations of cell lysates from these two lines (normalized for RasGAP levels) revealed that in the Src-transformed cells, accessibility of the RasGAP SH3 domain is increased by ~20-fold relative to that seen in untransformed Rat-1 cells (Figure 6E). These *in vivo* observations are consistent with the *in vitro* results, and support a specific regulatory role for p190 in the conformational changes in the RasGAP SH3 domain. It is likely that the apparently reduced effect of p190 on SH3 accessibility in the *in vivo* assay as compared with the *in vitro* assays (20-fold versus 100-fold) reflects the fact that the untransformed Rat-1 cells still contain detectable amounts of tyrosine-phosphorylated p190 as well as some co-immunoprecipitable RasGAP–p190 complex (data not shown). Therefore, it is expected that a fraction of the RasGAP in the Rat-1 cells will also have an exposed SH3 domain. It is also possible that the conformational change in the SH3 domain that we observe is in fact an underestimate of the actual change, since it is expected that the B4F8 antibody has to compete for RasGAP SH3 binding with the real cellular targets of this domain.

We wanted next to verify that the interaction of the SH3-specific B4F8 antibody with RasGAP reflects an interaction similar to that which normally occurs between the RasGAP SH3 domain and its cellular target(s). One of the most highly conserved amino acids in SH3 domains is a tryptophan (W317 in RasGAP) that participates directly in the interaction between the SH3 domain and the proline-rich SH3 binding site, as revealed by structural and mutational studies of several SH3-containing proteins (Musacchio *et al.*, 1992; Yu *et al.*, 1992, 1994; Kohda *et al.*, 1993; Koyama *et al.*, 1993; Mattingly *et al.*, 1994; Guruprasad *et al.*, 1995). Moreover, the NMR solution structure of the isolated RasGAP SH3 domain revealed that amino acid W317 is located in the apparent binding surface and is spatially arranged in a manner similar to that seen in PLC-γ, suggesting that this tryptophan residue participates in target binding (Yang *et al.*, 1994). Therefore, we used site-directed mutagenesis to change this tryptophan in RasGAP to lysine and then examined the ability of the B4F8 antibody to recognize the mutated RasGAP. As shown (Figure 7A), a GST–SH3 fusion protein containing the substituted tryptophan is not recognized by the B4F8 antibody, despite protein expression equivalent to the wild-type GST–SH3 (Figure 7B). This result indicates that B4F8 recognizes the target binding site of the RasGAP SH3 domain and suggests that the ability of B4F8 to bind RasGAP should closely approximate the interaction of the RasGAP SH3 domain with cellular targets. Consistent with this hypothesis, we have found that another monoclonal antibody which is also specific for the RasGAP SH3 domain (D4B4) is unaffected by p190 association in its

