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Shc adaptor proteins are key transducers of mitogenic signaling mediated by the G protein-coupled thrombin receptor

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The serine protease thrombin activates G protein signaling systems that lead to Ras activation and, in certain cells, proliferation. Whereas the steps leading to Ras activation by G protein-coupled receptors are not well defined, the mechanisms of Ras activation by receptor tyrosine kinases have recently been elucidated biochemically and genetically. The present study was undertaken to determine whether common signaling components are used by these two distinct classes of receptors. Here we report that the adaptor protein Shc, is phosphorylated on tyrosine residues following stimulation of the thrombin receptor in growth-responsive CCL39 fibroblasts. Shc phosphorylation by thrombin or the thrombin receptor agonist peptide is maximal by 15 min and persists for ⩾2 h. Following thrombin stimulation, phosphorylated Shc is recruited to Grb2 complexes. One or more pertussis toxin-insensitive proteins appear to mediate this effect, since (i) pertussis toxin pre-treatment of cells does not blunt the action of thrombin and (ii) Shc phosphorylation on tyrosine can be stimulated by the muscarinic m1 receptor. Shc phosphorylation does not appear to involve protein kinase C, since the addition of 4-β-phorbol-12,13-dibutyrate has no effect. Rather, thrombin-induced Shc phosphorylation is enhanced in cells depleted of phorbol ester-sensitive protein kinase C isoforms. Expression of mutant Shc proteins defective in Grb2 binding displays a dominant-negative effect on thrombin-stimulated p44 MAP kinase activation, gene induction and cell growth. From these data, we conclude that Shc represents a crucial point of convergence between signaling pathways activated by receptor tyrosine kinases and G protein-coupled receptors.

Keywords: G protein/Shc adaptor protein/signaling/thrombin

Introduction

Procoagulant α-thrombin (thrombin) has multiple biological effects in a variety of cell types and tissues (for reviews, see Carney, 1992; Coughlin *et al.*, 1992). In CCL39 hamster fibroblasts, thrombin is a potent mitogenic factor. When added alone, thrombin can induce arrested cells to re-enter the cell cycle and continuously proliferate at a near-maximal rate (Van Obberghen-Schilling and Pouysségur, 1983). The biological effect of thrombin is elicited by proteolytic cleavage of the thrombin receptor in its N-terminal extension which induces receptor activation and subsequent interaction with G proteins (Vu *et al.*, 1991). At least two classes of G proteins, pertussis toxin-sensitive ($G_{i/o}$-like) and pertussis toxin-insensitive (G_q-like), are coupled to the thrombin receptor (see Van Obberghen-Schilling and Pouysségur, 1993). It is likely that G_i plays a critical role in thrombin's mitogenic effect, since pre-treatment of cells with pertussis toxin inhibits up to 80% of DNA synthesis induced by thrombin in CCL39 cells, whereas the toxin has no effect on fibroblast growth factor (FGF)-stimulated cell proliferation (Chambard *et al.*, 1987). Furthermore, microinjection of specific anti-$G_{\alpha i}$ antibody was found to inhibit the proliferative response to thrombin in fibroblasts (LaMorte *et al.*, 1992). However, the means by which $G_{\alpha i}$ protein(s) transduce(s) mitogenic signals from the thrombin receptor to the nucleus are still largely unknown.

Recent findings suggest that Ras activation is an important early event in mitogenic signal transduction by G protein-coupled receptors (Cook *et al.*, 1993; Van Corven *et al.*, 1993). Indeed, Ras can be activated by thrombin in CCL39 cells in a pertussis toxin-sensitive manner (Van Corven *et al.*, 1993), as measured by the accumulation of Ras in its GTP-bound form. Further, expression of a dominant-negative form of Ras has been shown to inhibit thrombin-stimulated gene induction and DNA synthesis re-initiation in astrocytoma cells (LaMorte *et al.*, 1993), as well as proliferation of vascular smooth muscle cells (Irani *et al.*, 1994). This inhibition is supported by similar findings obtained by microinjecting Ras-interfering antibodies. Altogether, these results suggest a role for Ras in cell growth promotion by thrombin.

At present, the mechanism(s) of Ras activation by G protein-linked signaling pathways is (are) not clear. Our recent results indicated that thrombin and the thrombin receptor peptide agonist lead to activation of Src and Fyn in a pertussis toxin-sensitive manner (Chen *et al.*, 1994). These non-receptor tyrosine kinases have been identified as upstream activators of Ras. Although these findings may give us some indications concerning signaling components activated by the thrombin receptor, it is unlikely that Src-related kinases directly interact with Ras. Rather, one or more kinases or adaptor proteins are presumed to be located between Src kinases and Ras. The recently described Shc adaptor proteins are constitutively phosphorylated in cells harboring oncogenic variants of the non-receptor tyrosine kinases Src and Fps (McGlade *et al.*, 1992), suggesting that Shc may be a substrate of Src.

The Shc gene encodes overlapping proteins of 46, 52 and 66 kDa ($p46^{Shc}$, $p52^{Shc}$ and $p66^{Shc}$) (Pelicci *et al.*,

