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3BP-1, an SH3 domain binding protein, has GAP activity for Rac and inhibits growth factor-induced membrane ruffling in fibroblasts

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The SH3 binding protein, 3BP-1, was originally cloned as a partial cDNA from an expression library using the Abl SH3 domain as a probe. In addition to an SH3 binding domain, 3BP-1 displayed homology to a class of GTPase activating proteins (GAPs) active against Rac and Rho proteins. We report here a full length cDNA of 3BP-1 which extends the homology to GAP proteins previously noted. 3BP-1 functions *in vitro* as a GAP with a specificity for Rac-related G proteins. Microinjection of the 3BP-1 protein into serum-starved fibroblasts produces an inhibition of platelet-derived growth factor (PDGF)-induced membrane ruffling mediated by Rac. Co-injection of 3BP-1 with an activated Rac mutant that is unresponsive to GAPs, counteracts this inhibition. 3BP-1 does not show *in vitro* activity towards Rho and, in agreement with this finding, microinjection of 3BP-1 into fibroblasts has no effect on lysophosphatidic acid (LPA)-induced stress fiber assembly mediated by Rho. Thus 3BP-1 is a new and specific Rac GAP that can act in cells to counter Rac-mediated membrane ruffling. How its SH3 binding site interacts with its GAP activity remains to be understood.

Key words: cytoskeleton/GAP/Rac/SH3/3BP-1

Introduction

Src homology 3 (SH3) domains are non-catalytic regions of ~60 amino acids which were first identified as conserved sequences present in Src-related non-receptor tyrosine kinases and subsequently found in a variety of signaling molecules such as phospholipase C-γ (PLC-γ), the p85 subunit of phosphatidylinositiol 3- (PI 3-) kinase and also in cytoskeletal proteins such as myosin and spectrin (Koch *et al.*, 1991). Since SH3 domains are found in a wide variety of proteins present in single-cell and higher eukaryotes, important and at the same time diverse functions of these domains are likely (Musacchio *et al.*, 1992). While the full range of SH3 domain function is not yet clear, mutations or deletions in the SH3 domain of the Src and Abelson (Abl) non-receptor tyrosine kinases have been reported to activate their catalytic and transforming properties (Franz *et al.*, 1989; Jackson and Baltimore, 1989; Potts *et al.*, 1988; Hirai and Varmus, 1990) and there are indications that Abl kinase activity might be regulated through a cellular inhibitor which binds to its SH3 domain (Pendergast *et al.*, 1991).

The identification and functional analysis of proteins which bind to SH3 domains has provided new evidence concerning the functions of these conserved sequences. Through specific binding, SH3 domains may interact with a variety of related proteins which may function in different cellular processes. Several SH3 domain-containing proteins have recently been reported to bind or interact with GTPases or their regulators: the Src and PLC-γ SH3 domains, among others, have been shown to bind the dynamin GTPase and, in some cases, SH3 binding activates dynamin (Gout *et al.*, 1993; Herskovits *et al.*, 1993; Scaife *et al.*, 1994; Seedorf *et al.*, 1994). The SH3 domains of Src and the p85 subunit of PI 3-kinase have been shown to interact with proline-rich regions in cdc42 GTPase activating protein (GAP) (Barfod *et al.*, 1993) and the SH3 domains of Grb-2 have been shown to interact with mSos1, a guanine nucleotide exchange factor for Ras (Buday and Downward, 1993; Egan *et al.*, 1993; Gale *et al.*, 1993; Li *et al.*, 1993; Olivier *et al.*, 1993; Rozackis-Adcock *et al.*, 1993; Simon *et al.*, 1993). Finally, the yeast protein, BEM 1, which contains two SH3 domains, and is involved in the organization of the actin cytoskeleton through cell polarity and bud formation, interacts with the GDP–GTP exchanger, Cdc24 (Peterson *et al.*, 1994).

3BP-1 is a protein which was isolated through its ability to bind the SH3 domain of the Abl non-receptor tyrosine kinase in a cDNA expression library screen, and a short proline-rich segment of the protein was shown to mediate binding to the Abl SH3 (Cicchetti *et al.*, 1992; Cicchetti and Baltimore, 1995). Subsequently, a 10 amino acid proline-rich consensus motif derived from 3BP-1 and other proteins, was shown to be a target for SH3 binding (Ren *et al.*, 1993). 3BP-1 contains, in addition to an SH3 binding region, a segment with homology to the GAP region of Bcr, N-chimaerin, cdc42 GAP and p190. The GAP regions in these proteins stimulate the intrinsic GTPase activity of small Ras-related GTP binding proteins, converting them from their active GTP-bound state to their inactive GDP-bound form (Diekmann *et al.*, 1991; Settleman *et al.*, 1991; Barfod *et al.*, 1993; Lancaster *et al.*, 1994). The identification of SH3 binding proteins such as 3BP-1, cdc42 GAP and dynamin, among others, indicates that SH3 domains may mediate protein–protein interactions with signaling pathways involving GTP binding proteins implicated in cytoskeletal regulation.

Here we report the sequence of a full length cDNA of 3BP-1 and demonstrate that the central region of the

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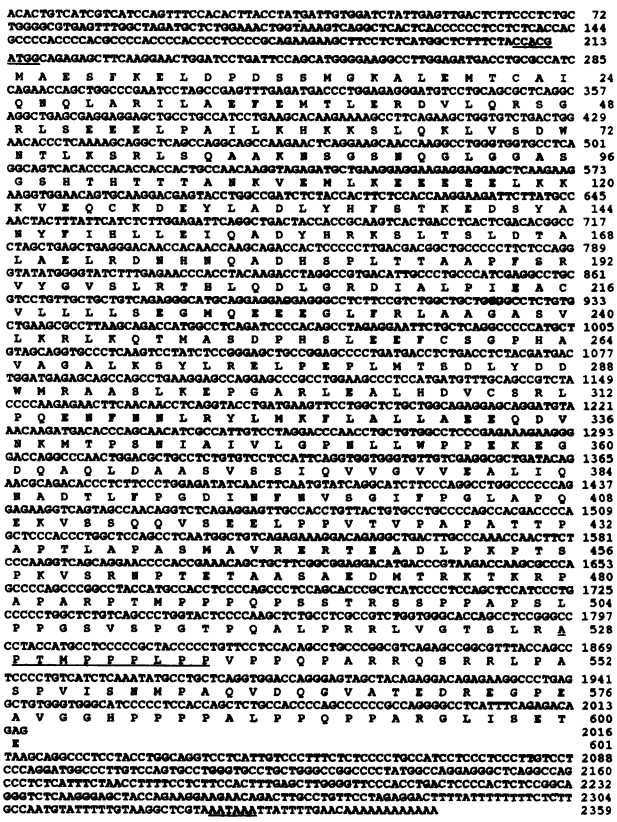

