Regulation of c-*myc* expression by PDGF through Rho GTPases

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Src family protein-tyrosine kinases have a central role in several biological functions, including cell adhesion and spreading, chemotaxis, cell cycle progression, differentiation and apoptosis. Surprisingly, these kinases also participate in mitogenic signalling by receptors that themselves exhibit an intrinsic protein-tyrosine kinase activity, including those for platelet-derived growth factor (PDGF), epidermal growth factor and colony-stimulating factor-1. Indeed, Src kinases are strictly required for the nuclear expression of the c-*myc* proto-oncogene and thus for DNA synthesis in response to PDGF. However, the nature of the signalling pathways by which Src kinases participate in the induction of c-*myc* expression by tyrosine kinase receptors is still unknown. Here we show that PDGF enhances c-*myc* expression and stimulates the c-*myc* promoter in a Src-dependent manner, and that neither Ras nor the mitogen-activated protein kinase pathway mediate these effects. In contrast, we present evidence that PDGF stimulates Vav2 through Src, thereby initiating the activation of a Rac-dependent pathway that controls the expression of the c-*myc* proto-oncogene.

The cytoplasmic tyrosine kinase Src was first identified as the protein product of a viral oncogene (v-*src*) responsible for the transforming ability of the Rous sarcoma virus[1]. It is now clear that its normal counterpart, c-Src, and its related kinases are involved in many key cellular processes, including cell proliferation, differentiation, survival, adhesion and migration[2]. These kinases can be stimulated by a large spectrum of cell-surface receptors, including immune-response receptors, cell adhesion receptors, cytokine receptors and G-protein-coupled receptors[2]. Many polypeptide growth factor receptors, although themselves exhibiting an intrinsic tyrosine kinase activity, can also recruit and activate tyrosine kinases of the Src family, which in turn act as integral components of a number of receptor-induced signalling pathways[2]. For example, stimulation of NIH 3T3 fibroblasts with platelet-derived growth factor (PDGF), which acts on its cognate receptor tyrosine kinase (RTK), results in the activation of several members of the Src family of non-receptor tyrosine kinases (NRTKs), including c-Src, Fyn and c-Yes[3–5]. Remarkably, the ability of neutralizing antibodies for Src kinases and dominant-negative mutants for c-Src and Fyn to inhibit DNA synthesis in response to PDGF[5,6] indicates that these kinases can act downstream from RTKs in a pathway that is strictly required for cell proliferation. Of interest, the expression of c-*myc* was found to be sufficient to overcome the requirement of c-Src for the induction of DNA synthesis by RTKs[7,8], indicating that Src kinases regulate a pathway controlling c-*myc* expression. However, the nature of the signalling molecules activated by Src on stimulation by PDGF has remained elusive.

Both PDGF receptors and activated Src can enhance the activity of the small GTP-binding protein Ras, thereby stimulating the mitogen-activated protein kinase (MAPK) pathway. However, available evidence[8] indicates that Ras proteins are not strictly required for the induction of c-*myc* expression by PDGF. Interestingly, these RTKs and oncogenic Src proteins are also able to activate GTP-binding proteins of the Rho family[9], and accumulating evidence indicates that these GTPases are important in cellular proliferation and invasiveness besides their best characterized effects on the organization of the cellular actin cytoskeleton[10–13]. Nevertheless, the mechanism by which Rho proteins exert their growth-promoting effects is also still poorly understood.

Here we show that Rho GTPases are able to stimulate potently the expression of the c-*myc* proto-oncogene. Furthermore, we found that inhibition of endogenous Rho proteins by bacterial to-xins or dominant interfering molecules for Rac and Cdc42 prevents expression from the c-*myc* promoter in response to PDGF and by activated Src. In search of the underlying mechanism, we obtained evidence of the existence of a novel signalling pathway linking PDGF receptors to the nucleus through Src. In this biochemical route, stimulation with PDGF leads to the phosphorylation of the ubiquitously expressed Vav2 Rac-guanine nucleotide exchange factor (GEF) through Src, and the consequent activation of a Rac-dependent pathway that culminates with the nuclear expression of the c-*myc* proto-oncogene.