1992). The three isoforms of Shc share common structural characteristics, all contain one Src homology 2 (SH2) domain, thought to direct their interaction with phosphotyrosine-containing proteins, and a region rich in glycine and proline residues with homology to the α1-chain of collagen (CH domain). An additional domain in the N-terminal region of Shc has also been identified which binds to tyrosine-phosphorylated proteins through a novel sequence motif (Kavanaugh *et al.*, 1995). It is believed that Shc proteins act as adaptor proteins in cellular signal transduction pathways generating from diverse stimuli. Growth factors that stimulate receptor tyrosine kinases, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), nerve growth factor (NGF) and insulin, induce tyrosine phosphorylation of Shc proteins (Pelicci *et al.*, 1992; Rozakis-Adcock *et al.*, 1992; Pronk *et al.*, 1993). Tyrosine phosphorylation of Shc proteins also occurs in cells following stimulation of cytokine receptors [including receptors for interleukin (IL)-2, IL-3, granulocyte–macrophage colony-stimulating factor (GM-CSF) and erythropoietin] (Burns *et al.*, 1993; Cutler *et al.*, 1993; Damen *et al.*, 1993; Zhu *et al.*, 1994) which are thought to exert their effects by recruitment of non-receptor tyrosine kinases. Recent reports indicate that the G protein-coupled receptors for thyrotropin-releasing hormone (TRH) (Ohmichi *et al.*, 1994) and endothelin (Cazaubon *et al.*, 1994) are also capable of inducing phosphorylation of Shc on tyrosine residues. Following phosphorylation on tyrosine, Shc proteins can associate with Grb2 and the Ras exchange factor Sos, and thus may participate in activation of Ras. The observation that Shc overexpression leads to cell transformation (Pelicci *et al.*, 1992), together with the strict correlation between growth factor-induced Shc phosphorylation and proliferation, has led to the proposal that Shc plays a fundamental role in the mitogenic response of cells to growth factors. This has been confirmed recently by studies showing that microinjection of Shc neutralizing antibodies inhibits DNA synthesis stimulation by insulin-like growth factor (IGF)-I, insulin and EGF receptors (Sasaoka *et al.*, 1994).

In the present study, we show that thrombin and the thrombin receptor peptide agonist stimulate a rapid and sustained tyrosine phosphorylation of $p46^{Shc}$ and $p52^{Shc}$, as well as the formation of Shc–Grb2 complexes in CCL39 cells. In addition, we observed that thrombin- and thrombin receptor peptide-mediated Shc phosphorylation is not sensitive to pertussis toxin, indicating that G_q, rather than G_i or G_o, may mediate this effect. Consistent with this observation, tyrosine phosphorylation of Shc was observed following stimulation of the muscarinic m1 receptor (coupled to G_q), indicating that G_q, rather than G_i or G_o may activate Ras by the Shc–Grb2 pathway. Finally, Shc phosphorylation appears to be an important link to downstream growth signals triggered by thrombin, since mutant Shc proteins defective in Grb2 binding are able to blunt thrombin-stimulated MAP kinase activity, induction of the c-*fos* gene and colony formation in CCL39 cells.

Results

Phosphorylation of Shc proteins in CCL39 cells

To examine the effect of thrombin on Shc phosphorylation in quiescent CCL39 fibroblasts, cells were first incubated

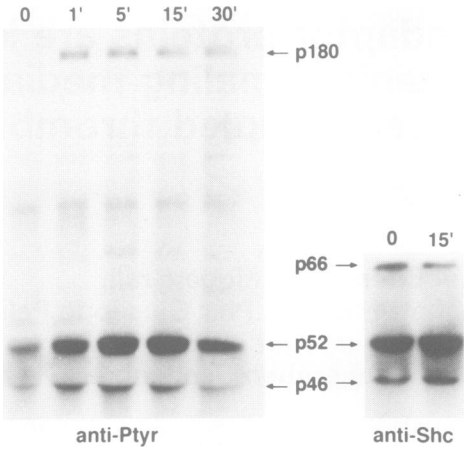

Fig. 1. Time course of Shc phosphorylation. Left: growth-arrested CCL39 cells were incubated for the indicated times with 1 U/ml thrombin prior to lysis, immunoprecipitation of lysates with polyclonal anti-Shc antibody and separation of proteins by SDS–PAGE on 8% gels. Western blotting was then performed with 4G10 anti-phosphotyrosine antibody. The positions of Shc isoforms ($p46^{Shc}$, $p52^{Shc}$ and $p66^{Shc}$) are indicated. Right: Western blot of Shc immunoprecipitates from cells treated with thrombin for 0 or 15 min was performed using anti-Shc antibody.

in serum-free medium for 16–20 h prior to treatment with 1 U/ml of the protease for various times. Following treatment, cells were solubilized and lysates were immunoprecipitated with polyclonal anti-Shc antibody. Tyrosine phosphorylation of Shc proteins was determined by immunoblotting using monoclonal anti-phosphotyrosine antibody, 4G10. As shown in Figure 1 (left panel), thrombin significantly increases Shc phosphorylation on tyrosine residues. Thrombin-induced tyrosine phosphorylation of Shc proteins is apparent within 1 min, it peaks after ~15 min of stimulation and persists for at least 2 h (not shown). Whereas all three Shc isoforms are present in anti-Shc immunoprecipitates from cells, as determined by immunoblot analysis (Figure 1, right panel), $p52^{Shc}$ is the major phosphorylated form detected with the 4G10 anti-phosphotyrosine antibody. Weak stimulation of $p46^{Shc}$ phosphorylation by thrombin can also be observed. An additional tyrosine phosphorylated protein of $M_r \approx 180\,000$ was also present in anti-Shc immunoprecipitates from thrombin-stimulated cells. A protein of similar size to this phosphoprotein was also recognized by antibodies directed against human SOS1 (results not shown), as discussed below.

The G protein-coupled thrombin receptor can be activated either proteolytically with thrombin, or with a peptide corresponding to the sequence of the receptor adjacent to the thrombin cleavage site, referred to as the 'tethered ligand', or thrombin receptor peptide (TRP). To confirm that the effect of thrombin is mediated by the thrombin receptor, we examined the effect of TRP on phosphorylation of Shc in CCL39 cells. As shown in Figure 2 (top), tyrosine phosphorylation of both $p52^{Shc}$ and $p46^{Shc}$ can be detected in anti-Shc immunoprecipitates from cells stimulated with 30 μM of TRP for 15 min. Both the magnitude of TRP-induced Shc phosphorylation and the time course of the effect (not shown) are similar to that observed with thrombin. It can be seen from the Western blot pictured in Figure 2 (bottom) that activation of the