Fig. 1. Primary sequence of 3BP-1 cDNA. The full length sequence contains 2359 nucleotides, including 213 nucleotides of 5′ untranslated sequence and 343 nucleotides of 3′ untranslated sequence. The presumed start site of the sequence is underlined as is the poly(A) signal sequence in the 3′ untranslated sequence. Also underlined is the 10 amino acid sequence essential for SH3 binding found in the C-terminal portion of the protein.

protein, which shares GAP domain homology, functions as a GAP *in vitro* and *in vivo*. 3BP-1 is shown to have *in vitro* GAP activity for Rac-related proteins but not for the Rho- or Ras-related small GTP binding proteins. 3BP-1 is also shown to function as a GAP in intact cells: microinjection of the purified GAP domain of 3BP-1 into serum-starved Swiss 3T3 fibroblasts inhibits platelet-derived growth factor (PDGF)-induced membrane ruffling mediated by Rac. Co-injection of 3BP-1 with an activated mutant of Rac, unresponsive to GAPs, does not inhibit membrane ruffling, indicating that 3BP-1-induced inhibition of membrane ruffling is mediated through endogenous Rac. In agreement with the *in vitro* data, microinjection of 3BP-1 does not inhibit lysophosphatidic acid (LPA)-induced stress fiber assembly mediated by Rho in fibroblasts.

Results

Full length cDNA cloning and sequence analysis of 3BP-1

A radiolabeled probe derived from the previously isolated partial 3BP-1 cDNA clone was used to screen a λgt10 cDNA library derived from the mouse pre-B cell line, 22D6. From this screening, several clones were isolated and the cDNA inserts were subcloned from the λgt10 vectors into pBluescript for sequencing. One of these

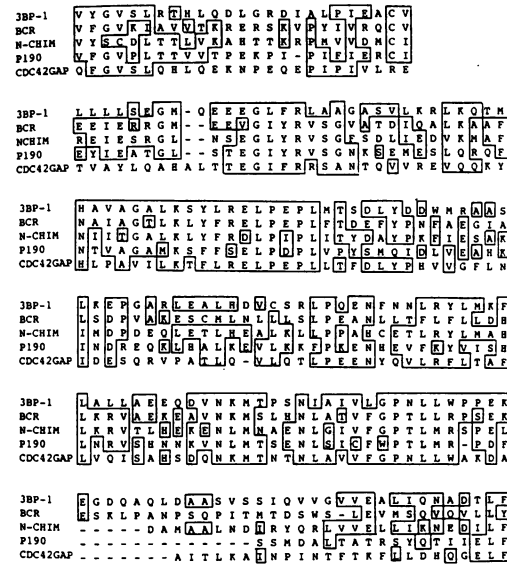

Fig. 2. Homology of 3BP-1 with GAPs. Boxed sequences show homology between 3BP-1, Bcr, N-chimaerin, p190 and cdc42 GAP sequences. '–' indicates an imposed one amino acid break in sequence for alignment purposes.

clones contained a cDNA encoding the full length 3BP-1 sequence. The 3BP-1 cDNA encodes a 601 amino acid protein with a predicted molecular weight of 65 kDa. The sequence in Figure 1 shows a 5′ untranslated region of 213 nucleotides which contains stop codons in all three reading frames before the first in-frame ATG. However, the second in-frame ATG, which occurs 18 nucleotides after the first, seems to contain a more optimal start site. The sequence surrounding this ATG initiation codon, ACCACGATGG is similar to the Kozak consensus start site, particularly at the important +4 and –3 sites (Kozak, 1991). The largest open reading frame follows, with the poly(A) signal, AATAAA, (Nevins, 1983) in the 3′ untranslated sequence, 12 nucleotides before the poly(A) tail begins.

Homology of 3BP-1 to GAPs

The previously isolated partial cDNA clone of 3BP-1 was shown to contain, in addition to an SH3 binding region in its C-terminal region, a domain with homology to GAP proteins (Cicchetti *et al.*, 1992). That homology is extended here and localized to the central portion of 3BP-1, from amino acid 193 to 358. Figure 2 depicts the sequence homology of the GAP domain of 3BP-1 with the GAP regions of cdc42 GAP, Bcr, N-chimaerin and p190. The GAPs which share homology with 3BP-1 all show activity for members of the Rho subfamily of Ras-related proteins, some of which have been demonstrated recently to be involved in regulating cytoskeletal organization (Hall, 1992; Nobes and Hall, 1994). In particular, Rho and the related Rac protein have been shown in fibroblasts to regulate LPA-induced stress fiber assembly and PDGF-induced membrane ruffling respectively (Ridley and Hall, 1992; Ridley *et al.*, 1992).

