Tec/Bmx non-receptor tyrosine kinases are involved in regulation of Rho and serum response factor by G α 12/13

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A transient transfection system was used to identify regulators and effectors for Tec and Bmx, members of the Tec non-receptor tyrosine kinase family. We found that Tec and Bmx activate serum response factor (SRF), in synergy with constitutively active α subunits of the G12 family of GTP-binding proteins, in transiently transfected NIH 3T3 cells. The SRF activation is sensitive to C3, suggesting the involvement of Rho. The kinase and Tec homology (TH) domains of the kinases are required for SRF activation. In addition, kinase-deficient mutants of Bmx are able to inhibit G α 13- and G α 12-induced SRF activation, and to suppress thrombin-induced SRF activation in cells lacking G α q/11, where thrombin's effect is mediated by G12/13 proteins. Moreover, expression of G α 12 and G α 13 stimulates autophosphorylation and transphosphorylation activities of Tec. Thus, the evidence indicates that Tec kinases are involved in G α 12/13-induced, Rho-mediated activation of SRF. Furthermore, Src, which was previously shown to activate kinase activities of Tec kinases, activates SRF predominantly in Rho-independent pathways in 3T3 cells, as shown by the fact that C3 did not block Src-mediated SRF activation. However, the Rho-dependent pathway becomes significant when Tec is overexpressed.

Keywords: gene regulation/G protein/serum response factor/small G protein/tyrosine kinase

Introduction

Hormones, neurotransmitters and many other biologically active molecules, such as thrombin, transduce signals through heterotrimeric G proteins. Molecular cloning has revealed at least four classes of G protein α subunits: G α s, G α i, G α q and G α 12 (Gilman, 1987; Birnbaumer *et al.*, 1990). G α s and G α i subunits regulate adenylyl cyclase activities, while G α q subunits regulate phospholipase C activities. However, the function of the G α 12 class of G proteins, which includes G α 12 and G α 13, remains unknown. Activated forms of G α 12 and G α 13 were shown to induce transformation phenotypes when transfected into fibroblast cells (Jiang *et al.*, 1993; Xu *et al.*, 1994), suggesting that this class of G proteins may

be involved in cell growth regulation. Moreover, G α 12 and G α 13 were shown to induce formation of stress fibers in fibroblast cells through the small G protein Rho (Buhl *et al.*, 1995). This observation was confirmed by a report that G α 12 activated serum response factor (SRF) through Rho (Fromm *et al.*, 1997). The *in vivo* function of G α 13 was also investigated using the gene targeting technique in mice. Mice lacking G α 13 are embryonic lethal, apparently due to failure to develop vasculature structures, indicating that G α 13 may be involved in the function of endothelial cells (Offermanns *et al.*, 1997). In this study, thrombin-mediated chemotaxis of fibroblasts lacking G α 13 was blocked, demonstrating that the thrombin receptor couples to G α 13. This is consistent with the observation that thrombin stimulates the binding of a photo-affinity GTP analog to G α 13 (Offermanns *et al.*, 1997).

Members of the Rho family of small GTP-binding proteins, including RhoA, Rac and Cdc42, play significant roles in cytoskeleton reorganization (Hall, 1998). Rac and Cdc42 have also been found to regulate gene transcription by activating the c-Jun N-terminal kinase (JNK) and p38 stress-induced kinase via a cascade of kinase-mediated phosphorylation events (Coso *et al.*, 1995; Minden *et al.*, 1995). However, Rho appears to be a weak activator of JNK and p38 kinase (Hill *et al.*, 1995). Nevertheless, all three small G proteins were able to stimulate SRF to induce serum response element (SRE)-dependent transcription (Hill *et al.*, 1995). Although Rac and Cdc42 appear to be able to activate RhoA, RhoA can also be regulated by Rac- and Cdc42-independent pathways, probably through G protein-mediated pathways (Coso *et al.*, 1995; Hill *et al.*, 1995; Hall, 1998).

Tec, Bmx, Btk and Itk belong to one of the non-receptor tyrosine kinase families (Rawlings and Witte, 1995; Neet and Hunter, 1996). This class of kinases is distinguished from other subfamilies by N-terminal pleckstrin homology (PH) domains and Tec homology (TH) domains located downstream of the PH domain. In addition, these kinases contain Src homology (SH) 2, SH3 and C-terminal kinase domains. Btk and Itk are mainly found in hematopoietic cells, while Tec and Bmx are found in a variety of tissues and cell types. Loss-of-function mutations in the Btk molecule are believed to be responsible for both human X-linked agammaglobulinemia (XLA) and murine X-linked B-cell immunodeficiency (XID) (Rawlings and Witte, 1995). However, regulatory mechanisms and cellular functions of these kinases remain ill defined. Studies have indicated that Tec/Btk kinases were substrates of the Src kinases and that Src-mediated phosphorylation of Tec/Btk molecules stimulated the kinase activities of the Tec/Btk kinases (Mano *et al.*, 1996; Rawlings *et al.*, 1996). In addition, a number of cytokine receptors, including c-kit, CSF, interleukin-3 and erythropoietin, were shown to stimulate kinase activity of Tec kinases (Machide *et al.*,

1995; Rawlings and Witte, 1995; Miyazato *et al.*, 1996). Moreover, G protein-coupled thrombin receptors were shown to stimulate tyrosine phosphorylation of Tec in platelets (Hamazaki *et al.*, 1998), suggesting that multiple pathways lead to Tec activation. Furthermore, purified G α q was shown to activate the kinase activities of purified Btk, suggesting that G α q directly regulates Btk (Bence *et al.*, 1997). In this report, we present evidence for the involvement of Tec and Bmx in G α 12/13-induced Rho- and SRF-activation.