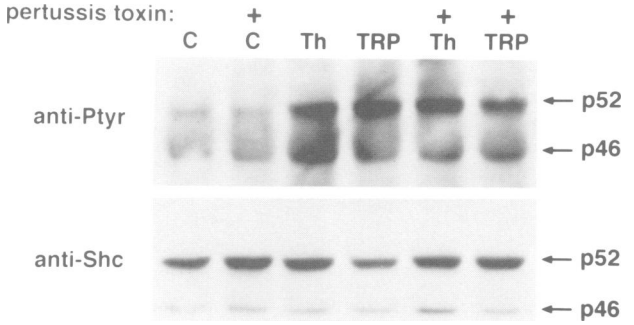

Fig. 2. Effect of pertussis toxin on thrombin receptor-induced Shc phosphorylation. Growth-arrested CCL39 cells were pre-treated for 4 h with 100 ng/ml pertussis toxin where indicated (+) prior to incubation with: serum-free medium (C), 1 U/ml thrombin (Th) or 30 μM TRP for 15 min. Following stimulation, cells were lysed and lysates were immunoprecipitated with anti-Shc antibody and analyzed by immunoblotting with anti-phosphotyrosine antibody (top) or with anti-Shc antibody (bottom).

thrombin receptor has no effect on the quantity of Shc proteins immunoprecipitated.

Pertussis toxin-insensitive G proteins regulate Shc phosphorylation

It is well established that the thrombin receptor is functionally coupled to at least to two classes of G proteins in CCL39 cells, sensitive and insensitive to pertussis toxin, respectively. In order to determine whether Shc phosphorylation is a direct consequence of G protein activation, rather than a secondary effect mediated by protein kinase C (PKC)- or Ca^{2+}-dependent processes, we used several approaches. First, we examined the effect of directly activating G proteins with fluoroaluminate AlF_4^- and GTPγS. It has previously been shown that ⩾1 mM GTPγS, a non-hydrolyzable GTP analog, can activate various G proteins in CCL39 cells, providing that cells are depolarized by incubation in a high K^+ medium (Paris and Pouysségur, 1990). Both agents induced a weak tyrosine phosphorylation of $p52^{Shc}$ in CCL39 cells following treatment for 15 min (results not shown), confirming that one or more G proteins are able to regulate Shc phosphorylation.

To determine whether a G_i-like protein may be involved in this effect, we pre-treated cells with 100 ng/ml of pertussis toxin for 4 h before adding thrombin (1 U/ml) or TRP (30 μM). Indeed, recent studies have shown that Ras and MAP kinase activation in CCL39 cells by thrombin and TRP are partially blocked by pre-treatment of cells with the toxin, indicating that a G_i-like protein may play a crucial role in the signal transducing pathway leading from the thrombin receptor to MAP kinase activation. As shown in Figure 2, Shc phosphorylation induced by thrombin or TRP treatment of cells for 15 min is not affected by prior inhibition of pertussis toxin-sensitive G proteins. Rather, we occasionally observed a slight elevation of Shc phosphorylation on tyrosine in control cells pre-treated with the toxin. The ability of the toxin to inhibit thrombin-induced DNA synthesis re-initiation in CCL39 cells was verified for each preparation of pertussis toxin used (data not shown). These results suggested to us that G_q, or other pertussis toxin-insensitive G proteins

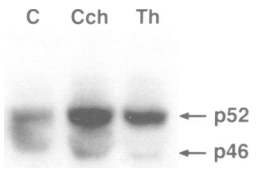

Fig. 3. Stimulation of Shc phosphorylation by carbachol in M1-81 cells. Growth-arrested M1-81 cells were incubated for 15 min with serum-free medium (C), 10^{-4} M carbachol (Cch) or 1 U/ml thrombin (Th) then lysed. Cell lysates were immunoprecipitated with anti-Shc antibody and immunoprecipitates were analyzed by Western blotting using anti-phosphotyrosine antibody.

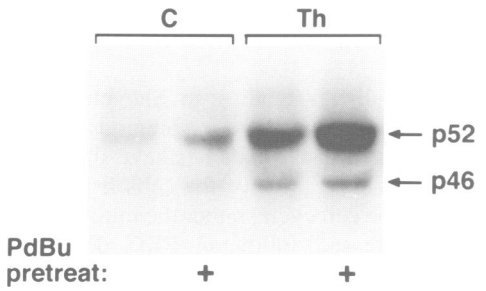

Fig. 4. Effect of thrombin in PKC-depleted cells. Growth-arrested CCL39 cells were incubated for 15 min with serum-free medium (C) or 1 U/ml thrombin (Th) prior to lysis. Where indicated (+), cells were incubated for 24 h with 100 ng/ml PdBu, prior to treatment in the presence or absence of thrombin. Following stimulation, cell lysates were immunoprecipitated with anti-Shc antibody and analyzed by anti-phosphotyrosine immunoblotting.

may be involved in Shc phosphorylation. This conclusion was supported by the experiments shown in Figure 3. Carbachol, a muscarinic receptor agonist, is able to stimulate Shc phosphorylation on tyrosine residues in a CCL39-derived line of fibroblasts (M1-81 cells) stably expressing the human muscarinic m1 receptor. It is noteworthy that thrombin stimulation of Shc phosphorylation is weaker in M1-81 cells than in CCL39 cells. Like the thrombin-induced effect, carbachol-stimulated phosphorylation of the 52 kDa Shc isoform, and pertussis toxin treatment did not blunt the signal (results not shown).