Tissue distribution of 3BP-1

To assess the expression pattern of 3BP-1, we probed a Western blot of mouse-derived tissue extracts with an

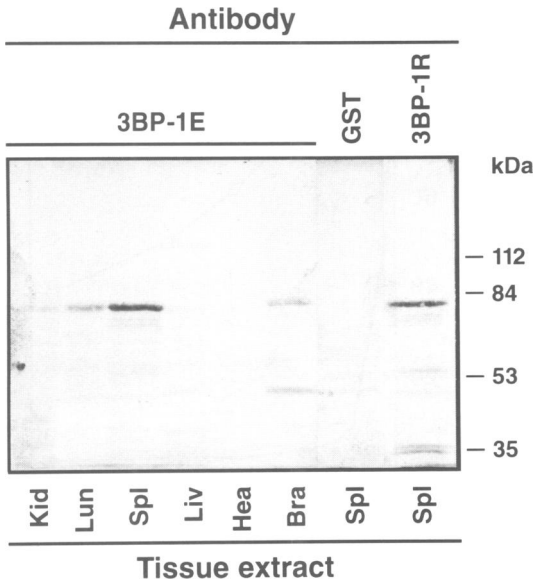

Fig. 3. Tissue distribution of 3BP-1. Organs were isolated from mice and prepared as tissue extracts. 100 μg of protein from each tissue extract, as determined by protein assay, was loaded onto a 10% SDS–PAGE for electrophoresis. The separated proteins on the gel were then transferred to nitrocellulose and blotted with the 3BP-1E antibody. Lanes: Kid: kidney; Lun: lung; Spl: spleen; Liv: liver; Hea: Heart; Bra: brain. Spleen extracts in the right two lanes were probed with either an anti-GST antibody or the 3BP-1R antibody as indicated.

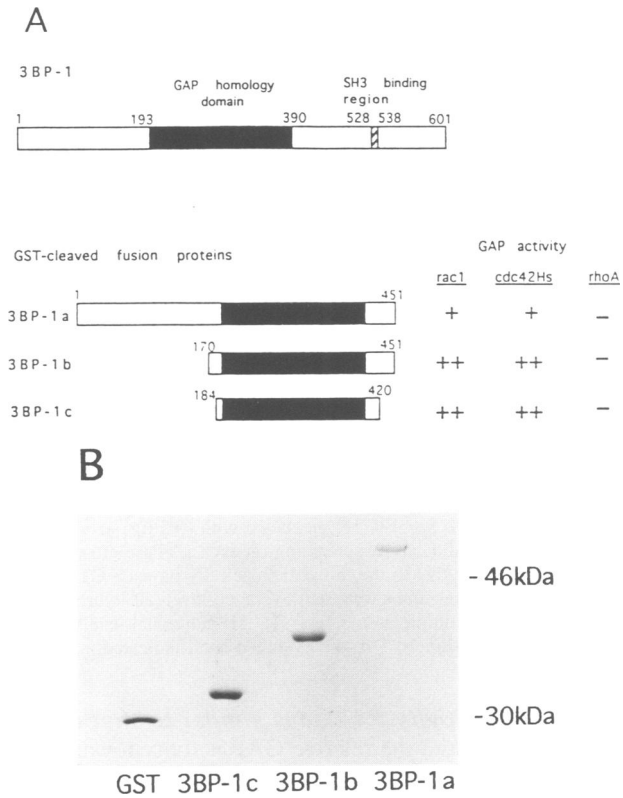

Fig. 4. The GAP domain of 3BP-1 is sufficient for *in vitro* GAP function. (**A**) Bacterially expressed, purifed 3BP-1 fusion proteins (cleaved from GST) are depicted, and the results of *in vitro* GAP assays using these proteins with either Rac 1, cdc42Hs or Rho A are shown. Purified recombinant Rac 1, cdc42Hs or Rho A (2 μg each) were bound to [γ-^{32}P]GTP (Amersham) and mixed with either 3BP-1a, b or c (1 μg each). The loss of radioactivity (^{32}P) during a time course at 25°C was measured by a filter binding method and the resulting levels of GTP hydrolysis over endogenous levels are depicted as '–' for no effect, '+' for significant effect and '++' for 2-fold greater effect than '+'. (**B**) The purified 3BP-1 proteins, along with GST, were loaded on a 12% SDS–PAGE and electrophoresed to demonstrate the purity of the preparations.

affinity-purified rabbit polyclonal antibody, 3BP-1E, directed against amino acids 263–467 of 3BP-1. A band of ~80 kDa was detected in all tissues analyzed, but was present at very low levels in heart and liver and was highly enriched in spleen and brain (Figure 3). The band migrated more slowly than would be predicted from the deduced molecular weight of 3BP-1. However, an anti-GST antibody showed no reactivity with a spleen extract, while an antibody directed against amino acids 394–513 of 3BP-1 (3BP-1R) reacted to a band of the same apparent molecular weight, strongly indicating that the band represents the 3BP-1 protein. Interestingly, antibody 3BP-1E, which is directed against the GAP homology region of 3BP-1, also recognized, only in the brain extract, a low molecular weight band which may be N-chimaerin. N-chimaerin is specifically expressed in brain and shares high homology in its GAP domain to 3BP-1. Antibody 3BP-1R, which is not directed against the GAP homology domain of 3BP-1, did not exhibit binding to the protein presumed to be N-chimaerin in brain extracts (data not shown).

3BP-1 is a GAP in vitro

The predicted GAP activity of 3BP-1 was analyzed *in vitro*. 3BP-1 deletion constructs, (Figure 4A), were expressed as GST fusion proteins in *Escherichia coli*, cleaved from GST and purified (Figure 4B). None of the constructs showed activity for Rho A, while 3BP-1a, b and c all showed GAP activity towards Rac and cdc42Hs. The 3BP-1 constructs tested here all exclude the C-terminal segment which contains the proline-rich SH3 domain binding region. The full length protein and constructs containing the C-terminal segment of 3BP-1 (and also including the GAP domain) were active for Rac and cdc42Hs, but significant degradation of the C-terminal segment of the proteins precluded a quantitative analysis (data not shown). The constructs 3BP-1b and c were ~2-fold more active than the larger construct, 3BP-1a, after allowing for molar concentration of protein, suggesting that the isolated GAP domain has a slightly higher activity than that flanked by surrounding sequences.

3BP-1 shows GAP specificity for Rac-related GTP binding proteins

The time course of GTP hydrolysis stimulated by the GAP region of 3BP-1 (3BP-1c) was analyzed with 10 small GTP binding proteins and is shown in Figure 5. 3BP-1c functions *in vitro* as a GAP for Rac 1, Rac 2, cdc42Hs and Rho G, being most active for Rac 1 and cdc42Hs. 3BP-1 did not show significant GAP activity towards Rho A, Rho B, Rho C, Ras or the Ras-related proteins, TC10 and Rap1a. Since all of the G proteins tested had previously been demonstrated to be functional substrates for one or more GAPs (data not shown), the activities displayed by 3BP-1 indicate its preference for one set of substrates over another.