Results

Non-receptor tyrosine kinases activate SRF

To better understand the cellular function of non-receptor kinases, we investigated the regulation of SRF-mediated transcription of a luciferase reporter gene by a number of non-receptor tyrosine kinases. In this reporter system, an altered c-fos SRE, SRE.L (Hill *et al.*, 1995), was placed in front of the luciferase gene. SRE.L binds only to the transcription factor SRF, and not to tertiary complex factor (TCF). Thus, SRE.L can be regulated by SRF independently of TCF (Hill *et al.*, 1995). A number of non-receptor tyrosine kinases from different subclasses, including Syk, Sik, Csk, Tec and Src, were tested for their abilities to activate SRF-mediated transcription. When these kinases were co-expressed with the SRE.L-luciferase reporter plasmid in NIH 3T3 cells, we found that activated Src and the wild-type Tec kinase were potent activators of reporter gene transcription, presumably through activation of SRF (Figure 1). Previous studies have demonstrated that the small G proteins Rho, Cdc42 and Rac were able to stimulate SRE.L-mediated transcriptional activation (Hill *et al.*, 1995). A Rho-specific inactivator (*Clostridium botulinum* C3 transferase), which specifically ADP-ribosylates Rho proteins at Asn41 (Hill *et al.*, 1995), was used to discriminate Rho-mediated pathways from those which are Cdc42- or Rac-mediated. Consistent with previous findings (Hill *et al.*, 1995), C3 inhibited transcriptional activation of the reporter gene by constitutively activated Rho (RhoV14), but not by constitutively activated Cdc42 (Cdc42V12) (Figure 1), suggesting that C3 specifically affects Rho-mediated activities. When C3 was co-expressed with Tec under the same experimental condition, C3 abolished Tec-mediated activation of reporter gene transcription (Figure 1). Additional evidence to support the activation of Rho by Tec was sought. Rho activation leading to cytoskeleton reorganization has been well studied (Hall, 1998). We tested whether Tec induces cytoskeleton reorganization using a previously reported approach (Gohla *et al.*, 1998). NIH 3T3 cells were co-transfected with an expression plasmid expressing both blue fluorescence protein (BFP) and Tec, or BFP alone. Cells expressing Tec, identified by expression BFP (Figure 1C), showed increases in actin polymerization and formation of stress fibers (Figure 1B). However, cells transfected with the plasmid expressing BFP alone showed no such phenotypes (Figure 1D and E). Activation of Rho by G protein-coupled receptors, including endothelin and LPA (Fleming *et al.*, 1996), and by PDGF (Fleming *et al.*, 1996) and insulin receptor (Karnam *et al.*, 1997), was also shown to translocate the RhoA proteins to particulate fractions. Therefore, we tested whether Tec can induce

Fig. 1. Regulation of SRF by non-receptor tyrosine kinases. (A) NIH 3T3 cells were co-transfected with 0.15 μ g SRE.L-luciferase reporter plasmid, 0.15 μ g GFP expression construct and 0.2 μ g β -galactosidase (LacZ), Syk, Sik, Csk, Tec, Src, and activated Cdc42V12 (Cdc*) or RhoV14 (Rho*) in the presence (open bars) or absence (black bars) of 0.02 μ g C3 expression plasmid. One day later cells were lysed, and GFP levels and luciferase activities were determined. The luciferase activities presented are normalized against the levels of GFP expression. Data show similar trends with or without normalization. Each experiment was carried out in triplicate, and error bars represent standard deviations. (B, C, D and E) NIH 3T3 cells were transfected with an expression plasmid that expresses both BFP and Tec (B and C), or BFP alone (D and E). FITC-phalloidin was viewed with a standard filter set for FITC (B and D) while BFP was viewed with a filter set using an excitation filter at 350 nm and an emission filter at 450 nm with a bandpass of 20 nm (C and E). (F) Cos-7 cells were transfected with Tec or LacZ. One day later the cells were fractionated into the cytosol (C) and particulate fractions (P), which were analyzed by Western blot using a RhoA-specific antibody.

translocation of the RhoA proteins. Expression of Tec increased the levels of Rho A proteins in particulate fractions by 75% (Figure 1F); therefore, Tec can activate Rho, which leads to SRF activation. Interestingly, Src-mediated SRF activation was largely unaffected by the expression of C3 (Figure 1), suggesting that Tec kinases may not be predominantly used for SRF activation.

Tec kinases activate SRF in synergy with Gα13

The activated form of Gα12, Gα12QL, was previously shown to activate SRE.L-mediated gene transcription (Fromm *et al.*, 1997). We confirmed that observation, and also found that the activated form of Gα13, Gα13QL, which is a member of the G12 family with a substitution of a Leu residue for Gln226, could activate SER.L-mediated gene transcription when transfected into 3T3 cells (Figure 2A). Moreover, co-expression of C3 blocked G protein-mediated SRF activation (Figure 2A), suggesting that these G proteins activate SRF via Rho. Once again, under our transfection conditions, expression of C3 did not significantly affect Cdc42V14-induced SRF activation (Figure 2A). Two G-protein βγ subunit complexes were also tested. Cells co-expressing Gβ1 and Gγ2 or Gβ1 and Gγ5 showed only slight SRF activation (Figure 2A). Furthermore, expression of activated Gαi2 and GαoA did not activate SRF (Figure 2A). The expression of these G protein subunits is shown in Figure 2C.