PKC activation is not necessary for G_q-mediated Shc phosphorylation in CCL39 cells

We have demonstrated previously that thrombin stimulates phosphatidylinositol-specific phospholipase C activity leading to inositol 1,4,5-trisphosphate and diacylglycerol production in CCL39 cells. Since diacylglycerol has been shown to be an activator of PKC, we set out to determine the possible contribution of PKC to thrombin-stimulated Shc phosphorylation. For this purpose, we examined Shc phosphorylation in PKC-depleted cells. Phorbol ester-sensitive PKC isoforms were down-regulated by pre-treatment of cells with 4-β-phorbol-12,13-dibutyrate (PdBu), a direct activator of PKC. As shown in Figure 4, thrombin is able to stimulate Shc phosphorylation in the absence of PKC, indicating that this event occurs independently of PKC activation. In fact, we observed an enhanced response to thrombin in cells pre-treated with PdBu, which suggests that PKC may exert an inhibitory

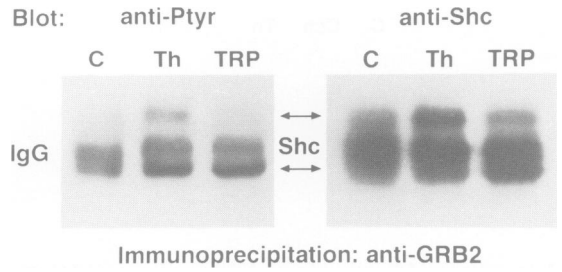

Fig. 5. Thrombin receptor stimulation leads to Shc–Grb2 complex formation. Serum-starved CCL39 cells were treated with: no addition (C), 1 U/ml thrombin (Th) or 30 μM TRP for 15 min before immunoprecipitating cell lysates with anti-Grb2 antibody. Anti-Grb2 immunoprecipitates were analyzed by Western blotting using anti-phosphotyrosine (anti-Ptyr) or anti-Shc antibodies, as indicated above the autoradiogram. The positions of $p46^{Shc}$ and $p52^{Shc}$ are indicated by arrows.

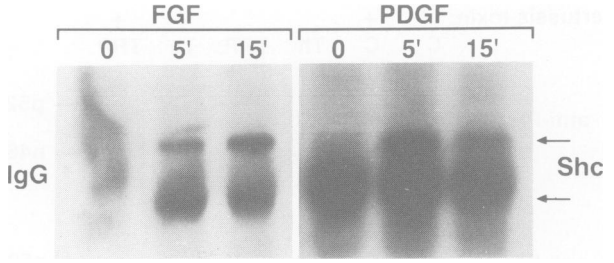

Fig. 6. Time course of FGF- and PDGF-stimulated Shc phosphorylation. Quiescent CCL39 cells were stimulated with 30 ng/ml of FGF or PDGF for the indicated times prior to lysis and immunoprecipitation of lysates with anti-Shc antibody. Phosphotyrosine phosphorylated proteins in immunoprecipitates were detected by immunoblotting. Arrows indicate positions of $p46^{Shc}$ and $p52^{Shc}$.

effect on the pathway leading to Shc phosphorylation. Consistent with this observation, previous studies using CCL39 cells and various subclones have shown that PKC alone is not sufficient to drive the cells from a quiescent state back into the cell cycle, rather the mitogenic response of cells is increased following PKC down-regulation (Seuwen *et al.*, 1990; Van Obberghen-Schilling *et al.*, 1991; Kahan *et al.*, 1992).

Thrombin stimulates Shc–Grb2 complex formation

It has been well documented that, following stimulation of certain receptor tyrosine kinases, phosphorylated Shc becomes associated to an additional adaptor protein, Grb2. The Shc–Grb2 complex binds to Sos, a Ras guanine nucleotide exchange factor involved in Ras activation. Furthermore, the formation of Shc–Grb2 complexes has been found recently to be necessary for the neoplastic transformation associated with overexpression of Shc proteins (Salcini *et al.*, 1994). To examine whether Shc, phosphorylated in response to G protein-coupled receptor agonists, can complex with Grb2, we analyzed Grb2 immunoprecipitates from thrombin- or TRP-treated cells. Western blots, shown in Figure 5, confirm that Shc is present following agonist stimulation and that it is phosphorylated on tyrosine residues. These results, together with the finding that a protein of 180 kDa immunologically related to SOS co-precipitates with phosphorylated Shc (Figure 1 and unpublished results), demonstrate that, in CCL39 cells, thrombin induces phosphorylation of $p52^{Shc}$/$p46^{Shc}$ on tyrosine residues, presumably via a G_q-mediated pathway, and tyrosine-phoshorylated Shc associates with Grb2 and SOS.

FGF and PDGF stimulation of Shc phosphorylation

We have demonstrated recently that both thrombin and PDGF stimulate the activity of Src family kinases in CCL39 fibroblasts. By contrast, FGF was found to inhibit Src-like kinase activity in these cells (Chen *et al.*, 1994). Since Shc has been proposed to be a substrate of Src, we examined whether the divergent effects of PDGF and FGF on Src kinase activation were reflected in the ability of these two mitogens to stimulate Shc phosphorylation. As shown in Figure 6, both FGF and PDGF stimulate Shc phosphorylation, although the time course of the PDGF effect is more rapid than that of the FGF effect. At the same concentration (30 ng/ml), FGF and PDGF both stimulate DNA synthesis re-initiation in quiescent CCL39 cells as effectively as 1 U/ml thrombin (Chen *et al.*, 1994). Thus, Shc phosphorylating activity correlates well with the mitogenic activity of these growth factors, not with their ability to activate Src-like kinases.

Role of Shc in MAP kinase activation and gene induction

In an attempt to define the role of Shc proteins in the growth response pathway stimulated by thrombin, we transiently expressed Shc, or a competitive inhibitory mutant of Shc, in CCL39 cells and examined the consequences on thrombin-stimulated MAP kinase and transcriptional activation.

The effects of the Shc gene product on MAP kinase activation were studied by co-transfecting an Shc expression vector (SHC) and a reporter plasmid ($p44^{mapk}$), encoding an epitope-tagged p44 MAP kinase, in CCL39 cells. The epitope tag on MAP kinase corresponds to a sequence from the influenza hemagglutinin HA1 protein which allows the transfected protein to be immunologically distinguished from endogenous MAP kinase, thereby allowing p44 MAP kinase activity to be monitored selectively in transfected cells. The epitope-tagged kinase has been shown previously to function identically to the endogenous p44 MAP. Following thrombin stimulation of transfected cells, the tagged p44 MAP kinase was immunoprecipitated and assayed for activity using myelin basic protein (MBP) as a substrate. As shown in Figure 7A, thrombin stimulates MBP phosphorylation by nearly 5-fold in cells transfected with the vector alone. In cells transfected with the Shc cDNA construct which encodes both $p52^{Shc}$ and $p46^{Shc}$ proteins, both basal and thrombin-stimulated levels of MBP phosphorylation are increased. In each case, the level of p44 MAP kinase expression was equivalent (not shown). Thus, overexpression of Shc proteins enhances MAP kinase activation. We next examined the effect of expressing a dominant-interfering Shc mutant on thrombin-stimulated MAP kinase activity. The Shc mutant used, referred to as TM (tyrosine mutant), has a substitution of Tyr317 with phenylalanine which abolishes its ability to form a complex with Grb2 and transform cells (Salcini *et al.*, 1994). Results shown in Figure 7B indicate that the TM construct blocks thrombin-stimulated MAP kinase activation in a dose-dependent