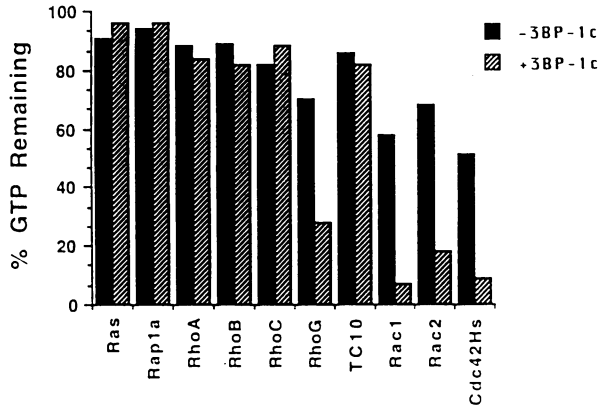

Fig. 5. 3BP-1 shows *in vitro* specificity for Rac-related GTP binding proteins. GAP activity of 3BP-1c on Ras, Rap1a and Rho family GTPases: each recombinant low molecular mass GTP binding protein (2 μg) was bound to [γ-^{32}P]GTP, incubated with (0.5 μg) or without 3BP-1c, and the radioactivity remaining on each GTPase after 8 min was determined relative to the initial radioactivity on each GTP binding protein. Bar graphs represent averaged levels of hydrolysis as determined from multiple assays (n = 4). All individual levels of hydrolysis were within 10% ± the averaged level indicated.

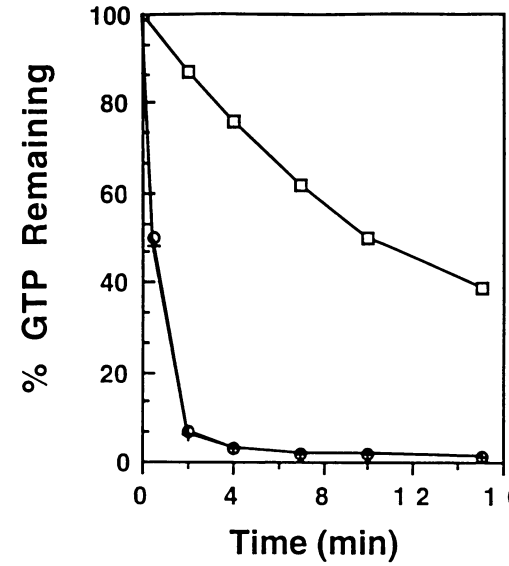

Fig. 6. *In vitro* GAP activity of 3BP-1 is similar to that of Bcr. Purified recombinant Rac 1 (2 μg) was bound to [γ-^{32}P]GTP (Amersham) and mixed with GST (F, 5 μg); 3BP-1c (O, 1 μg); or BcrGAP, a 28 kDa C-terminal fragment of Bcr, (+, 1 μg) in reaction buffer. The loss of the bound radioactivity (^{32}P) over the indicated time at 25°C was measured by a filter binding method. The percentage of GTP remaining is relative to the initial radioactivity on Rac 1.

3BP-1 GAP activity for Rac is similar to that of Bcr

We next compared the relative GAP activity towards Rac of 3BP-1c with that of an equimolar amount of the GAP domain of Bcr which had previously been shown to act as a GAP for Rac *in vitro* (Diekmann *et al.*, 1991). Figure 6 shows that the GAP domains of the 3BP-1 (3BP-1c) and Bcr proteins showed almost identical activity toward Rac *in vitro,* while the GST control protein did not produce any GTPase stimulation.

3BP-1 inhibits PDGF-induced membrane ruffling mediated by Rac

Rac proteins mediate PDGF-induced membrane ruffling in fibroblasts (Ridley *et al.*, 1992) and their activitiy is inhibited by the GAP domain of Bcr (Ridley *et al.*, 1993). We therefore investigated whether microinjected 3BP-1 GAP was able to act as a GAP for Rac in intact cells. First, we examined if serum-starved Swiss 3T3 fibroblasts were perturbed by microinjection of GST or 3BP-1c proteins at concentrations of 1 mg/ml (purified proteins shown in Figure 4B). Cells were fixed and stained 30 min after injection with TRITC-labeled phalloidin for actin visualization and anti-3BP-1 or anti-GST affinity-purified rabbit polyclonal antibodies followed by FITC-labeled goat anti-rabbit IgG, for visualization of injected cells. (In the figures, left hand panels show actin in the same field of cells as the right hand panels which were stained with antibody.) The actin stains in Figure 7A and C show that membrane ruffling was not stimulated by microinjection of either protein, which are shown in Figure 7B and D.

Serum-starved Swiss 3T3 cells were next injected with 3BP-1c or GST proteins as above and, 30 min following injection, treated with 3 ng/ml PDGF for 10 min to stimulate membrane ruffling. Cells were then fixed and stained as above. Antibody staining identified the cells in Figure 7F and H injected with GST and 3BP-1 respectively. Cells injected with 3BP-1c, compared with non-injected cells or GST-injected cells, show an inhibition of membrane ruffling, as can be seen in the actin staining of the outer membrane of the cells injected with 3BP-1c in Figure 7G compared with those injected with GST in Figure 7E. 3BP-1c also significantly inhibited membrane ruffling stimulated by epidermal growth factor and the tumor promoter phorbol 12-myristate 13-acetate (data not shown). At a concentration of 1 mg/ml, the percentage of 3BP-1c-injected cells which showed ruffling inhibition was 91.7 ± 4.6 (n = 3, total number of cells analyzed = 208), whereas 6% of non-injected cells did not exhibit membrane ruffling (number of cells analyzed = 67). Strong inhibition of ruffling by 3BP-1c was observed when it was injected at concentrations of 1 mg/ml and 0.5 mg/ml, was seen to decrease at 0.3 mg/ml and was not detected at 0.1 mg/ml (data not shown).