Results

Src-related kinases mediate the induction of c-*myc* expression upon stimulation of NIH 3T3 cells with PDGF. Stimulation of quiescent fibroblasts with PDGF results in the rapid activation of a number of intracellular signalling pathways, many of which converge in the nucleus to regulate the expression of growth-promoting genes. Indeed, exposure of NIH 3T3 fibroblasts to PDGF provoked a sustained accumulation of c-*myc* mRNA; high levels of c-*myc* and its protein product, c-Myc, were maintained throughout the G1/S transition and early S phase (Fig. 1a). In agreement with previous observations[5,7,8], PDGF induction of c-*myc* mRNA was dependent on the activity of Src-related kinases, as judged by the ability of an inhibitor for this family of protein kinases, PP1 (ref. 14), to abolish this response in a concentration-dependent manner (Fig. 1b). In contrast, PP1 had a very weak effect on the PDGF-induced autophosphorylation of the PDGF receptor[15,16], even at the highest doses tested (5–10 μM), which completely abolished the PDGF-induced increase in c-*myc* mRNA (Fig. 1b, upper panels). To investigate the molecular mechanisms involved in the regulation of c-*myc* expression by PDGF receptors and Src in more detail, we used a reporter plasmid carrying the *luciferase* gene under the control of the human c-*myc* promoter (pMyc-Luc). This reporter construct contains the c-*myc* P1/P2 promoter from position –157 to +500

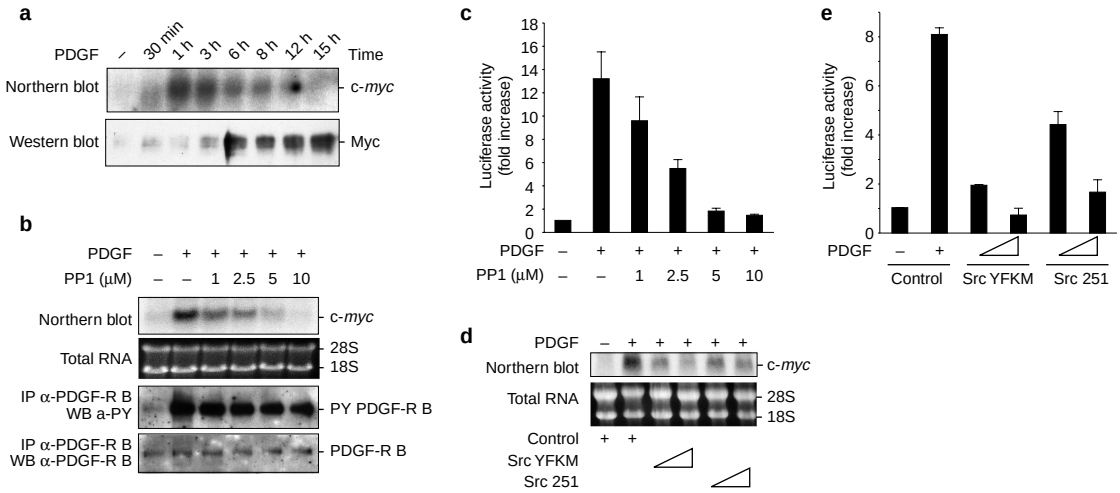

Figure 1 **PDGF stimulation of c-*myc* expression is dependent on the activity of Src-related kinases. a**, Analysis of c-*myc* mRNA and protein expression in NIH 3T3 cells unstimulated or treated with PDGF for the indicated durations. **b**, Upper panels, analysis of c-*myc* mRNA expression in NIH 3T3 cells pretreated for 20 min with the indicated concentrations of the PP1 inhibitor and then stimulated for 1 h with PDGF (12.5 ng ml^{-1}). Lower panels, in parallel, PDGF receptor B (PDGF-R B) was immunoprecipitated from total cellular lysates and analysed for tyrosine phosphorylation with anti-phosphotyrosine (α-PY) and anti-PDGF receptor (α-PDGF-R) antibodies as a control. **c**, Stimulation of c-*myc* promoter in NIH 3T3 cells transfected with pMyc-Luc, pretreated for 20 min with the indicated concentrations of PP1 and then stimulated for 4 h with PDGF (12.5 ng ml^{-1}). **d**, Analysis of c-*myc* mRNA expression in NIH 3T3 cells transfected with increasing concentrations (2 and 4 μg) of Src YFKM and Src 251 and then stimulated for 1 h with PDGF. Equal loading of total RNA was confirmed. **e**, Stimulation of pMyc-Luc transfected together with increasing concentrations (1 and 2 μg) of Src YFKM and Src 251 and then stimulated for 4 h with PDGF (12.5 ng ml^{-1}). Results in **c** and **e** represent luciferase activity in each sample, normalized for the corresponding efficiency of transfection, and are means ± s.e.m. for triplicate samples from a typical experiment. IP, immunoprecipitation; WB, western blot; –, no treatment.