Since both Tec and Gα13 can activate SRF in a C3-dependent manner, it is possible that there is an interaction between Gα13 and Tec. If this is the case, Tec may activate SRF synergistically with Gα13. In fact, cells co-transfected with cDNAs encoding Gα13QL and Tec produced more reporter gene products than those transfected with the G protein or kinase cDNA alone (Figure 2B). We also tested another member of the Tec family, Bmx (Tamagnone *et al.*, 1994). Although Bmx showed a little basal activation of SRF, the presence of Bmx markedly increased Gα13QL-induced SRF activation (Figure 2B), suggesting that Gα13 may activate Bmx and lead to SRF activation. C3 was able to abrogate the activation (Figure 2B). When Syk, Sik and Csk were tested with Gα13, none of the kinases had an effect on Gα13QL-mediated activation of SRF (Figure 2B). The expression levels of Gα13QL (Figure 2C) and Tec kinases (data not shown) were similar in various transfectants; thus, the synergistic effects between Gα13 and Tec kinases are unlikely to be due to variation in transfection efficiencies. Gα12 showed similar synergism with Tec and Bmx (data not shown).

Synergistic effect with Gα13 requires the kinase domain of Bmx

To test the importance of the kinase activity of Tec class kinases in synergistic activation of SRF by Gα13, two kinase-deficient Bmx mutants were constructed: BmxK, with a deletion of the C-terminal kinase domain; and BmxKR, containing a point mutation with substitution of an Arg residue for the ATP-binding Lys425 (Tamagnone *et al.*, 1994). The expression of BmxK or BmxKR showed no synergistic effect on Gα13QL-induced activation of SRF (Figure 3A). Thus, the kinase activity of Bmx is required for activation of SRF with Gα13. More importantly, both mutants appeared to inhibit Gα13-mediated

Fig. 2. Regulation of SRF by G proteins. (A) NIH 3T3 cells were co-transfected with 0.15 μg SRE.L-luciferase reporter plasmid, 0.15 μg GFP expression construct, 0.2 μg β-galactosidase (LacZ), Cdc42V12 (Cdc*) and RhoV14 (Rho*), or 0.01 μg of various G protein subunits in the presence (open bars) or absence (closed bars) of 0.02 μg C3 expression plasmid. (B) NIH 3T3 cells were co-transfected with 0.15 μg SRE.L-luciferase reporter plasmid, 0.15 μg GFP expression construct and 0.2 μg β-galactosidase (LacZ), and tyrosine kinases in the presence or absence of Gα13 (0.01 μg per transfection) and/or C3 (0.02 μg per transfection). LacZ expression plasmid was used to make the total amount of DNA in all transfections equal. One day after transfection cells were lysed, and GFP levels and luciferase activities was determined as in Figure 1. (C) The expression of Gαi2 and GαoA proteins was detected using an antibody (Santa Cruz, CA) recognizing a common sequence within Gαi and Gαo. All other G protein subunits were detected with specific antibodies indicated in the figure. 'C' represents samples derived from cells expressing LacZ.

SRF activation (Figure 3A). We also generated two other Bmx mutants: BmxN, encompassing the N-terminal half of the Bmx molecule; and BmxC, covering the C-terminal half (Figure 3C). The expression of these Bmx mutants is shown in Figure 3B. Neither BmxN nor BmxC showed stimulatory or inhibitory effects on Gα13-mediated SRF activation (Figure 3A). This suggests that the inhibitory effects observed with BmxK and BmxKR are specific, and that more than one structural domain is required for synergistic effects of Bmx and Gα13.

The inhibitory effects of the kinase-deficient Bmx on Gα13QL-mediated SRF activation do not appear to be robust. It is possible that the kinase-deficient Bmx molecules are weak dominant-negative mutants and thus cannot efficiently suppress the effects of overexpressed

Fig. 3. Involvement of Bmx in Gα13-mediated SRF activation. (A) NIH 3T3 cells were co-transfected with 0.15 μg SRE.L-luciferase reporter plasmid, 0.15 μg GFP expression construct and 0.2 μg β-galactosidase (LacZ), Bmx or its mutants, in the presence (open bars) or absence (closed bars) of 0.02 μg Gα13QL. One day later, cells were lysed and GFP levels and luciferase activities were determined as in Figure 1. (B) NIH 3T3 cells were co-transfected with 0.15 μg SRE.L-luciferase reporter plasmid, 0.15 μg GFP expression construct and 0.02 μg wild-type Gα12 or Gα13 in the presence of 0, 0.1 or 0.2 μg BmxKR. LacZ was used to make up the total DNA concentration to be equal in all transfections. One day later, cells were lysed and GFP levels and luciferase activities were determined as in Figure 1. Gα12 and Gα13 showed 250 and 330% increases, respectively, over cells expressing LacZ. (C) The expression of Bmx and its mutants in COS-7 cells were detected with an antibody recognizing the flag tag, which was carried by Bmx and its mutants. (D) Schematic representation of the Bmx molecule and its mutants. Various structural domains are also labeled. PH, pleckstrin domain; TH, Tec homology domain; SH, Src homology domain.