3BP-1c inhibited PDGF-induced membrane ruffling for up to 4 h following injection. However, by 16 h post-injection, its inhibitory effect was no longer evident. 3BP-1c protein was less visible by antibody immunofluoresence 4 h after injection, and was not detectable by 16 h after injection. We presume that 3BP-1c was substantially degraded by 16 h post-injection and was therefore not functionally active as a GAP.

Interestingly, a significant proportion of cells injected with 3BP-1c displayed a distinctly rounded morphology with no indications of toxicity or cell death (data not shown). This phenotype was not seen in cells injected with N17Rac1, a dominant inhibitor of Rac (Ridley *et al.*, 1992) and may be due to inhibition by 3BP-1 of Rac-related proteins such as cdc42Hs. Such proteins may be involved in the maintenance of typical flattened cell morphology; this phenotype is currently under further investigation.

Constituitively activated Rac is not inhibited by 3BP-1

Microinjection of a constituitively activated Rac mutant, which is unresponsive to GAPs and thus always in the

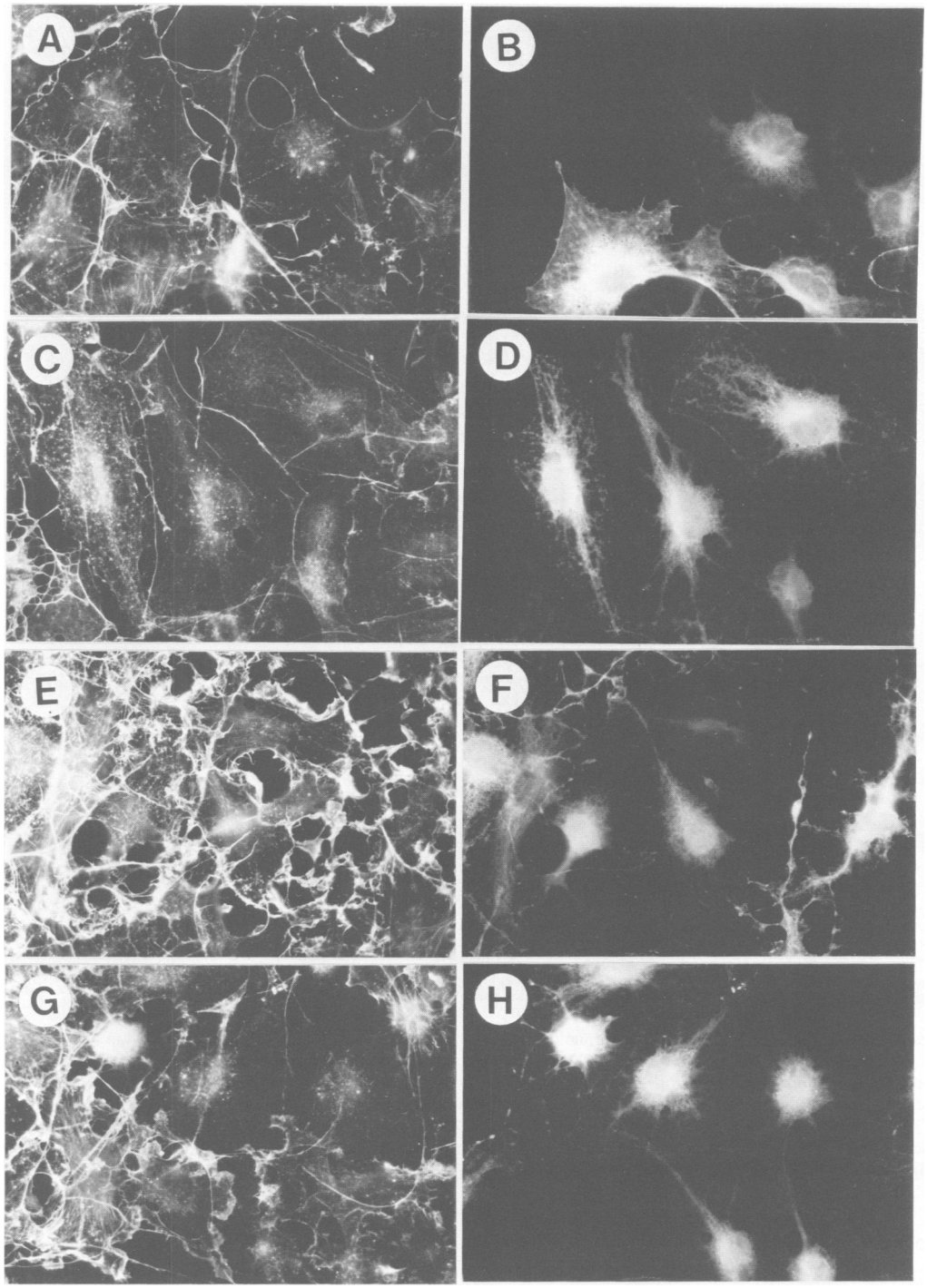

Fig. 7. 3BP-1 inhibits PDGF-induced membrane ruffling mediated by Rac. GST or 3BP-1c proteins at a concentration of 1 mg/ml were microinjected into serum-starved Swiss 3T3 cells. (**A**) and (**C**) show actin staining of GST- or 3BP-1-injected cells respectively, unstimulated by growth factors. (**B**) and (**D**) show antibody staining of microinjected GST or 3BP-1 in these unstimulated cells respectively. (**F**) and (**H**) show antibody staining of cells injected with GST or 3BP-1 respectively, stimulated with 3 ng/ml PDGF for 10 min (30 min following injection). (**E**) and (**G**) show actin staining of these PDGF-stimulated cells.

active, GTP-bound 'on' state, has been shown to stimulate membrane ruffling in Swiss 3T3 cells (Ridley *et al.*, 1992). Serum-starved Swiss 3T3 cells were microinjected with this activated mutant, V12Rac1, at a concentration of 0.2 mg/ml, together with a rat immunoglobulin marker protein (0.5 mg/ml). Antibody staining shows injected cells in Figure 8D. An examination of actin-stained filaments in these injected cells compared with the uninjected cells (Figure 8C) shows that membrane ruffling was stimulated in the V12Rac1-injected cells compared with the uninjected background cells. To verify that the mechanism whereby 3BP-1 inhibits membrane ruffling is through its ability to inhibit Rac, 3BP-1c at a concentration of 1 mg/ml and the activated Rac mutant, V12Rac1 (0.2 mg/ml), were co-injected into serum-starved Swiss 3T3 cells and fixed and stained 20 min after injection. An