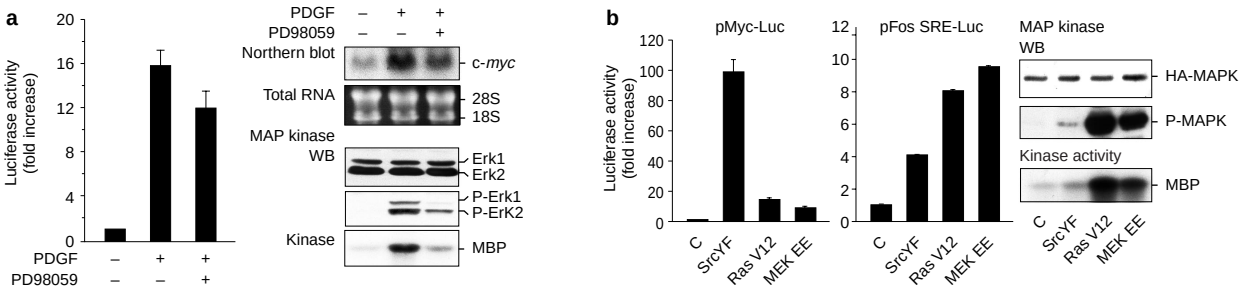

Figure 2. **Stimulation of the *myc* promoter by the PDGF-receptor–Src pathway is independent of Ras–MAPK activation. a**, Left panel, stimulation of c-*myc* promoter in cells transfected with pMyc-Luc and pretreated with PD98059 (60 μM) 20 min before stimulation with PDGF (12.5 ng ml^{-1}; 4 h). Right panels, analysis of c-*myc* mRNA expression (upper panels) and of endogenous Erk1/Erk2 kinase activity (lower panels) in NIH 3T3 cells pretreated for 20 min with PD98059 (60 μM) and then stimulated for 1 h with PDGF (12.5 ng ml^{-1}). Endogenous Erk1/Erk2 activity was analysed by kinase assay *in vitro* and specific anti-phospho-MAPK antibodies, as indicated. PD98059 (60 μM) partly diminished the protein levels of c-Myc in PDGF-treated cells (data not shown), probably owing to the contribution of Ras–MAPK to regulation of the stability of c-Myc protein[49,50]. **b**, Left panel, stimulation of c-*myc* promoter in cells cotransfected with pMyc-Luc and expression vectors for Src YF, Ras V12 and MEK EE, as indicated. Middle panel, stimulation of c-*fos*-SRE in cells cotransfected with pFos-SRE-Luc and expression vectors for Src YF, Ras V12 and MEK EE, as indicated. Right panel, stimulation of MAPK activity by activated forms of Src, Ras and MEK1 in NIH 3T3 cells transfected with HA–MAPK together with the indicated plasmids. HA–MAPK activity was measured by kinase assay *in vitro* or phospho-MAPK antibodies. Histograms show luciferase activity in each sample, normalized for the corresponding efficiency of transfection, and are means ± s.e.m. for triplicate samples from a typical experiment. –, no treatment; C, control; WB, western blot; MBP, myelin basic protein.

(position +1 corresponds to the beginning of the first exon of the human c-*myc* gene), which includes recognition sites for E2F, STAT3 (signal transduction and activators of transcription-3) and other transcription factors, on the basis of experimental evidence and the analysis of the nucleotide sequence from available databases[17,18]. As expected, PDGF strongly induced c-*myc* promoter activity in NIH 3T3 cells transfected with the pMyc-Luc reporter plasmid, and this response was inhibited by PP1 (Fig. 1c). As a complementary approach to ascertaining the role of Src kinases in the activation of c-*myc* expression, we used the overexpression of two dominant-negative mutants of Src, Src YFKM and Src 251, which lack enzymatic activity but can still bind upstream molecules through their N-terminal non-catalytic domain, thereby interfering with Src activation[19]. As shown in Fig. 1d, e, both Src-inhibiting constructs prevented the