constitutively activated Gα13. Since the wild-type Gα12 and Gα13 were also able to induce significant increases in production of reporter gene product, we tested whether the kinase-free Bmx mutants show more significant inhibitory effects. Significant activity of the wild-type Gα12/13 has been observed by others (Voyno-Yasenetskaya *et al.*, 1994; Buhl *et al.*, 1995; Fromm *et al.*, 1997; Gohla *et al.*, 1998), and it was thought to be caused by the low intrinsic GTPase activities of these proteins (Offermanns *et al.*, 1994). BmxKR inhibited Gα12-mediated SRF activation by >50% and Gα13-mediated SRF activation by ~45% (Figure 3B). In addition, the inhibitory effects depended on the amounts of transfected DNA.

Kinase-deficient Bmx mutants inhibit thrombin-induced SRF activation in Gαq/11-deficient cells

The dominant-negative mutants of Bmx were also tested in systems where ligands for G protein-coupled receptors were used to induce SRF activation. Both thrombin and norepinephrine are capable of stimulating SER.L-mediated transcription of the luciferase reporter gene through endogenous thrombin receptor (TR) and transfected α1B-adrenergic receptor (AR), respectively (Figure 4A). The transcriptional activities mediated by both receptors were blocked by co-expression of C3 (Figure 4A), suggesting that these two receptors can activate SRF through Rho proteins. Since both receptors were known to couple to Gq proteins (Wu *et al.*, 1992a; Offermanns *et al.*, 1994),

Fig. 4. Effects of Bmx mutants on SRF activation by G protein-coupled receptors. (A) NIH 3T3 cells were co-transfected with 0.15 μg SRE.L-luciferase reporter plasmid, 0.15 μg GFP expression construct and 0.2 μg β-galactosidase (LacZ), or α1-AR in the presence or absence of 0.02 μg C3 expression plasmid. (B) Fibroblast cell line established from Gαq/11-deficient mouse embryo was co-transfected with 0.15 μg SRE.L-luciferase reporter plasmid, 0.15 μg GFP expression construct and 0.2 μg β-galactosidase (LacZ), or α1-AR in the presence or absence of 0.02 μg of C3 expression plasmid. (C) Gαq/11-deficient fibroblast cells were co-transfected with 0.15 μg SRE.L-luciferase reporter plasmid, 0.15 μg GFP expression construct and 0.2 μg β-galactosidase (LacZ), or Bmx mutants. For (A), (B) and (C), ligands [20 U thrombin (Thr) or 1 μM norepinephrine (Nor)] were added the next day. Eight hours later, luciferase activities were determined as described in Figure 1. (D) Gαq/11-deficient fibroblast cells were co-transfected with 0.15 μg SRE.L-luciferase reporter plasmid, 0.15 μg GFP expression construct and 0.2 μg β-galactosidase (LacZ), or Bmx mutants in the presence or absence of 0.05 μg activated RhoV14 or 0.1 μg Tec. The luciferase activities were determined the next day as described in Figure 1.

the abilities of these two receptors to activate SRF were also tested in a fibroblast cell line that was derived from mice lacking Gαq and Gα11 (S.Offermanns and M.I.Simon, unpublished data). While thrombin can still activate SRF through endogenous thrombin receptors, recombinant α1-AR loses its ability to activate SRF (Figure 4B). α1-AR regained the ability to activate SRF after Gαq was reintroduced into the Gαq/11-deficient cells by transfection (Figure 4B). This result indicates that α1-AR-mediated SRF activation is completely dependent on Gαq/11 proteins, whereas the thrombin receptor is also able to activate SRF via other G proteins. Since α1-AR can couple to all five members of the Gαq family, including Gαq, 11, 14, 15 and 16 (Wu *et al.*, 1992a), the inability of α1-AR receptors to activate SRF in the Gαq/11-deficient cells also indicates that thrombin-induced SRF activation cannot be mediated by any of the five members of the Gq class of G proteins. Thrombin has been previously demonstrated to couple to Gα12/13

(Offermanns *et al.*, 1994, 1997), and 3T3 cells contain endogenous G α 13 (Figure 2C) and G α 12 (data not shown). Thus, thrombin-induced activation of SRF in the G α q/11-deficient cells should at least in part be mediated by G α 12/13. Therefore, this is an excellent system for testing the effects of Bmx kinase-deficient mutants BmxK and BmxKR on endogenous G α 12/13-mediated SRF activation. When the mutants were co-transfected into the G α q/11-deficient cells with the reporter gene, cells expressing either kinase-deficient mutants yielded <50% of thrombin-induced activities compared with those expressing the control β -galactosidase (Figure 4C). Expression of BmxK and BmxKR had no effect on Tec-, activated RhoA- (Figure 4D), Cdc42-, Rac1- or PDGF-induced Rho activation (data not shown), suggesting that the inhibitory effects of BmxK and BmxKR are not attributed to their non-specific effects. Thus, we believe that Bmx is likely to lie downstream of G α 12/13 but upstream of RhoA, and may mediate thrombin- and G α 12/13-induced activation of Rho, eventually leading to activation of SRF.