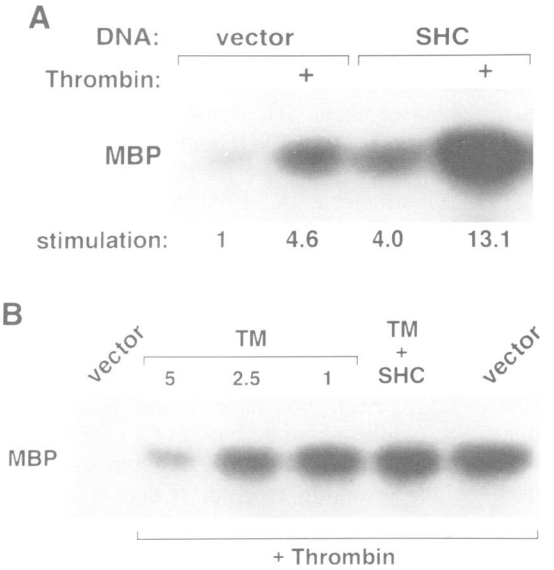

Fig. 7. Effect of Shc on MAP kinase activation in transiently transfected CCL39 cells. (**A**) CCL39 cells were co-transfected with 0.3 μg of $p44^{mapk}$ and 10 μg of L-SHC-SN encoding $p46^{Shc}$ and $p52^{Shc}$ proteins (or the LXSN expression vector). Forty-eight hours later, cells were treated for 5 min with 1 U/ml thrombin, where indicated, prior to lysis and determination of MAP kinase activity, as described in Materials and methods, using MBP as a substrate. Quantification of MAP kinase activity was performed by densitometric scanning of the autoradiogram. (**B**) CCL39 cells were transfected with a total of 20 μg of DNA per well. The indicated quantity (1, 2 or 5 μg) of L-TM-SN (TM) plasmid was completed to 20 μg with vector DNA. TM+SHC corresponds to 5 μg of L-TM-SN plus 15 μg of L-SHC-SN DNA. Forty-eight hours following transfection, cells were stimulated with 1 U/ml thrombin and MAP kinase activity was determined.

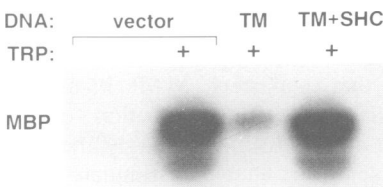

Fig. 8. Effect of wild-type Shc and dominant-interfering Shc Tyr317 mutant on MAP kinase activation by TRP. CCL39 cells were co-transfected with $p44^{mapk}$ (0.3 μg) and a total of 40 μg of DNA corresponding to either the LXSN vector alone (vector), 10 μg of L-TM-SN (TM) or 10 μg of L-TM-SN plus 30 μg of L-SHC-SN (TM+SHC). Forty-eight hours later, 100 μM TRP was added where indicated and MAP kinase activation was determined as described in Materials and methods.

manner. Importantly, the inhibition by TM can be reversed by expression of the wild-type Shc proteins, indicating that it is not due to a non-specific inhibitory effect of Shc overexpression. Potentiation of MAP kinase activation by Shc was also observed in cells stimulated with the thrombin receptor peptide agonist, TRP. Similarly, the dominant-interfering Shc proteins inhibited MAP kinase activation by TRP (Figure 8). Altogether, these results indicate a functional role for Shc in thrombin receptor-mediated activation of the MAP kinase cascade.

Similar findings concerning the implication of Shc proteins in thrombin-induced transcriptional activation were obtained using luciferase as a reporter gene driven

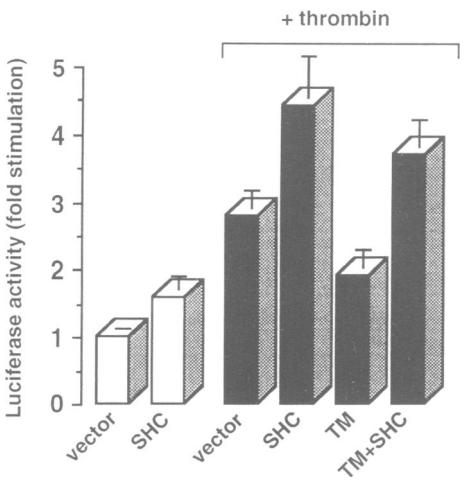

Fig. 9. Effect of Shc expression on c-*fos* promoter activity. CCL39 cells were co-transfected with pADneo-fosluci (50 ng) and the indicated Shc expression vector prior to the determination of luciferase activity as described in Materials and methods. A total of 4 μg of DNA was transfected per well, corresponding to LXSN (vector), 1 μg of L-SHC-SN (SHC), 1 μg of L-TM-SN (TM) or 1 μg of L-TM-SN plus 3 μg of L-SHC-SN (TM+SHC). In the case of SHC and TM, the amount of DNA transfected was completed to 4 μg using vector DNA. Cells were stimulated for 6 h with 1 U/ml thrombin, where indicated. Mean values of quadruplicate determinations (± SEM) from nine independent experiments are shown.

by c-*fos* promoter sequences. Growth factor activation of the c-*fos* promoter has been shown previously to occur, principally, through phosphorylation of TCF/Elk-1 by MAP kinases (reviewed in Treisman, 1994). Figure 9 shows that thrombin stimulates luciferase activity 3-fold above basal values in cells transfected with the empty expression vector. Transfection of the SHC vector increases this effect, indicating that Shc proteins can enhance thrombin-induced transcription. The TM Shc mutant blocks thrombin-stimulated luciferase activity, as observed for MAP kinase activation, presumably by blocking formation of Shc–Grb2 complexes. This inhibitiory effect of TM can be reversed by co-expressing an excess of wild-type Shc proteins.