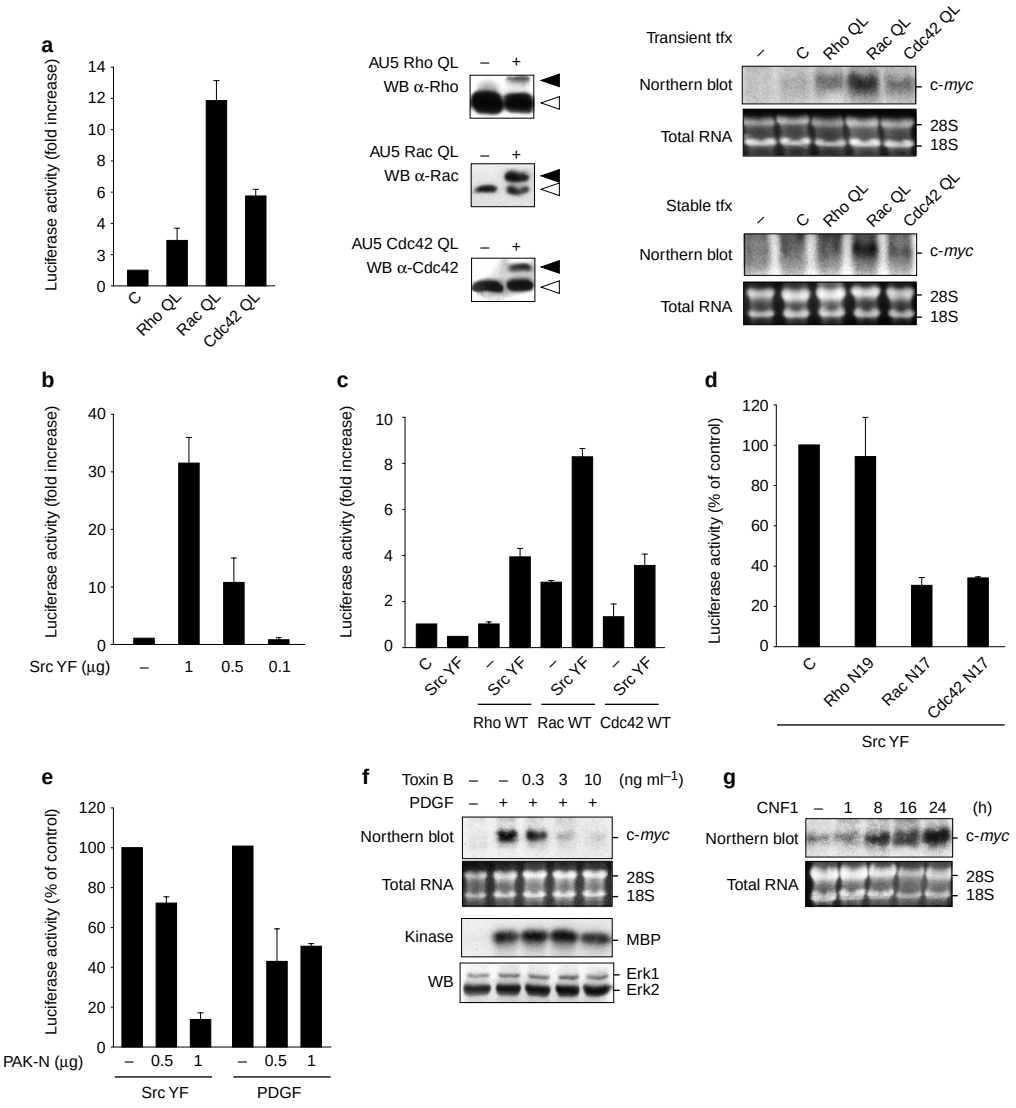

Figure 3. **Rho GTPases participate in the Src-dependent activation of the c-*myc* promoter. a**, Left panel, stimulation of the c-*myc* promoter by Rho QL, Rac QL and Cdc42 QL in NIH 3T3 cells cotransfected with pMyc-Luc. Right panels, analysis of c-*myc* mRNA expression in NIH 3T3 cells transiently (upper panels) or stably (lower panels) expressing activated forms of these Rho GTPases, as indicated. Middle panels, analysis of the expression of AU5 Rho QL (upper panel), Rac QL (middle panel) and Cdc42 QL (lower panel) proteins. Open arrowheads indicate endogenous proteins, and closed arrowheads indicate the AU5-tagged proteins. **b**, Dose–response stimulation of pMyc-Luc by decreasing concentrations of Src YF. **c**, Cooperation of Src-dependent stimulation of the c-*myc* promoter with wild-type Rho GTPases. NIH 3T3 cells were transfected with Src YF (0.1 μg) and Rho wt, Rac wt, and Cdc42 wt (1 μg each), as indicated. **d**, Inhibition of Src-induced stimulation of the c-*myc* promoter by dominant-negative interfering forms of Rho GTPases (Rho N19, Rac N17 and Cdc42 N17) (1 μg) in NIH 3T3 cells cotransfected with pMyc-Luc, as indicated. **e**, Inhibition of Src- and PDGF-induced stimulation of the c-*myc* promoter by increasing concentrations of an expression vector for a GST fusion protein containing the CRIB domain of PAK (PAK-N), in cells cotransfected with pMyc-Luc, as indicated. **f**, Upper panels, analysis of c-*myc* mRNA expression in NIH 3T3 cells pretreated with increasing concentrations of *Clostridium difficile* toxin B (24 h) and then stimulated for 1 h with PDGF. Lower panels, stimulation of endogenous Erk1/Erk2 kinase activity in response to PDGF in NIH 3T3 cells treated as above. **g**, Analysis of c-*myc* mRNA expression in cells treated with *Escherichia coli* CNF-1 (300 ng ml^{-1}). Results in **a**, **b** and **c** represent luciferase activity in each sample normalized for the corresponding efficiency of transfection, expressed as fold induction with respect to control transfected cells, and are means ± s.e.m. for triplicate samples from a typical experiment. Luciferase activities in **d** and **e** were expressed as percentages of the stimulation obtained by Src YF and PDGF, as indicated, when cotransfected with vector control. Data are means ± s.e.m. for triplicate samples from a typical experiment. –, no treatment; C, control; α-, antibody against; WB, western blot.