Tec-mediated SRF activation is independent of SH3 and PH domains but dependent on TH and kinase domains

The high basal activity of Tec kinase in SRF activation (Figure 1) allows us to determine which domains of the Tec kinases are important in activation of the downstream effector SRF. A number of deletion mutants lacking various structural domains were generated: TecK, lacking the C-terminal kinase domain; PH, lacking the N-terminal PH domain; SH3, with a deletion of the SH3 domain; and TH domain, containing a deletion in the TH domain (the Ras GAP homologous region was deleted but the Pro-rich region remained). The TH domain is unique to the Tec family kinases and shares homology with Ras GAP (Rawlings and Witte, 1995). The SH3 domain contains the major autophosphorylation site (Park *et al.*, 1996). The expression of wild-type Tec and its mutants, detected with an antibody specific to a Tec N-terminal sequence, is shown in Figure 5B. TecK, when expressed in NIH 3T3 cells, lost its basal SRF activation activity (Figure 5A). In addition to the results associated with Bmx kinase-deficient mutants, we conclude that the kinase domain is essential for SRF activation. The TH mutant also failed to show any basal activation of SRF when expressed in NIH cells (Figure 5A). Furthermore, neither TH nor K mutants showed synergistic SRF activation with G α 13QL (Figure 5A). However, deletion of the PH or SH3 domains did not affect either basal activity or synergistic activation of SRF with G α 13 as measured by SRE.L-mediated production of luciferase (Figure 5A). Thus, PH and SH3 domains do not appear to be essential for Rho and SRF activation.

G α 13 and Src stimulate phosphorylation and kinase activities of Tec

Src and its related kinases have been demonstrated to stimulate the kinase activity of Tec and Btk (Mano *et al.*, 1996; Rawlings *et al.*, 1996) when co-expressed in a number of mammalian cell lines. Co-expression of Src and Tec in Cos-7 cells resulted in increases in the phosphotyrosine content in immunoprecipitated Tec proteins as detected by anti-phosphotyrosine antibodies

Fig. 5. Involvement of Tec in SRF activation by G α 13. (A) NIH 3T3 cells were co-transfected with 0.15 μ g SRE.L-luciferase reporter plasmid, 0.15 μ g GFP expression construct and 0.2 μ g β -galactosidase (LacZ), Tec or its mutants in the presence (open bars) or absence (closed bars) of 0.01 μ g G α 13QL. The luciferase activities were determined the next day as described. (B) The expression of Tec and its mutants were detected by antibodies raised against a Tec peptide located at the TH domain. (C) COS-7 cells were co-transfected with 0.5 μ g β -galactosidase (LacZ), or Tec in the presence or absence of 0.01 μ g G α 13QL, G α 12QL or Src. Two days later, cells were lysed and Tec was immunoprecipitated with the anti-Tec antibody in complex with protein A/G-Agarose beads. Immunocomplexes were divided into two portions and separated by electrophoresis. Proteins were blotted to two nitrocellulose membranes, which were probed with the anti-Tec antibody (upper panel) and an anti-phosphotyrosine antibody (middle panel). Similar immunocomplexes were also prepared for kinase assay, where the immunocomplexes were incubated with a peptide substrate in the presence of [^{32} P]ATP as described in the Materials and methods. The peptides were separated by 20% SDS-PAGE, and the results were recorded on an X-ray film (lower panel).

(Figure 5C). Co-expression of G α 13 also increased phosphotyrosine content in immunoprecipitated Tec proteins (Figure 5C). These results indicated that G α 13, like Src, could also stimulate autophosphorylation of the Tec proteins. G α 13-induced increases in Tec phosphotyrosine contents apparently depends on the Tec kinase domain, since G α 13 failed to increase phosphotyrosine contents in the Tec mutant lacking the kinase domain (data not shown). G α 12 can also stimulate autophosphorylation of the Tec molecule (Figure 5C). These results are consistent with the finding that G α 12/13-coupled thrombin receptor can induce Tyr phosphorylation of Tec (Hamazaki, 1998). The high basal phosphotyrosine content in wild-type Tec may explain the smear banding seen in the Western blot (Figure 5B). The lack of smear banding in Western detection of the SH3-deletion mutant is consistent with the idea that SH3 domains of Tec kinases contain major autophosphorylation sites (Park *et al.*, 1996).

Fig. 6. Regulation of Tec kinases by Src. (A) NIH 3T3 cells were co-transfected with 0.15 μ g SRE.L-luciferase reporter plasmid, 0.15 μ g GFP expression construct and 0.01 μ g β -galactosidase (LacZ), G α 13QL or Src in the presence (open bars) or absence (closed bars) of 0.2 μ g Csk. (B) NIH 3T3 cells were co-transfected with 0.15 μ g SRE.L-luciferase reporter plasmid, 0.15 μ g GFP expression construct and 0.01 μ g β -galactosidase (LacZ), G α 13QL or Src in combination with 0.2 μ g LacZ (open bars), and dominant-negative small G proteins RacN17 (stripped bars) or Cdc42N17 (closed bars). (C) NIH 3T3 cells were co-transfected with 0.15 μ g SRE.L-luciferase reporter plasmid, 0.15 μ g GFP expression construct and 0.2 μ g β -galactosidase (LacZ), Tec, Bmx, BmxKR (KR) in the presence (open bars) or absence (closed bars) of Src (0.01 μ g) and/or C3 (0.02 μ g). The luciferase activities were determined the next day as described in Figure 1.