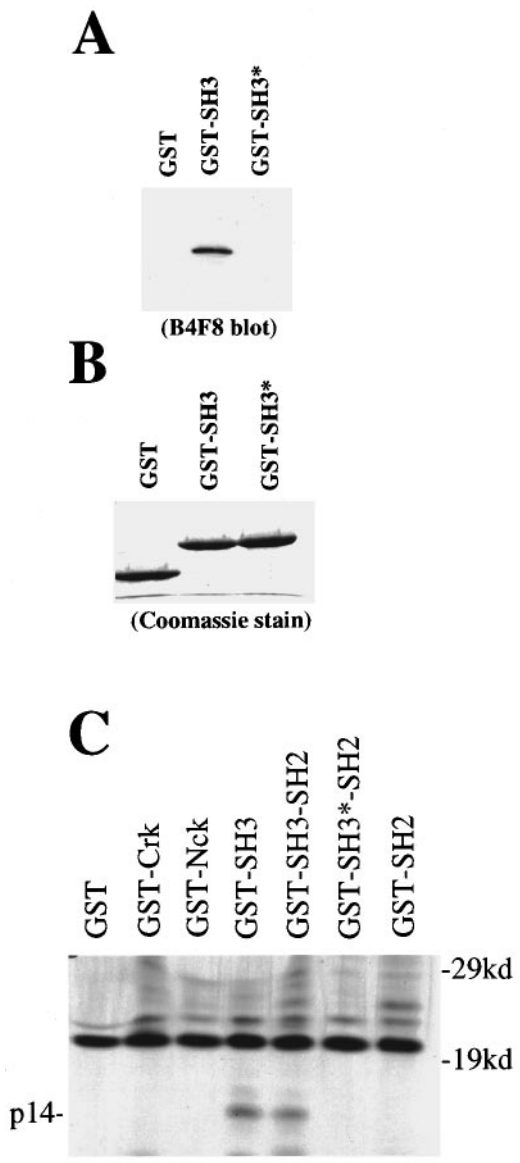

Fig. 7. Detection of a RasGAP SH3 binding protein. GST, GST–SH3 and the lysine-substituted GST–SH3 (GST–SH3*) fusion proteins were subjected to SDS–PAGE followed by either immunoblotting with B4F8 antibody (**A**) or Coomassie Blue staining (**B**). (**C**) ^{35}S-Labeled Rat-src cells were incubated with either GST beads alone or GST fusions containing the RasGAP SH3, the SH3–SH2 domain, the C-terminal SH2 domain or the SH3-containing Crk and Nck proteins, as indicated. In addition, the lysine-substituted RasGAP SH3 domain was tested in the SH3–SH2 fusion (GST–SH3*–SH2). Bound proteins were analyzed by 15% SDS–PAGE followed by fluorography. The RasGAP SH3 binding protein is indicated as p14.

ability to bind RasGAP and is still able to recognize the mutated SH3 domain (data not shown). Therefore, it appears that the increased accessibility of the RasGAP SH3 domain by p190 is limited to the target binding surface of the SH3 domain.

Detection of a RasGAP SH3 binding protein

To determine whether we could detect a specific interaction between the RasGAP SH3 domain and any cellular protein, we used the GST–SH3 fusion protein immobilized on beads to capture putative SH3 binding proteins from lysates of metabolically radiolabeled fibroblasts. A single reproducibly detected protein of ~14 kDa was found to associate with the RasGAP SH3 domain or with a RasGAP SH3–SH2 fusion, but not with GST alone, a GST–SH2 fusion protein or SH3-containing fusions of the Crk and Nck proteins (Figure 7C). In addition, the RasGAP SH3 mutant containing the tryptophan substitution is defective in its ability to bind the 14 kDa protein. Together with the previous observations, these results indicate that there is at least one specific cellular target of the RasGAP SH3 domain which exhibits SH3 binding properties very similar to those seen with the B4F8 antibody, and is likely, therefore, to be influenced by p190. Due to relatively low levels of the 14 kDa SH3 target, it was not possible to detect an association between this protein and the endogenous RasGAP protein by co-immunoprecipitation from Rat-src cells (not shown).

Discussion

We have investigated the structural basis for the RasGAP–p190 protein complex. This analysis revealed that tandem SH2 binding sites in p190 RhoGAP mediate a high-affinity interaction with RasGAP upon tyrosine phosphorylation of p190. Moreover, we have found that the tyrosines in these two binding sites of p190 (Y1087 and Y1105) are the major substrates *in vivo* for both Abl and Src family tyrosine kinases, suggesting that an important role for p190 phosphorylation by these kinases is to promote the association of p190 with RasGAP. It has been demonstrated previously that p190 is a major substrate for tyrosine phosphorylation in cells that are oncogenically transformed by tyrosine kinases, suggesting that formation of the RasGAP–p190 complex may be a critical step in the transformation process (Ellis *et al.*, 1990; Moran *et al.*, 1991). Notably, p190 is a prominent tyrosine-phosphorylated protein in human chronic myelogenous leukemia cells (Druker *et al.*, 1992).

Although the biological consequences of the RasGAP–p190 interaction are unknown, we have shown that the SH2-dependent association of RasGAP with p190 results in a local conformational change in RasGAP that exposes the target binding surface of the SH3 domain. It is possible that the the sole function of p190 in the RasGAP–p190 complex is to promote this specific conformational change in RasGAP, thereby allowing RasGAP to undergo additional protein interactions via its SH3 domain (Figure 8). However, the fact that RasGAP and p190 exhibit catalytic activities for Ras and Rho GTPases, respectively, and that p190 possesses its own GTPase activity (Foster *et al.*, 1994), raises the possibility that the RasGAP–p190 complex may be subject to additional levels of regulation or may have additional signaling properties. In fact, it has been reported that RasGAP catalytic activity is reduced 3- to 4-fold when complexed with p190 (Moran *et al.*, 1991).