activity of the c-*myc* promoter and the induction of c-*myc* expression by PDGF in a concentration-dependent manner, as judged by luciferase assays using the pMyc-Luc plasmid as a reporter and by northern blot analysis. Thus, in agreement with previous reports[5,7,8], Src family kinases are required for the induction of c-*myc* expression upon stimulation of NIH 3T3 cells with PDGF.

Stimulation of c-*myc* expression by PDGF involves a Ras–MAPK-independent pathway. The Ras–MAPK pathway seems to be central for the proliferative response to many growth factors[20,21]. However, available evidence indicates that PDGF receptors might also use a parallel Ras-independent but Src-dependent pathway that is also involved in regulating the expression of the c-*myc* proto-oncogene[8,22]. In support of this, treatment of NIH 3T3 cells with PD98059, which blocks MAPK activation by preventing its phosphorylation by the upstream stimulating kinase, MAP-kinase kinase (MEK)[23], only slightly affected the induction of the c-*myc* promoter and c-*myc* expression by PDGF (Fig. 2a, left and upper right panels), although, as a control, it effectively blocked the PDGF-induced MAPK activity (Fig. 2a, lower right panel). Furthermore, a constitutively active form of Src, Src YF, was far more effective than the activated forms of Ras (Ras V12) or MEK1 (MEK EE) in stimulating the transcriptional activity of the c-*myc* promoter (Fig. 2b, left panel), although Src YF stimulated the activity of MAPK to a much more limited extent (Fig. 2b, right panels). As an additional control, Src YF was also less effective than Ras V12 and MEK EE in stimulating the transcriptional activity of a reporter plasmid in which *luciferase* expression is under the control of the serum response element (SRE) from the c-*fos* promoter (Fig. 2b, middle panel). Together, these results clearly indicate that PDGF receptors and Src can use a Ras–MAPK-independent pathway to stimulate the c-*myc* promoter.