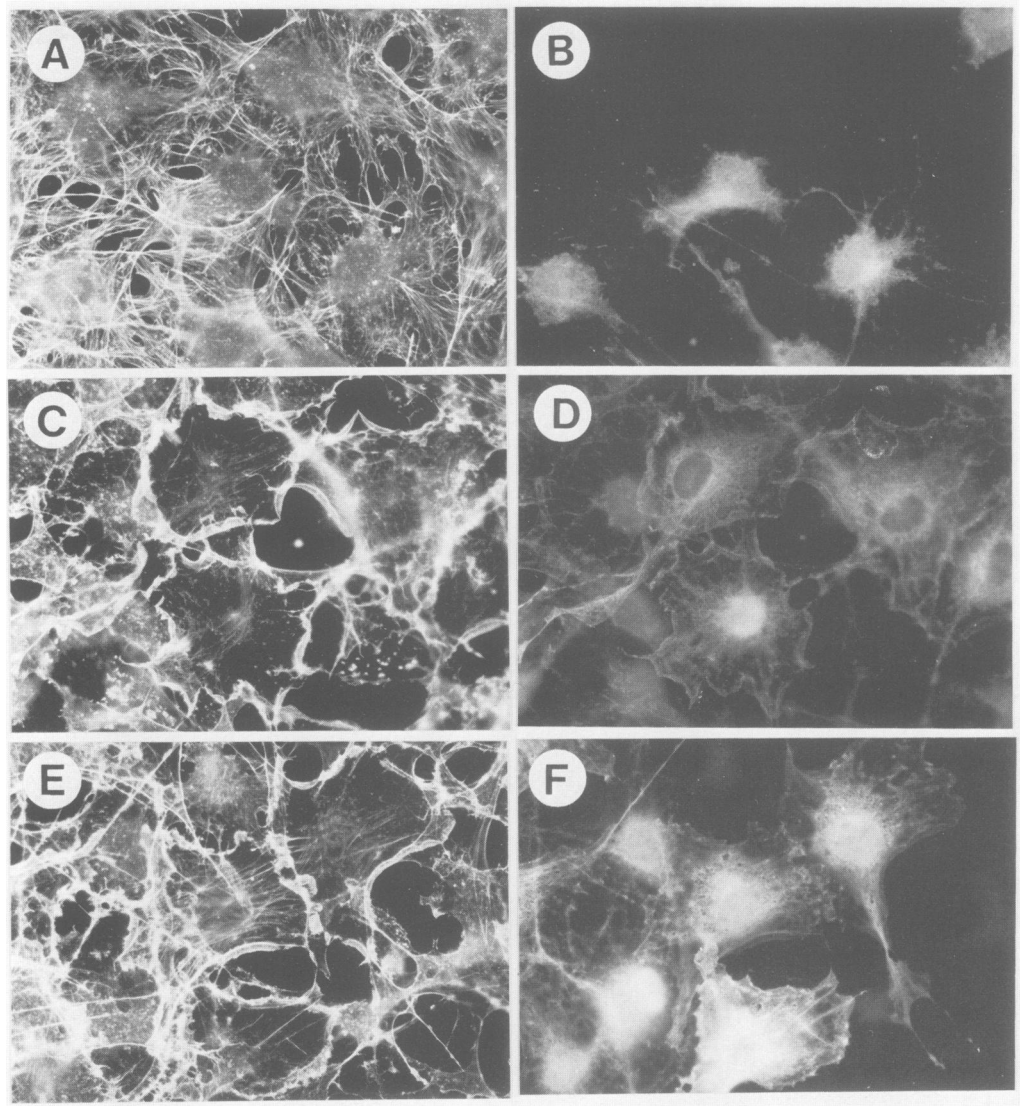

Fig. 8. 3BP-1 does not inhibit LPA-induced stress fiber formation mediated by Rho or membrane ruffling induced by an activated Rac mutant. (**A** and **B**) 3BP-1c was injected into serum-starved Swiss 3T3 cells and cells were stimulated 15 min after injection with LPA. Phalloidin-stained cells in (A) demonstrate actin stress fiber induction and (B) shows 3BP-1 antibody visualization of 3BP-1c-injected cells. (**C** and **D**) Activated V12Rac1 was microinjected into serum-starved Swiss 3T3 cells, which were fixed 20 min after injection and stained with phalloidin to visualize actin filaments (C) or anti-rat IgG to identify injected cells (D). (**E** and **F**) 3BP-1c at a concentration of 1 mg/ml was co-injected with V12Rac1 (0.2 mg/ml), and cells were fixed 20 min after injection and stained with phalloidin for actin visualization (E) or anti-3BP-1 antibody for visualization of injected cells (F).

examination of the actin filament localization of the injected cells (Figure 8E) demonstrates that V12Rac1-induced membrane ruffling was not inhibited by 3BP-1c. The inability of 3BP-1c to inhibit membrane ruffling induced by a Rac mutant unresponsive to GAPs indicates that the inhibiting action of 3BP-1 on PDGF-induced membrane ruffling is the result of its ability to act as a GAP on endogenous Rac.

3BP-1 does not affect LPA-induced, Rho-mediated stress fiber assembly

The Ras-related Rho proteins mediate LPA-induced actin stress fiber assembly in fibroblasts (Ridley and Hall, 1992). 3BP-1c was shown here to be inactive towards Rho proteins *in vitro*, with the exception of Rho G. To determine whether 3BP-1c was active *in vivo* as a GAP for Rho, 3BP-1c at a concentration of 1 mg/ml was microinjected into serum-starved Swiss 3T3 cells which were stimulated 15 min after injection with 20 ng/ml LPA for 10 min. An examination of actin-containing filaments in injected versus non-injected cells (Figure 8A and B), shows that 3BP-1c-injected cells did not display any inhibition of LPA-induced, Rho-mediated actin stress fiber assembly. These results indicate that 3BP-1 does not inhibit Rho function and therefore is not a functional GAP for Rho in intact cells. Thus, the *in vitro* specificity of 3BP-1 as a GAP for Rac but not Rho is in agreement with its *in vivo* activity for these proteins.