The *trans*-phosphorylation activity of Tec was also determined. Tec was co-expressed with the control plasmid β -galactosidase, Src or G α 13QL in COS-7 cells, and then immunoprecipitated with the anti-Tec-specific antibody. The abilities of the immunoprecipitants to phosphorylate a peptide substrate derived from the major autophosphorylation site of Btk were determined. Tec immunoprecipitated from cells co-expressing Src or G α 13QL showed significantly higher kinase activities than that from cells co-expressing the control (Figure 5C). These results indicated that G α 13, like Src, can stimulate Tec kinase activity. Similar results were observed with G α 12 (Figure 5C).

Regulation of Tec kinases by Src

Src can stimulate Tec kinase activity and activate SRF. The question is, what is the role of Tec kinases in Src-mediated SRF activation? It is unlikely that Src mediates G α 13's effect since Src-induced SRF activation is largely insensitive to C3. In addition, the finding that Csk inhibits Src- but not G α 13-mediated activation of SRF (Figure 6A) and tyrosine phosphorylation of Tec (data not shown)

confirms the idea that G α 13 is not the upstream regulator of Src in SRF activation. Csk is a non-receptor tyrosine kinase, which was previously shown to phosphorylate Src at Tyr527, causing inhibition of Src (Neet and Hunter, 1996).

The C3-resistance of Src-induced SRF activation suggests that Src may predominantly use other pathways to activate SRF in NIH 3T3 cells. Src was previously demonstrated to activate JNK and p38 kinase via Cdc42 and Rac (Minden *et al.*, 1995), which are also potent activators of SRF. When dominant-negative forms of Rac1 and Cdc42 were co-transfected with Src and SRE.L-luciferase reporter gene, these dominant-negative mutants significantly inhibited Src-induced SRF activation (Figure 6B) but had little effect on G α 13- and Tec-mediated SRF activation. This result, together with the data shown in Figure 2A, indicates that Src-induced SRF activation is predominantly mediated by Cdc42/Rac1 and/or other proteins, rather than Rho, in NIH 3T3 cells.

Another important question is whether Src can use Tec to activate SRF. We approached this question by determining whether Src and Tec kinases can synergistically activate SRF when Tec is overexpressed. When Tec or Bmx was overexpressed with Src, Tec kinases and Src showed synergistic activation of SRF (Figure 6C). The synergism depends on the Tec kinase domain, since TecK did not act synergistically with Src (data not shown). Consistently, C3 could only block the part contributed by Tec kinases, i.e. C3 returned the SRF activation level to that induced by Src alone (Figure 6C). These data imply that the Src–Tec–Rho pathway may play a significant role in cells that express higher levels of Tec kinases.

Discussion

Downstream effectors for Tec kinases

We described the discovery of novel downstream effectors (Rho and SRF) for the Tec family non-receptor kinases. Although we do not understand the nature of the high basal activity shown by wild-type Tec in the activation of SRF, the inhibition of this basal activity by C3 (Figure 1) provides an evidence for Tec acting through Rho in the activation of SRF. Since SRF activation by Tec is almost completely inhibited by C3, while Cdc42V12- and Src-mediated SRF activation is largely unaffected by C3 under the same conditions (Figures 1 and 2A), Rho rather than Cdc42 or Rac1 is the major downstream effector for Tec kinases in 3T3 fibroblast cells. The findings that Tec enhanced Rho translocation and induced cytoskeleton reorganization further confirm the idea that Tec activates RhoA. This is consistent with previous observations that tyrosine kinase inhibitors inhibited cytoskeleton reorganization by G protein-coupled receptors LPA and thrombin (Gohla, 1998).

It is not clear how Tec regulates Rho. It is likely that nucleotide exchange factors are involved in Rho activation by Tec kinases. There are several known Rho-specific nucleotide exchange factors, including Lbc (Toksoz and Williams, 1994), RhoGEF (Gebbinck *et al.*, 1997), Lfc and Lsc (Glaven *et al.*, 1996). These exchange factors may be good candidates for direct or indirect regulation by Tec kinases. Two structural domains of the Tec kinases were found to be essential for activation of SRF: the kinase

and TH domains. It is reasonable to postulate that the kinase domain may be involved in phosphorylation of downstream effectors. The TH domain shares homology with the Ras GAP protein (Rawlings and Witte, 1995). The Tec TH domain was found to bind to Vav (Machide *et al.*, 1995), a hematopoietic, specific nucleotide exchange factor for Rho, Rac and Cdc42. Thus, the TH domain may be involved in interaction with exchange factors that might be substrates of Tec kinases. The finding that Tec interacts with Vav also raises a possibility that Tec may activate effectors in addition to Rho in other cell types. This may explain the observations of JNK and p38 kinase activation in hematopoietic cells (Bence *et al.*, 1997; Kawakami *et al.*, 1997), where Vav may be expressed.