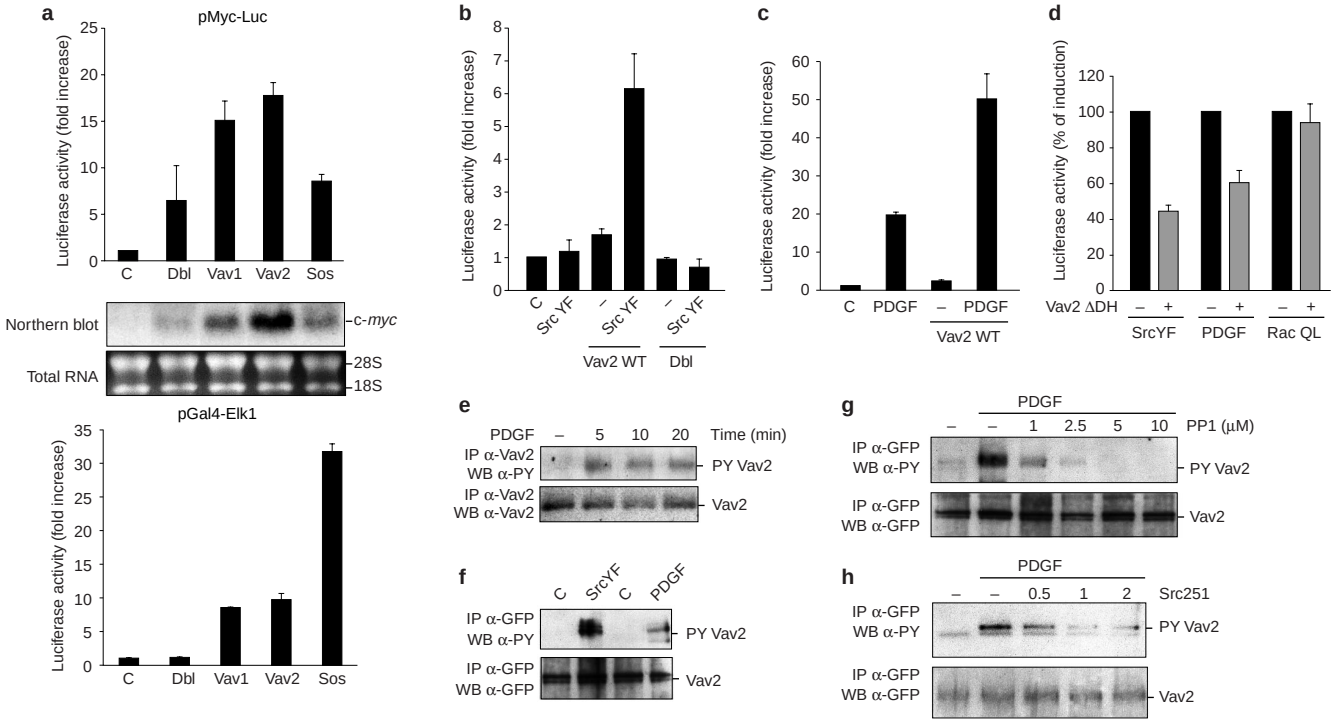

Figure 4. **Role of Vav2 in the Src-dependent activation of the c-*myc* promoter by PDGF. a**, Left panels, stimulation of c-*myc* promoter by activated forms of DH-containing exchange factors. NIH 3T3 cells were cotransfected with pMyc-Luc and onco-Dbl, onco-Vav1, onco-Vav2 or myristoylated Sos (0.5 μg each). Lower panels, analysis of c-*myc* mRNA expression in NIH 3T3 cells expressing activated forms of different exchange factors, as indicated. Right panel, as in the left panel but using a Gal4-driven luciferase-expressing reporter plasmid to score the activation of a chimaeric Gal4-Elk1 protein. **b**, Cooperation of Src with wild-type Vav2 to stimulate the c-*myc* promoter. NIH 3T3 cells were transfected with pMyc-Luc, 0.1 μg of Src YF and 1 μg of wild-type Vav2, or proto-Dbl, as indicated. **c**, Cooperation of PDGF with wild-type Vav2 to stimulate the c-*myc* promoter. Cells were transfected with pMyc-Luc and with 0.5 μg of wild-type Vav2, as indicated. Cells remained unstimulated or were treated with PDGF for 4 h. **d**, Inhibition of PDGF- and Src-induced stimulation of the c-*myc* promoter by a Vav2 protein lacking its DH domain (Vav2 ΔDH; 1 μg) in NIH 3T3 cells cotransfected with pMyc-Luc, using Rac QL (1 μg) as a control. Luciferase activities were expressed as percentages of the stimulation obtained by Src YF and Rac QL, respectively, and are means ± s.e.m. for triplicate samples from a typical experiment. **e**, Time-course analysis of endogenous Vav2 tyrosine phosphorylation in NIH 3T3 cells treated with PDGF. Cellular lysates were immunoprecipitated with anti-Vav2 antisera and blotted with specific anti-phospho-tyrosine antibodies. Blotting of parallel samples with anti-Vav2 antisera confirmed equal expression of endogenous Vav2. **f**, Analysis of Vav2 tyrosine phosphorylation in NIH 3T3 cells transfected with GFP-Vav2 wild-type (1 μg), unstimulated, treated with PDGF for 5 min, or transfected with Src YF. Total cellular lysates were immunoprecipitated with anti-GFP antisera and blotted with specific anti-phospho-tyrosine antibodies. Equal expression of GFP-Vav2 wild-type was confirmed by blotting parallel samples with anti-Vav2 antisera. **g**, As in **f**, but analysing Vav2 tyrosine phosphorylation in NIH 3T3 cells pretreated for 20 min with the indicated concentrations of PP1 and then stimulated for 5 min with PDGF. **h**, Analysis of Vav2 tyrosine phosphorylation in NIH 3T3 cells transfected with GFP-Vav2 wild-type (1 μg) and with increasing concentrations of dominant-negative Src, Src 251, as indicated. Cells were left unstimulated or treated with PDGF for 5 min. Total cellular lysates were immunoprecipitated with anti-GFP antiserum and blotted with specific anti-phosphotyrosine antibodies. Results in **a**, **b** and **c** represent luciferase activity in each sample, normalized for the corresponding efficiency of transfection, and are means ± s.e.m. for triplicate samples from a typical experiment, expressed as fold increase with respect to control cells. Luciferase activities in **d** were expressed as percentages of the stimulation obtained by Src YF, PDGF and Rac QL, respectively, when transfected with vector control, and are means ± s.e.m. for triplicate samples from a typical experiment. –, no treatment; C, control; α-, antibody against; IP, immunoprecipitation; WB, western blot; PY, phosphotyrosine.