Expression of Shc TM supresses cell growth

The role of Shc in thrombin-stimulated cell proliferation was examined in a colony formation assay following transfection of the constructs encoding wild-type or TM Shc proteins. To do so, the Shc plasmids (or vector alone) were transfected into CCL39 cells together with a selection vector which codes for an amiloride-resistant Na^+/H^+ exchanger isoform, NHE3. Non-transfected cells were eliminated by two successive acid-load selection tests, as described in Materials and methods, and colonies that emerged in serum-free defined medium containing thrombin were stained and counted. As shown in Figure 10, expression of wild-type Shc enhanced thrombin-dependent colony formation, whereas the Grb2 binding-deficient Shc mutant, TM, inhibited cell growth in a dose-dependent manner. At the highest concentration tested, TM markedly reduced thrombin-stimulated colony formation. Expression of wild-type Shc enhanced thrombin-stimulated colony formation in CCL39 cells.

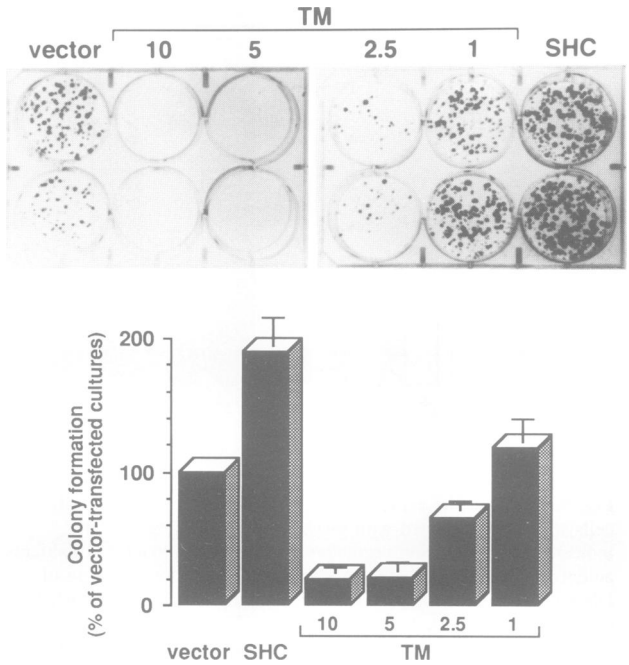

Fig. 10. Dominant-interfering Shc mutant inhibits thrombin-stimulated cell proliferation. CCL39 cells were co-transfected with 2.5 μg of the NHE3 Na^+/H^+ antiporter expression plasmid and 10 μg of the test plasmid corresponding to: LXSN (vector); 1, 2.5, 5 or 10 μg of L-TM-SN completed to 10 μg with the LXSN vector (TM); or 10 μg of L-SHC-SN alone (SHC). The colony formation assay was performed as described in Materials and methods. Giemsa-stained colonies from a representative experiment are shown in the top panel. Mean values of colony formation from duplicate determinations (± SEM) obtained in six independent experiments are plotted in the bottom panel.

Discussion

In CCL39 fibroblasts, proliferation following thrombin stimulation is dependent upon activation of Ras and the MAP kinase cascade, yet little is known about the events preceding Ras activation by the protease. Biochemical and genetic studies on receptor tyrosine kinases show that the Shc/Grb2/Sos pathway is essential for Ras activation (reviewed in Pawson, 1995). The present studies were designed to determine whether a similar pathway leads to Ras activation by the G protein-coupled thrombin receptor. In this report, we show that thrombin stimulates tyrosine phosphorylation of Shc proteins, $p46^{Shc}$ and $p52^{Shc}$, which results in their association with Grb2. In mammalian cells Grb2 appears to be constitutively associated with the Ras exchange factor Sos (Buday and Downward, 1993; Chardin *et al.*, 1993; Egan *et al.*, 1993; Li *et al.*, 1993). Indeed, we find that Sos is present in anti-Grb2 immunoprecipitates from thrombin-treated CCL39 cells, in addition to phosphorylated Shc proteins. Evidence that the Shc–Grb2 complex is involved in Ras activation by thrombin was obtained using a Shc mutant (TM) deficient in Grb2 binding. Indeed, the TM mutant was found to blunt very efficiently two Ras-dependent effects of thrombin, MAP kinase stimulation and transcriptional activation of the c-*fos* promoter. Further, expression of the TM Shc mutant markedly inhibits thrombin-stimulated CCL39 cell proliferation, indicating that Shc proteins play a determinant role in mitogenic signal transmission by thrombin in fibroblasts.

Which kinase(s) lie(s) upstream of Shc? Shc may be a suitable substrate for multiple tyrosine kinases, both receptor and non-receptor. Shc binding to transmembrane receptor tyrosine kinases via its SH2 domain has been reported, including receptors for EGF, NGF and PDGFβ, suggesting that it may be a direct substrate for these kinases (Pelicci *et al.*, 1992; Obermeier *et al.*, 1993; Segatto *et al.*, 1993; Yokote *et al.*, 1994). In the case of non-receptor tyrosine kinases, Shc has been implicated in the coupling of Src kinases to Ras (Pelicci *et al.*, 1992; Obermeier *et al.*, 1993; Segatto *et al.*, 1993; Yokote *et al.*, 1994). Consistent with this, Shc is phosphorylated on tyrosine residues and complexed to Grb2 in cells transformed by v-*src* (Rozakis-Adcock *et al.*, 1992). We have found that thrombin stimulates Src family kinase activity in CCL39 fibroblasts in a recent study aimed at identifying tyrosine kinases activated by the thrombin receptor (Chen *et al.*, 1994). However, the time course of Src kinase activation is quite different from the time course of Shc phosphorylation by thrombin. Src-like kinase stimulation is very rapid (≤1 min) and transient. Further, Src-like kinase activation by thrombin in CCL39 cells was found to be partially sensitive to pertussis toxin pre-treatment of cells, suggesting that a G_i-like protein is implicated in this effect. The fact that Shc phosphorylation stimulated by thrombin is not inhibited by pertussis toxin argues against a casual link between activation of Src family kinases by thrombin and Shc phosphorylation. Studies are in progress to identify signaling components activated by thrombin which lie upstream of Shc and Src.