Discussion

3BP-1 was originally identified as an Abl SH3 binding protein encoded by a partial cDNA from an expression library. It had a region of homology to Rho GAP proteins.

Here we show that, in a full length clone, the homology continues and covers the full active site of previously characterized Rac GAP and Rho GAP proteins. The GAP domain is in the center of the protein, distinct from the C-terminal SH3 binding site. Direct biochemical analysis shows that the GAP region of 3BP-1 is able to catalyze GTP hydrolysis mediated by Rac 1, Rac 2, cdc42Hs and Rho G, but not that catalyzed by Ras, Rap1a, TC10, Rho A, Rho B or Rho C. Rho G and cdc42Hs share significantly higher amino acid sequence homology to Rac than to other Rho and Ras family members, (Vincent *et al.*, 1992; Bush *et al.*, 1993) and thus the data presented here indicate that 3BP-1 GAP activity has *in vitro* specificity for those GTP binding proteins which share strong amino acid sequence homology to Rac.

Because Rac and Rho regulate actin cytoskeletal organization in fibroblasts, we determined whether the GAP-containing region of 3BP-1 could counter the intracellular effects of Rac and Rho. Microinjection studies showed that 3BP-1 inhibits PDGF-induced membrane ruffling mediated by Rac. Co-injection with an activated Rac mutant, unresponsive to GAPs, effectively blocked this inhibition. In contrast, 3BP-1 did not inhibit Rho-mediated, LPA-induced stress fiber assembly, showing that its biochemical specificity for Rac and not Rho is mirrored by its *in vivo* activity. 3BP-1 therefore acts similarly to the GAP domain of Bcr which also shows specificity for Rac but not Rho, *in vitro* and *in vivo* (Ridley *et al.*, 1993).

It will be important to determine whether 3BP-1 acts as a GAP *in vivo* for other members of the Rho subfamily of GTPases for which it has been shown to have *in vitro* activity. While cdc42 has been shown to be important for maintaining cell polarity and cortical actin assembly in yeast (Johnson and Pringle, 1990; Chant and Herskowitz, 1991; Li *et al.*, 1995), it is not yet clear what role its mammalian homolog, cdc42Hs, plays. While the functional role of Rho G is not yet known, the *in vitro* data presented here suggest that 3BP-1 may also function *in vivo* to stimulate GTP hydrolysis on this protein.

The 3BP-1 protein contains two regions of identifiable homology with other proteins, the GAP domain and the C-terminal proline-rich region that binds to the Abl SH3 domain. Whether the protein actually interacts with Abl in cells is not known, and there is sufficient overlap of binding specificity among SH3 domains such that the proline-rich region could easily bind to some other SH3 region with higher affinity than to that of Abl. We have been unable thus far to co-immunoprecipitate 3BP-1 and Abl from cell lysates, indicating that, if an interaction does occur, it is quite transient in nature or not tight enough to co-precipitate. However, the proline-rich region of 3BP-1 is quite likely from its sequence to bind one or more SH3 regions. While we have shown here that the SH3 binding region of 3BP-1 is not necessary for the *in vivo* or *in vitro* GAP activity of this protein for Rac, it is nonetheless possible that 3BP-1's activity is controlled by SH3 binding. We were unable to test the activity of the full size protein because of degradation of the C-terminal region (including the SH3 binding region) in bacteria, indicating that further work will be required to settle this question.

The role of 3BP-1 in cell physiology remains unclear. Presumably the GAP activity of 3BP-1 is regulated in cells and is not constituitively active. Its GAP domain interacts with a number of Rac homologs, including cdc42Hs and Rho G whose function is undetermined, making it difficult to know what pathways might interact with 3BP-1 and whether its demonstrable effect on membrane ruffling is its true or sole intracellular mode of action. However, there are some promising recent developments concerning signaling molecules which interact directly or functionally with cdc42Hs and Rac, and which leave open the possibility of a role for 3BP-1 in such interactions.

Recently, PI 3-kinase activity was demonstrated to be necessary for PDGF-induced membrane ruffling (Wennstrom *et al.*, 1994). The SH3 domain of p85 has been shown to bind to 3BP-1 *in vitro* (Gout *et al.*, 1993), which may indicate a link between 3BP-1 inhibition of Rac-mediated ruffling and PI 3-kinase involvement in the ruffling response. Similarly, proline-rich regions in cdc42 GAP have been shown to bind *in vitro* to the SH3 domains of the p85 regulatory subunit of PI 3-kinase (Barfod *et al.*, 1993). An advance into the possible pathways involved in cdc42Hs signaling came from the identification of $p120^{ACK}$, a novel hippocampal non-receptor tyrosine kinase which binds to the GTP-bound form of cdc42Hs and inhibits its intrinsic GTPase activity (Manser *et al.*, 1993). $p120^{ACK}$ contains an SH3 domain, and this domain is not involved in the binding interaction with cdc42Hs. It was reported that $p120^{ACK}$ inhibits the *in vitro* GAP activity of Bcr for cdc42Hs (Manser *et al.*, 1993). It is interesting to speculate that $p120^{ACK}$ might associate in cells with 3BP-1 through an SH3 domain interaction and thereby inhibit its GAP activity for cdc42Hs.

It will be interesting to identify the *in vivo* interactions of 3BP-1 and to demonstrate how such interactions regulate the GAP activity of this protein. The specificity of 3BP-1 GAP activity for Rac and Rac-like G proteins indicates likely signaling pathways in which this protein is involved. The identification of components of these pathways such as PI 3-kinase and $p120^{ACK}$ provide interesting candidate regulators for the GAP activity of 3BP-1.