Rho GTPases participate in the induction of c-*myc* expression by PDGF and Src. Previous studies have indicated that the stimulation of a variety of cell types by growth factors can result in the activation of members of the Rho family of small GTP-binding proteins[13]. These observations prompted us to test whether representative Rho proteins—Rho, Rac and Cdc42—could regulate c-*myc* expression. As shown in Fig. 3a, the activated form of Rac, Rac QL, strongly induced the transcriptional activity of the c-*myc*-promoter reporter plasmid (left panel), even when expressed at a level comparable to that of the endogenous Rac protein (middle panels). In line with these observations, Rac QL strongly increased c-*myc* mRNA levels when expressed both transiently and stably in NIH 3T3 cells (Fig. 3a, right panels). Constitutively active Cdc42 and Rho, Cdc42 QL and Rho QL, respectively, also stimulated the c-*myc* promoter and c-*myc* expression, albeit to a more limited extent (Fig. 3a).

On the basis of these observations, we next examined whether overexpression of the wild-type forms of these GTPases could cooperate with Src in the stimulation of the c-*myc* promoter. For these experiments, we coexpressed limited amounts of Src that were not themselves able to induce the c-*myc* promoter (Fig. 3b) with the wild-type form of each Rho GTPase. As shown in Fig. 3c, under these conditions we observed a very strong cooperation between Src and wild-type Rac. A more limited cooperation was also observed between Src and the small GTPases Cdc42 and Rho (Fig. 3c). Furthermore, as shown in Fig. 3d, the dominant-negative forms of Rac and Cdc42 strongly inhibited the Src-dependent activation of the c-*myc* promoter; together, these results indicate that these small GTPases are probably mediators in the pathway connecting Src to nuclear events that culminate in the induction of c-*myc* expression.

To confirm the involvement of endogenous Rho-GTPases in the activation of c-*myc* expression, we next overexpressed a glutathione *S*-transferase (GST) fusion protein containing the Cdc42/Rac-interactive-binding (CRIB) domain of PAK (PAK-N). The CRIB domain mediates the specific binding of PAK to the GTP-bound forms of Rac and Cdc42 (ref. 24), and its overexpression prevents cellular Rac and Cdc42 proteins from transmitting downstream signals, as we have recently documented in this cellular system[25]. Indeed, the expression of PAK-N abolished the stimulation of the c-*myc* promoter by Src YF and strongly decreased the activation of the reporter by PDGF (Fig. 3e), respectively. In addition, we took advantage of the recent observation that certain bacterial toxins act by modifying the activity of Rho, Rac and Cdc42 (ref. 26) to examine the contribution of the endogenous GTPases in signalling to *myc*. In particular, *Clostridium difficile* toxin B glucosylates Rho, Rac and Cdc42, thereby inhibiting their function[26,27], whereas cytotoxic necrotizing factor-1 (CNF-1) from *Escherichia coli* constitutively activates these GTPases by deaminating a critical glutamine residue, thus blocking both intrinsic and GTPase-activating protein (GAP)-induced GTP hydrolysis[26,28,29]. We found that exposure of NIH 3T3 cells to *C. difficile* toxin B inhibited potently the induction of c-*myc* expression in response to PDGF, in a dose-dependent manner (Fig. 3f). As a control, under these experimental conditions *C. difficile* toxin B did not affect the ability of PDGF to induce the autophosphorylation of PDGF receptors (data not shown) and to stimulate MAPK (Fig. 3f). In contrast, exposure of these cells to *E. coli* CNF-1 toxin was sufficient to provoke the expression of the c-*myc* proto-oncogene, which peaked after 24 h, at which time the maximal activation of Rac would be expected[29] (Fig. 3g). Thus, the activation or blockade of endogenous Rho GTPases by interfering molecules and bacterial toxins provided strong evidence for a key role of these GTP-binding proteins in the regulation of c-*myc* expression by PDGF and Src kinases.