Maximal tyrosine phosphorylation of $p46^{Shc}$ and $p52^{Shc}$ induced by thrombin occurs by 15 min of stimulation, and levels of the phosphorylated protein remain elevated for at least 4 h. This persistent effect observed on Shc phosphorylation is reminiscent of the sustained activation of MAP kinase seen in these cells. We have found previously that persistent MAP kinase activation is required for mitogenic stimulation of CCL39 cells (Meloche *et al.*, 1992b). Interestingly, the thrombin receptor peptide agonist is unable to stimulate persistent activation of MAP kinase, or cell cycle re-entry when added alone to cells (Vouret-Craviari *et al.*, 1992, 1993). The fact that the thrombin receptor peptide is a full agonist of Shc phosphorylation suggests that Shc phosphorylation may be an essential step, but not sufficient for signaling cell cycle re-entry of quiescent CCL39 cells. TRP activation of Shc phosphorylation confirms that the thrombin effect is mediated by the cloned G protein-coupled receptor. This is supported by our findings that AlF_4^- (a direct activator of G proteins) stimulates Shc phosphorylation, albeit weakly. Shc phosphorylation induced by direct activation of G proteins with AlF_4^- or via the endothelin receptor has also been observed recently in astrocytes (Cazaubon *et al.*, 1994). The addition of angiotensin II to cultures of neonatal cardiac fibroblasts has also been found to stimulate $p46^{Shc}$ and $p52^{Shc}$ phosphorylation (Schorb *et al.*, 1994). This effect of angiotensin II appears to be mediated by the angiotensin I receptor that couples to both G_i and G_q, however the nature of the G protein involved in the stimulation of Shc phosphorylation by angiotensin II was not determined.

The thrombin receptor modifies the activity of at least two distinct biological effectors (adenylyl cyclase and

phospholipase C), via G_i-like and G_q proteins, respectively. The conclusion that a pertussis toxin-sensitive G protein appears to be crucial for thrombin mitogenic effects was drawn from the finding that pertussis toxin blunts ~80% of the DNA synthesis stimulated by thrombin in CCL39 cells, together with results of microinjection experiments in which specific anti-G_i antibody was found to block thrombin-stimulated DNA synthesis. Baffy and colleagues have reported recently that G_q and G_o, rather than G_i, are the G proteins involved in mediating thrombin receptor effects in CCL39 cells (Baffy *et al.*, 1994). Data presented above that Shc tyrosine phosphorylation is insensitive to pertussis toxin, and that carbachol stimulates tyrosine phosphorylation of Shc proteins in M1-81 cells stably expressing the m1 receptor would indicate that G_q is involved in this effect.

G_q is known to stimulate phosphatidylinositol-specific phospholipase C activity generating IP_3 and diacylglycerol, which in turn induces Ca^{2+} release from intracellular stores and stimulates PKC. Although PLC/PKC activation is not sufficient for stimulation of proliferation in CCL39 cells (Seuwen *et al.*, 1990), it may contribute to the pertussis toxin-insensitive component of thrombin-induced DNA synthesis. In the present study, down-regulation of PdBu-sensitive PKC isoforms did not blunt the thrombin-stimulated effect, and we were unable to detect significant Shc phosphorylation on tyrosine residues in response to phorbol ester. This indicates that Shc phosphorylation is not mediated by PKC in thrombin-treated cells, although phorbol ester-insensitive PKC isoforms, such as PKCζ, may be involved. Consistent with our findings, Ohmichi *et al.* (1994) have observed that TRH stimulates the tyrosine phosphorylation of the $p52^{Shc}$ protein and induces association of phosphorylated Shc with a Grb2 fusion protein in GH3 cells. The TRH receptor is a G protein receptor presumably coupled to G_q in these cells. Neither phorbol esters nor the calcium ionophore A23187 had any effect on TRH-mediated Shc phosphorylation, suggesting that the effect of TRH is independent of PKC.

An inhibitory effect of pertussis toxin was observed on thrombin-stimulated luciferase activity (data not shown), raising the possibility that additional signals mediated by G_i or G_o proteins may contribute to the transcriptional response and subsequent cell cycle re-entry.

In conclusion, the thrombin receptor, similar to receptors with intrinsic tyrosine kinase activity, is capable of triggering the phosphorylation of Shc proteins on tyrosine residues and their subsequent association with Grb2. Shc phosphorylation by thrombin appears to be mediated by a pertussis toxin-insensitive G protein and to represent a crucial link between G_q-coupled receptors and the activation of Ras/MAP kinases. However, a pertussis toxin-sensitive signaling component which is crucial for growth promotion remains to be identified. In this system, the protein tyrosine phosphatase PTP1D has also been found to play a positive role in linking the thrombin receptor to mitogenic signaling (Rivard *et al.*, 1995). Therefore, the G protein-coupled thrombin receptor and receptor tyrosine kinases share at least two adaptor/signaling molecules leading to Ras activation.

Materials and methods

Materials

Highly purified human α-thrombin (3200 NIH units/mg) and human recombinant basic FGF were generous gifts of J.W.Fenton II (New York State Department of Health, Albany, NY) and D.Gospodarowicz (University of California Medical Center, San Francisco, CA), respectively. Triton X-100, pertussis toxin, carbachol, PdBu, bovine myelin basic protein and luciferin were from Sigma (St Louis, MO). Hybon™-C and the enhanced chemiluminescence (ECL) immunodetection system were purchased from Amersham, and Pansorbin from Calbiochem (San Diego, CA). The thrombin receptor peptide agonist (TRP) of seven residues (SFFLRNP) was synthesized by Neosystem (Strasbourg, France). The amiloride analog, HOE694, was provided by W.Scholz and H.J.Lang (Hoechst AG, Frankfurt, Germany).