Upstream regulators of Tec kinases

In this report, we also revealed the involvement of the α subunits of the G12 family of G proteins in Tec-mediated SRF activation. Four lines of evidence are presented to support the idea that G α 13 acts upstream of Tec in activation of SRF. First, overexpression of wild-type Tec, like G α 13QL, activated SRF in a C3-dependent manner (Figure 1), suggesting that both Tec and G α 13 may act through Rho. Secondly, G α 13 and Tec acted synergistically in regulation of SRF (Figure 2B). More importantly, the Tec isoform Bmx, which showed little basal activity, can enhance G α 13-induced SRF activation (Figure 3A). This suggests that G α 13 may stimulate the activity of Bmx. Thirdly, kinase-deficient mutants were able to inhibit G α 12/13-mediated SRF activation in NIH 3T3 cells and thrombin-induced SRF activation in the G α q/11-deficient fibroblast cells (Figures 3A and 4C). The inhibitory effects of kinase-deficient mutants appear to be specific since they did not inhibit RhoV14-, Tec-, Cdc42-, Rac1- or PDGF-mediated SRF activation (data not shown; Figure 4D). In addition, the inhibitory effect requires the entire non-kinase portion of the molecule because neither BmxN nor BmxC, which divided the Bmx molecule between the SH3 and SH2 domains, showed inhibitory effects (Figure 3A). Finally, expression of G α 13 and G α 12, like Src, stimulates autophosphorylation and transphosphorylation activities of the Tec molecules (Figure 5C). Therefore, it is reasonable to conclude that Tec kinases are involved in regulation of Rho and SRF by G α 13. However, we do not know whether G α 12/13 directly or indirectly activates Tec kinases. Therefore, the mechanisms by which G α 12/13 regulates Tec kinase need to be further investigated.

Function of other Tec structural domains

Although deletion of the PH domain does not appear to affect its ability to activate SRF in the overexpression system, this result can not exclude its importance under normal physiological conditions, where the expression of Tec kinases may be lower. Moreover, the PH domain may be involved in other mechanisms of Tec activation. Recently, it was shown that Btk could be activated by phosphoinositide 3-kinase (Li *et al.*, 1997), and the PH domain is thought to be involved. In addition, an earlier report showed that G $\beta\gamma$ can activate kinase activity of Btk (Langhans-Rajasekaran *et al.*, 1995). In fact, we found that co-expression of G $\beta\gamma$ worked synergistically with G α 13QL in SRF activation (J.Mao, unpublished data),

suggesting possibilities of multiple mechanisms of regulation of Tec kinases.

Our observation that deletion of the SH3 domain of Tec does not affect Rho and SRF activation is rather consistent with findings from previous works. Although we could not exclude the possibility that the SH3 domain may be involved in interactions with upstream regulators or in regulation of other Tec effectors, the idea that SH3 may have a self-regulatory function is plausible. The SH3 domain of Btk was shown to contain the major autophosphorylation site (Park *et al.*, 1996). This may also be true for Tec and Bmx since the tyrosine residues are conserved, and the peptide derived from this region of Btk can be phosphorylated by Tec (Figure 5C). In addition, the Tec SH3 mutant, unlike the wild-type and other deletion mutants, does not show polymorphic electrophoretic motility (Figure 5B), which is characteristic of phosphorylated proteins. The Tec SH3 deletion mutant was previously shown to have increased kinase activity (Yamashita *et al.*, 1996); the deletion mutation, and the point mutation in the Btk SH3 domain which replaces the autophosphorylation site, showed increased activation of downstream effectors, as measured by focus formation assay of transfected fibroblast cells (Li *et al.*, 1995; Park *et al.*, 1996). However, given the low expression level of Tec SH3 when compared with the wild-type (Figure 5B), it is difficult to compare the specific activities of Tec SH3 and its wild-type in SRF activation, although both showed similar apparent activities (Figure 5A).

Roles of Tec kinases in Src-mediated SRF activation

It is apparent that in NIH 3T3 cells Tec kinases may not play a significant role in Src-mediated SRF activation, since Src-mediated SRF activation is largely insensitive to C3. However, the Tec-linked pathway gains significance with the increases in the expression levels of Tec (Figure 6C). As demonstrated previously (Minden *et al.*, 1995), and verified in this report, Src can act through Cdc42 and Rac to regulate downstream effectors since Cdc42 and Rac1 dominant-negative mutants inhibited Src-mediated activation of SRF (Figure 6B) and JNK (Minden *et al.*, 1995). Unless the dominant-negative mutants fail to block SRF activation efficiently, the incomplete blockage by these mutants suggests that there are other pathways contributing to SRF activation.

Signal pathways leading to SRF activation

In this report, we have demonstrated that Tec and Bmx play a role in G α 13-mediated SRF activation. Previously, the G α q protein was shown to directly activate Btk (Bence *et al.*, 1997). We also found that activated G α q (J.Mao and D.Wu, unpublished data) and Gq-coupled α 1-AR (Figure 4A) in the presence of ligand could stimulate SRE.L-mediated transcription of the reporter gene in a C3-dependent way. Thus, it may be generally true that G protein α subunits from two distinct classes, the Gq and G12 classes, are involved in regulation of the Tec family non-receptor tyrosine kinases, although activation of Btk and Itk by G α 13 and of Itk, Tec and Bmx by G α q needs to be tested. The Tec kinases could also play a significant role in Src-mediated SRF activation, provided that there are high levels of the Tec kinase expression. Furthermore,

this report reveals an important effector for Tec kinases: the small G protein Rho. The information regarding the roles of various structural domains of Tec kinases in SRF activation may help us to better understand the roles of Tec kinases in pathogenesis of diseases including XLA, which is associated with Btk deficiency (Rawlings and Witte, 1995).