Materials and methods

Cloning and sequence analysis

A DNA probe derived from the partial 3BP-1 sequence was labeled with [^{32}P]ATP (Amersham) and used to screen a λgt10 cDNA library derived from the mouse pre-B cell line 22D6. A positive reacting clone containing the entire 3BP-1 sequence was isolated. The λ DNA was purified using λ phage adsorbent (Promega) according to the manufacturer's protocols. The cDNA insert DNA was excised with *Not*I, subcloned into the *Not*I site of pBluescript (Stratagene) and sequenced using Sequenase (USB) as per the manufacturer's instructions. The full length sequence was assembled by matching up overlapping sequences generated by 3′ deletion constructs of the full length clone in pBluescript (Stratagene) using Erase a Base (Promega) according to the manufacturer's instructions. Sequence homology was identified using the GenBank library and the FASTA application program.

Tissue distribution

Organs were isolated from mice, and tissue extracts were prepared according to standard procedures: 1.5 w/v in 1% SDS, 20 mM Tris–HCl pH 7.5, homogenized on ice. Protein levels of the extracts were determined using the BCA protein determination system (Pierce). Standardized extracts containing ~100 μg of protein per lane were run on a 10% SDS–PAGE, transferred to nitrocellulose and subjected to Western blot analysis using affinity-purified rabbit polyclonal antibodies directed against 3BP-1 or GST.

Antibody production

GST fusion proteins derived from 3BP-1 sequences were grown in *E.coli* and purified as described (Ausebel *et al.*, 1993). Polyclonal rabbit antisera against these proteins were generated by HRP Inc. (PA). 3BP-1 fusion peptides and free GST protein were coupled to Affigel-10 resins (Bio-Rad) according to the manufacturer's protocols and loaded into disposable columns (Bio-Rad). Antiserum was immunopurified on these columns basically according to protocols described (Harlow and Lane, 1988). Antiserum was first purified on the columns containing GST–3BP-1 antigens. This antibody solution was then loaded onto Affigel–GST-bound columns, collecting the flow through for the 3BP-1 peptide antibody and the eluate for anti-GST antibodies. These antibodies were then concentrated using Amicon 10 Centripreps and Centricons as per the manufacturer's instructions. Western blot analysis of GST–3BP-1 bacterially expressed fusion proteins, as well as NIH3T3 lysates, confirmed that the anti-3BP-1 antibodies specifically recognized 3BP-1 (data not shown).

Purification and thrombin cleavage of GST-3BP-1 proteins

Deletion constructs of 3BP-1 were prepared by using synthetic oligonucleotides for PCR amplification of the 3BP-1 cDNA. Oligo sequences contained *Xba*I and *Sal*I restriction sites preceded by six nucleotide overhang sequences, for efficiency of restriction enzyme digest, and followed by internal 3BP-1 sequences which allowed for subcloning of the isolated, and restriction enzyme-digested PCR product fragments into the *Xba*I and *Sal*I sites in the polylinker of the pGEX-KG vector (Guan and Dixon, 1991). The plasmid constructs were introduced into *E.coli* and the 3BP-1 fragments were expressed as GST fusion proteins. The fusion proteins were grown and purified as described (Ausebel *et al.*, 1993) and, in this case, the GST fusion protein was cleaved with thrombin while bound to the glutathione–agarose beads. Before addition of thrombin, the beads were washed with thrombin cleavage buffer, TCB (2.5 mM $CaCl_2$, 50 mM Tris–HCl pH 7.5 and 150 mM NaCl). Beads were then resuspended in TCB (1 ml buffer/1 ml beads) and plasma thrombin (Calbiochem, #605195) was added to the slurry at a ratio of ~1/200, thrombin/GST fusion protein, inverting for 1 h at room temperature. The treated beads were centrifuged briefly and the supernatant was incubated on an inverter for 30 min at 4°C with benzamidine–Sepharose 6B (Pharmacia) which had been loaded into a disposable column (Biorad) and washed with buffer at 4°C according to the manufacturer's instructions. The thrombin-free eluate from the column was collected and concentrated using Amicon 10 Centripreps. Buffer was exchanged using PD-10 columns (Pharmacia) as per the manufacturer's instructions using 100 mM NaCl, 50 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$ for the microinjection assays and 20 mM HEPES pH 7.6, 0.5 mM DTT, 0.5% NP-40, 25 mM NaCl for *in vitro* GAP assays.

In vitro GAP assays

The low molecular mass GTP binding proteins were cloned as PCR fragments into the *Bam*HI–*Eco*RI sites of the pGEX-2T vector and expressed as GST fusion proteins as described (Hart *et al.*, 1994). The C-terminal fragment of Bcr (amino acids 962–1227) was expressed and purified similarly by PCR cloning and glutathione–agarose chromatography. The GST was removed from the fusion proteins by thrombin (Sigma) digestion. The apparent loss of γ-^{32}P as an outcome of the hydrolysis or dissociation of the [γ-^{32}P]GTP or [α-^{32}P]GTP bound to Rac 1 or other low molecular weight GTP binding proteins was determined as described previously (Zheng *et al.*, 1994) by measuring the radioactivity remaining on the nitrocellulose filters at 25°C in the presence or absence of GAPs.

Cell culture, microinjection and immunofluoresence

Swiss 3T3 cells were maintained in Dulbecco's-modified Eagle's medium containing 10% fetal calf serum. Quiescent serum-starved cells were obtained as described (Ridley and Hall, 1992). Proteins were microinjected into the cytoplasm and cells were subsequently stimulated with PDGF (UBI) or LPA (Sigma Chemical Co.). Cells were fixed and permeabilized, and actin filaments localized with TRITC-labeled phalloidin as described (Ridley and Hall, 1992). For identification of 3BP-1- or GST-injected cells, affinity-purified polyclonal rabbit antibodies against these proteins were used at a concentration of 1:100, followed by a 1:400 dilution of FITC-conjugated goat anti-rabbit immunoglobulin G (Pierce). A rat IgG marker protein (0.5 mg/ml) was co-injected with the V12Rac1 mutant as the primary antibody for visualization of V12Rac1-injected cells.

Accession number

The sequence reported in this paper has been deposited in the EMBL database under accession number X87671.

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