Cell culture

The Chinese hamster lung fibroblast line CCL39 (American Type Culture Collection) was maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 7.5% fetal calf serum, penicillin (50 U/ml) and streptomycin (50 μg/ml) in 5% CO_2/95% air at 37°C. Growth-arrested cells were obtained by total serum deprivation for 16–24 h. Pre-treatment of cells for 4 h with pertussis toxin (100 ng/ml) was carried out to ADP-ribosylate and functionally uncouple pertussis toxin-sensitive G proteins from the thrombin receptor.

Antibodies

Polyclonal and monoclonal anti-Shc antibodies were generated by immunizing New Zealand white rabbits with a GST–Shc fusion protein as described earlier (Pelicci *et al.*, 1992). Anti-Grb2 antibody against GST fusion protein containing the N-terminal SH3 domain of Grb2 was a generous gift of Dr J.Schlessinger (New York University Medical Center, NY). Monoclonal anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY), and 12CA5 monoclonal antibody directed against a sequence of influenza hemagglutinin HA1 protein (Wilson *et al.*, 1984) was from Babco (Emeryville, CA). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgGs were from Amersham (France).

Plasmids

The constructs L-SHC-SN and L-TM-SN, which correspond to wild-type and Tyr317 mutant Shc cDNAs cloned in the LXSN retroviral expression vector, have been described previously (Salcini *et al.*, 1994). The plasmid $p44^{mapk}$ encodes an epitope-tagged p44 MAP kinase, as reported (Meloche *et al.*, 1992a). pADneo-fosluci containing the entire c-*fos* promoter region cloned upstream of the *Photinus pyralis* luciferase gene was kindly provided by Drs C.Stratowa and A.P.Czernilofsky (Boehringer Ingelheim). The NH3 expression plasmid (pCMVNHE3) (Orlowski *et al.*, 1992) was kindly provided by Dr J.Orlowski (McGill University, Montreal, Canada).

Immunoprecipitation and Western blotting analysis

Confluent CCL39 cells in 100 mm dishes were incubated in serum-free DMEM for 20 h before addition of agonists for the indicated times at 37°C. Stimulation was terminated by washing twice with ice-cold phosphate-buffered saline (PBS). Cells were then solubilized in lysis buffer containing 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 1 mM NaF, 1% Triton X-100, 0.5% NP-40 and protease inhibitors (5 μg/ml aprotinin, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and 10 μg/ml pepstatin A) for 30 min at 4°C. Cells were scraped from dishes and passed several times through a 26 gauge needle to disperse large aggregates. After centrifugation at 10 000 *g* for 15 min at 4°C, the supernatants were transferred to tubes containing anti-Shc, anti-Grb2 or anti-phosphotyrosine antibodies, vortexed and incubated at 4°C for 2.5 h with constant rotating. Fifty microlitres of washed 10% Pansorbin was added to each tube and the incubation was continued for another 2 h. After centrifugation at 10 000 *g* for 4 min, the immunoprecipitates were washed four times with lysis buffer and resuspended in 25 μl of reducing electrophoresis sample buffer. Samples were resolved on an 8% SDS–polyacrylamide gel and transferred to Hybond-C nitrocellulose membrane (Amersham).

For immunoblotting, membranes were blocked and probed (1 h at room temperature) with 3% milk in PBS (for monoclonal anti-phosphotyrosine antibody 4G10) or with 5% milk in 10 mM Tris (pH 7.5), 100 mM NaCl and 0.1% Tween-20 (for monoclonal anti-Shc antibody). After three washes, blots were incubated with the appropriate second antibodies conjugated to horseradish peroxidase. Immunoreactive bands were detected with the enhanced chemiluminescence reagent (Amersham).

Transient transfection

MAP kinase assay. CCL39 cells inoculated in 6-well culture plates were transfected with the indicated SHC expression vectors and 0.3 μg of the

$p44^{mapk}$ reporter plasmid using a calcium phosphate co-precipitation protocol. After 16 h, transfected cells were rinsed twice with PBS and placed in DMEM containing 0.5% fetal calf serum. Twenty-four hours later, cells were treated with the indicated agonist in serum-free DMEM for 5 min prior to lysis, and determination of MAP kinase activity in an immune complex kinase assay was as described previously (Meloche *et al.*, 1992a).

Luciferase assay. Cells in 24-well culture plates were co-transfected with the indicated SHC constructs and 50 ng of the pADneo-fosluci reporter plasmid and growth arrested, as described above. Agonists were added for 6 h and quadruplicate determinations of luciferase activity in cell lysates were carried out in the presence of coenzyme A, according to the method described in the Promega 'Protocols and Applications Guide'.

Colony formation

CCL39 cells in 6-well plates were co-transfected with 2.5 μg of the NHE3 expression plasmid and 10 μg of the indicated test plasmid as described above. Cultures were subjected to two successive acid-load selection tests, 48 and 72 h following transfection, to kill non-transfected cells. Acid-load selection of cells expressing the transfected Na^+/H^+ antiporter isoform NHE3 was carried out in the presence of the amiloride analog HOE694 (Counillon *et al.*, 1993) at a concentration of 0.1 mM. Surviving cells were allowed to recover from the second acid-load in complete culture medium for 24 h before placing them in a previously described serum-free medium (Van Obberghen-Schilling and Pouysségur, 1983) containing insulin (10 μg/ml) and thrombin (0.1 U/ml). Colonies were stained with Giemsa ~2 weeks after transfer to the defined medium.

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Note added in proof

While this paper was being reviewed, van Biesen *et al.* (1995) (*Nature*, **376**, 781–784) reported an increase in Shc phosphorylation following stimulation of G_i-coupled (LPA and α_{2A}AR) receptors that appears to be mediated by G protein βγ subunits.