The isolated RasGAP SH2–SH3–SH2 domain (but not full-length RasGAP) has been found to be active in several biological assays when overexpressed. These include cytoskeletal reorganization (McGlade *et al.*, 1993), adhesion to extracellular matrix (McGlade *et al.*, 1993), transcriptional activation (Medema and de Laat, 1992), oncogenic transformation (McGlade *et al.*, 1993; Mattingly *et al.*, 1994), uncoupling of G-proteins from muscarinic receptors (Martin *et al.*, 1992) and neuronal differentiation (Nakata and Watanabe, 1996). Notably, we find that the SH3

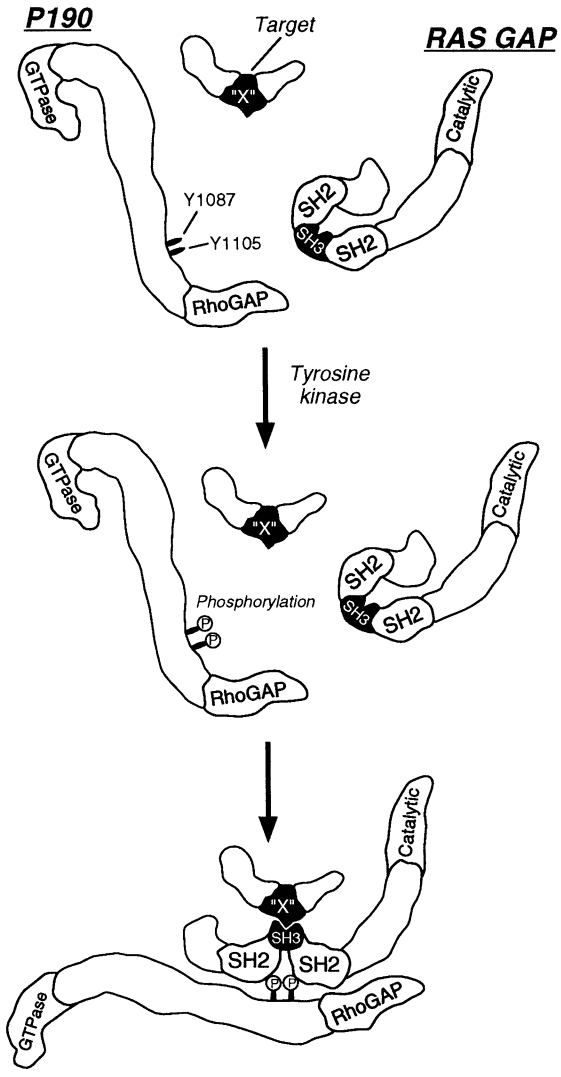

Fig. 8. Model depicting the postulated role of p190 in the RasGAP–p190 complex. Upon tyrosine phosphorylation, p190 binds RasGAP via the two RasGAP SH2 domains. This binding results in a conformational change that exposes the target binding surface of the RasGAP SH3 domain, thereby allowing RasGAP to undergo additional protein interactions.

domain in this isolated region of RasGAP is fully accessible, and is unaffected by association with p190 (our unpublished observation). This result suggests that the apparently activated state of this isolated region of RasGAP may be due to constitutive exposure of the SH3 domain by relief from conformational constraints that normally occur in the context of the full-length protein, where it appears to be buried. Consistent with this hypothesis is the finding that the biological activities of the isolated SH2–SH3–SH2 domain are mediated by the SH3 domain (Duchesne *et al.*, 1993; Mattingly *et al.*, 1994).

Although the precise nature of the effector signal from the RasGAP SH3 domain has not been established in mammalian cells, a RasGAP SH3-mediated signal from Ras to the cdc2 kinase appears to be required for *Xenopus* oocyte maturation (Pomerance *et al.*, 1996). This signal is independent of MAP kinase activation, suggesting that there may be a distinct Ras-mediated effector signal that is transmitted via the RasGAP SH3 domain. The results described here suggest that such a signal may depend on the activation of p190 by tyrosine phosphorylation. Since Src family kinases are able to promote specific phosphorylation of the RasGAP binding site in p190, it appears that the RasGAP–p190 complex may be a point of convergence for Ras- and Src-mediated signaling pathways. This hypothesis is also supported by the observation that a myristylation-defective Src protein cooperates with the isolated SH2–SH3–SH2 domain of RasGAP to transform cultured fibroblasts to an oncogenic state (DeClue *et al.*, 1993).