Materials and methods

Cell culture, transfection and luciferase assay

COS-7 and NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum at 37°C in 5% CO₂. For transfection, cells (5×10⁴ cells/well) were seeded into 24-well plates the day before transfection. Cells were transfected with 0.25 µg DNA per well for COS-7 cells and 0.5 µg DNA per well for NIH 3T3 cells using Lipofectamine Plus (Life Technologies, MD), as suggested by the manufacturer. The transfection was stopped after 3 h by switching to culture medium containing 0.5% fetal bovine serum. Cell extracts were collected 24 h later for luciferase assays, kinase assays and Western analysis.

Gαq and Gα11-deficient mice were generated by standard gene targeting procedure. Mice lacking Gαq and Gα11, respectively, were backcrossed for two rounds to generated mice lacking both Gαq and Gα11. Fibroblasts from a 10-day embryo were isolated and cultured. Cells were subject repeated subcultures (every 2 days) for 3 months. The cells were then used for transfection.

Luciferase assays were performed using Boehringer Mannheim constant light luciferase assay kit as instructed. Cell lysates were first taken for determining fluorescence intensity emitted by green fluorescent proteins (GFP) in a Wallac multi-counter, which measures fluorescence and luminescence. Luciferase substrate was added to the cell lysates and luciferase activities were determined by measuring luminescence intensity with the Wallac multi-counter. Luminescence intensity was normalized against fluorescence intensity. DNA concentrations were adjusted if transfection of any of the cDNAs resulted in significant differences between normalized and non-normalized data.

Construction of expression plasmids and mutagenesis

Wild-type flag-tagged Bmx (kindly provided by Dr Dominique Weil) and its mutants were carried by CMV expression vectors. Tec and its mutants are carried by pSRα mammalian expression vectors. The Bmx and Tec mutants were generated by PCR using the high fidelity thermostable DNA polymerase *Pfu* (Stratagene, CA). BmxK, BmxN and BmxC consist of residues 1–420, 1–255 and 256–655, respectively. TecK, Tec TH, Tec SH3 and Tec PH contain deletion for residues 370–630, 123–143, 186–233 and 1–116, respectively.

cDNAs encoding the activated Gα proteins and Gβγ subunits are carried by CMV expression vectors as previously described (Wu *et al.*, 1992b, 1993; Jiang *et al.*, 1993). Expression plasmids for Csk, Sik, Syk and Src dominant-negative mutants, activated forms of Cdc42 and Rac, and C3 were kindly provided by Masato Okada, Angela L.Tyner, Lakhu Keshvara, Owen Witte, Solvio Gutkind and Alan Hall, respectively.

For the construction of the reporter gene plasmid, SRE derivative, SRE.L, was synthesized exactly as described in Hill *et al.* (1995). SER.L was inserted in front of a TK minimal promoter–luciferase gene.

Immunoprecipitation and Western analysis

Cells were lysed with the lysis buffer containing 1% NP-40, 137 mM sodium chloride, 20 mM Tris pH 7.4, 1 mM DTT, 10% glycerol, 10 mM sodium fluoride, 1 mM pyrophosphate, 2 mM sodium vanadate and Complete™ protease inhibitors (Boehringer Mannheim, following manufacturer's instructions). The cell lysates were precleared with 20 µl of protein A/G–Sepharose beads (Santa Cruz Biotech, CA) for 0.5 h at 4°C, and then incubated with 0.5 µl of antibodies and 20 µl of protein A/G–Sepharose beads for 3.5 h on ice. The immunocomplexes were pelleted and washed three times with cold lysis buffer in the absence of protease or phosphatase inhibitors and twice with cold kinase buffer containing 25 mM Hepes pH 7.4, 10 mM MgCl₂ and 1 mM DTT. The immunocomplexes were then used either for Western analysis or kinase assay.

In vitro kinase assay

The kinase reactions were performed for 30 min at 30°C in the presence of 10 µCi [γ-³²P]ATP, 10 µM ATP and an excess of a peptide

substrate derived from the Btk SH3 sequence containing the major autophosphorylation site (Park *et al.*, 1996; Bence *et al.*, 1997). The reactions were terminated by addition of 4× SDS sample buffer. The samples were boiled and loaded on 20% SDS–PAGE gels. The results were visualized by exposure to X-ray film.

RhoA translocation assay

Cos-7 cells were transfected with Tec or the control LacZ. The next day, cells were collected by incubation with 5 mM EDTA and spun down. The cell pellets were incubated with hypotonic buffer (20 mM Tris plus protease inhibitors) and lysed by repeated freezing and thaw cycles. Then the cytosol fraction was separated by high-speed centrifugation (26 000 g) for 30 min at 4°C. The pellets were washed twice by lysis buffer and dissolved in sample buffer. The cytosol fractions were also added with 2× sample buffer to the same total volume as the particulate fractions. Equal volumes of cytosol and particulate fractions were loaded onto SDS–PAGE. RhoA proteins were detected by a RhoA-specific antibody (Santa Cruz, CA) after the proteins were transferred onto a nitrocellulose membrane.

Cytoskeleton reorganization assay

NIH 3T3 cells were transfected with an expression plasmid that express both BFP and Tec or with a expression plasmid expressing BFP alone. Three days after transfection, cells were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton. Cells were then stained with 0.5 µg/ml FITC-phalloidin (Sigma) as described in Gohla *et al.* (1998). BFP and FITC were viewed under an Olympus IX70 fluorescence microscope.

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