Unlike most previously described SH2-mediated interactions, which are detected at cellular membranes or cytoskeletal structures, the RasGAP–p190 complex is predominantly cytoplasmic (Moran *et al.*, 1991). In addition, the complex is quite abundant relative to other reported SH2-mediated complexes, and nearly all of the cellular RasGAP is associated with p190 in Src-transformed cells (Moran *et al.*, 1991). Together, these observations raise the possibility that the RasGAP–p190 complex may function as a cytoplasmic 'sink' for some RasGAP SH3 binding protein, potentially neutralizing its activity by making it unavailable. Recently, a 68 kDa RNA binding protein has been identified as a specific interactor with the RasGAP SH3 domain *in vitro* (Parker *et al.*, 1996). Thus far, this is the only reported target of the RasGAP SH3 domain; however, the biological relevance of this interaction has not yet been established. Moreover, we have been unable to detect this protein complex in the Src-transformed Rat-1 cells described here. Thus, determining the precise role of the RasGAP SH3 domain for the biological function of RasGAP will probably have to await the identification of additional SH3 targets. The 14 kDa RasGAP SH3 binding protein identified in these studies is a strong candidate for a relevant interactor, since the interaction is specific for the SH3 domain of RasGAP and it requires the target binding surface of the SH3 domain, suggesting that its interaction with RasGAP will probably be influenced by p190 association. Therefore, efforts are underway to identify this putative SH3 target protein.

Although the structural basis for several SH3-mediated protein interactions has now been established, the regulatory mechanism for such interactions has not been elucidated previously. The results described here indicate that local conformational changes can play an important regulatory role in such interactions. This type of indirect regulatory mechanism for SH3 interactions potentially provides additional specificity to protein complex formation. That is, unlike many SH2-regulated interactions, which depend only on activation of a particular tyrosine kinase and the presence of a relevant substrate, it appears that the interaction between RasGAP and putative SH3 binding target proteins requires activation of a tyrosine kinase as well as the presence of both RasGAP and p190. Since the expression of p190 is restricted to the nervous system during embryogenesis (M.Brouns and J.Settleman, unpublished observation), whereas RasGAP is widely expressed (Henkemeyer *et al.*, 1995), it is conceivable that the RasGAP SH3 domain is only 'activated' in a subset of cells that express both RasGAP and p190.

Our studies of the RasGAP–p190 complex suggest that

tyrosine kinase activity can regulate SH3-mediated protein interactions indirectly. Thus, like SH2-directed interactions, at least some SH3-dependent interactions are likely to mediate the biological effects of activated tyrosine kinases. Although RasGAP has a somewhat unique arrangement of SH2 and SH3 domains, there is evidence that other proteins may utilize a similar regulatory mechanism to direct protein interactions. For example, phosphorylation of the regulatory tyrosine (Y527) of c-Src results in an intramolecular interaction involving the Src SH2 domain that also appears to influence the properties of the Src SH3 domain (Superti-Furga *et al.*, 1993). In addition, the SH3-mediated Grb2–Sos complex is influenced by the kinase-dependent association of Shc with the Grb2 SH2 domain in activated T cells (Ravichandran *et al.*, 1995). This might be explained by a SH2-dependent Shc interaction with Grb2 that promotes a conformational change that is conducive to the SH3-mediated Grb2–Sos interaction. Since several signaling proteins have now been identified that contain tandemly arranged SH2 and SH3 domains, it is possible that a similar conformational mechanism is used widely to regulate SH3-mediated interactions.

Materials and methods

Plasmid construction and mutagenesis

GST fusion constructs were prepared by PCR-directed subcloning of the indicated regions of RasGAP or p190 into pGex plasmids (Pharmacia). Inserts were confirmed by DNA sequencing. Sequences from the RasGAP SH2–SH3–SH2 region were obtained from a human RasGAP cDNA clone (kindly provided by F.McCormick). GST–GBD contains amino acids 1055–1143 of rat p190. GST–Crk and GST–Nck expression vectors were kindly provided by B.Mayer. To express p190 in transfected cells, an HA epitope was added to the 5′ end of the rat p190 coding sequence (Settleman *et al.*, 1992b) by PCR and the entire coding sequence was subcloned into the RcCMV expression plasmid (Invitrogen). The plasmid is referred to as RcHA190. Tyrosine kinase expression constructs were kindly provided by Joan Brugge (Src), Shiv Pillai (Blk) and Rick Van Etten (Abl). The mammalian expression vector form of GST–GBD was prepared by subcloning into the vector PEBG, which expresses GST fusion proteins under the control of the EF-1α promoter. To generate carboxy-terminal truncation mutants of p190 in the RcHA190 expression construct, the Erase-a-base system (Promega) was used. The 3′ ends of deletion mutants were confirmed by DNA sequencing and expression of truncated protein was confirmed by immunoblotting of lysates from transfected Cos cells with the D2D6 anti-p190 monoclonal antibody. For single amino acid substitution mutations in the p190 coding sequence and the tryptophan to lysine substitution of the RasGAP SH3 domain, the pSELECT (Promega) mutagenesis system was used according to the manufacturer's specifications. The region of p190 containing the mutation(s) was sequenced to confirm that only the expected substitution was introduced and the mutated fragment was then subcloned into the full-length p190 expression vector. To generate the M1, M2 and M3 deletion mutations in p190, an overlapping PCR strategy was used to generate specific in-frame deletions of the indicated peptide sequences, and deletions were confirmed by DNA sequencing. GST–GBD-M1 and GST–GBD-M2 are mutant forms of GST–GBD prepared by PCR subcloning of deletion mutants M1 and M2, respectively.

Cell culture and transfections

Cos-7, Rat-1 and Rat-src cell lines were maintained at 37°C in Dulbecoo's modified Eagle's medium supplemented with antibiotics and 10% heat-inactivated fetal bovine serum (or 10% calf serum for Rat-1). Cells were passaged regularly by trypsinization. High five insect cells were maintained at 28°C in Grace's complete medium supplemented with 10% fetal calf serum. For production of p190 and RasGAP proteins in insect cells, baculoviruses encoding RasGAP or p190 were used to infect 15 cm plates of High five cells at 70% confluence and infected cells were collected 48 h post-infection for preparation of protein lysates. For Cos transfections, cells at 80% confluence in 10 cm plates were incubated with 5 or 10 μg of plasmid DNA, mixed with DEAE–dextran for 4–5 h, and then subjected to a 45 s shock with 10% dimethylsulfoxide. Cells were collected for protein analysis by scraping 48 h after transfection. For metabolic labeling experiments, Rat-src cells were incubated in methionine-free medium supplemented with [^{35}S]methionine (NEN) at a concentration of 0.1 mCi/ml for 6 h.

Immunoprecipitation and immunoblotting

Cells for protein analysis were collected by scraping and lysed in a solution containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 5 mM EGTA, 1% Triton X-100, 10% glycerol, and protease inhibitors phenylmethylsulfonyl fluoride (PMSF), aprotinin and leupeptin, and phosphatase inhibitors sodium fluoride (20 mM) and sodium orthovanadate (1 mM). Lysates were clarified by centrifugation at 13 000 *g*. For immunoprecipitation, lysates were incubated for 1 h at 4°C with either anti-RasGAP monoclonal antibody or anti-HA epitope monoclonal antibody 12CA5 followed by a 1 h incubation with protein A–Sepharose. Samples were washed three times with a solution containing 20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA and 0.1% Triton X-100, and then resolved by SDS–PAGE (7.5% acrylamide). Proteins were then electroblotted by semi-dry transfer at 10 V for 1 h onto nitrocellulose. Filters were blocked with either 5% milk (for B4F8, 12CA5, GST and D2D6 antibodies) or 3% bovine serum albumin (for PY20) in TBST for 1 h and then incubated for 1 h in primary antibody. After six washes of 5 min each in TBST, filters were incubated for 30 min in horseradish peroxidase-conjugated secondary antibody (Bio-Rad), washed again, and developed by enhanced chemiluminescence (NEN) and autoradiography. The carboxy-terminal RasGAP antibody was kindly provided by Onyx Pharmaceuticals.

GST fusion protein binding assays

GST fusion proteins were expressed in XA90 bacteria by 2 h of IPTG induction of log-phase cultures. Proteins were purified from lysed cells on glutathione–agarose beads (Sigma) as previously described (Foster *et al.*, 1996). Protein expression was confirmed by SDS–PAGE followed by Coomassie Blue staining. For binding assays, ~2 μg of GST fusion protein immobilized on beads were incubated with cell lysate for 1 h at 4°C with gentle rocking. Beads were washed as for immunoprecipitation and analyzed by immunoblotting as described above.

Peptide competition studies

Four peptides were custom synthesized in milligram quantities and HPLC purified by Quality Controlled Biochemicals, Inc. Peptide P1 has the sequence DPSDYAEPMD (single letter code), and corresponds to p190 amino acids 1083–1092. Peptide P2 has the sequence ENIYSVP-HDS, and corresponds to p190 amino acids 1102–1111. Peptides P1* and P2* are identical in sequence to P1 and P2, respectively, but contain phosphotyrosine at the tyrosine position. For competition studies with GST–RasGAP SH2–SH3–SH2 fusion protein, GST fusion protein immobilized on beads (~2 μg) was pre-incubated for 30 min at 4°C with the various peptides at the indicated concentrations and then inbubated for 1 h with baculovirus-produced p190 (~200 ng). For studies with full-length RasGAP, lysate from RasGAP baculovirus-infected insect cells was pre-incubated with the various peptides as above and then mixed with an equivalent amount of lysate from p190 baculovirus-infected insect cells. RasGAP and associated p190 were then immunoprecipitated with RasGAP antibody and analyzed by immunoblotting RasGAP and p190 simultaneously.

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