A role for Vav2 in the Src-dependent activation of the c-*myc* promoter by PDGF. The activity of Rho-family GTPases is regulated by GEFs, which catalyse the exchange of GDP for GTP bound to the G proteins, rendering them active[11,30]. These GEFs share the presence of a Dbl-homology (DH) domain, which binds small GTPases of the Rho family and is involved in promoting nucleotide exchange[31].

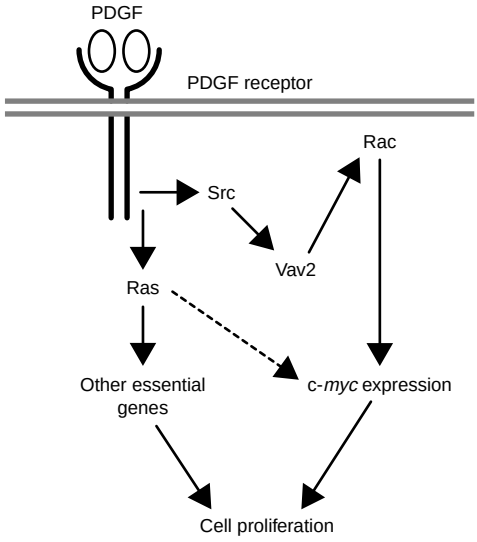

Figure 5. **Schematic representation of the pathway connecting PDGF receptors to the stimulation of the c-*myc* promoter, through the Src-dependent phosphorylation of Vav2 and the activation of Rac and its related GTP-binding proteins.**

As shown above, the expression of activated forms of Rho GTPases, and in particular Rac1, can potently stimulate the expression of c-*myc*. Furthermore, exposure of cells to bacterial toxins stimulating Rho proteins was sufficient to enhance the levels of c-*myc* mRNA. Thus, we next examined whether the overexpression of different DH-domain-containing exchange factors could also regulate c-*myc* expression by acting on endogenous Rho GTPases. Using the activated forms of Vav1 and Vav2 as an example, we observed that these oncogenic GEFs strongly induced the activity of the c-*myc* promoter (Fig. 4a), clearly indicating that endogenous Rho-like GTP-binding proteins are able to control the transcriptional machinery involved in the expression of the c-*myc* proto-oncogene. Activated forms of Dbl and Sos, respectively, also stimulated the c-*myc* promoter, albeit to a more limited extent (Fig. 4a). In line with these observations, activated forms of both Vav1 and Vav2 strongly increased c-*myc* mRNA levels when expressed in NIH 3T3 cells (Fig. 4a). As expected, all exchange factors also activated the expression from the c-*fos* promoter effectively (data not shown), as this promoter exhibits responsive elements for transcription factors whose activity is controlled by small GTPases of the Ras, Rho and Rac family[11]. In contrast, oncogenic forms of Vav1 and Vav2 were less effective than activated Sos in stimulating the transactivation of a Gal4–Elk1 fusion protein (Fig. 4a, right panel), which served as a control for specificity. Interestingly, Vav1 and Vav2 share the characteristic feature of being activated upon tyrosine phosphorylation[32,33], and although Vav1 is expressed primarily in haematopoietic cells of the T and B lineages, Vav2 exhibits a ubiquitous distribution[33,34]. We therefore decided to investigate whether this protein is able to act as a link between Src and the stimulation of c-*myc*. As a first approach, we explored whether the overexpression of wild-type Vav2 protein could cooperate with Src in the stimulation of the c-*myc* promoter. As shown in Fig. 4b, the ability of limited amounts of Src to induce the c-*myc* promoter was strongly enhanced by coexpression of the wild-type Vav2 protein. The lack of cooperation with Dbl, which is not phosphorylated by Src (data not shown), supported the specificity of this approach. Similarly, overexpression of the wild-type Vav2 protein but not Dbl strongly enhanced the stimulation of the c-*myc* promoter by PDGF (Fig. 4c, and data not shown), thus indicating that Vav2 can participate in a signal transduction pathway connecting the PDGF receptor to the c-*myc* promoter.

As the DH domain is strictly required for the ability of GEFs to activate Rho proteins, we specifically deleted this domain from Vav2 and observed that expression of this mutated molecule can effectively decrease the ability of activated Src and PDGF to stimulate the c-*myc* promoter. However, it did not affect *luciferase* expression when provoked by a molecule acting downstream of Vav2, Rac QL (Fig. 4d). Together, these results indicated that Vav2 could mediate the stimulation of c-*myc* expression downstream of PDGF receptors and Src. Indeed, in agreement with previous reports[35], we confirmed that PDGF induces the tyrosine phosphorylation of the endogenous Vav2 protein in NIH 3T3 cells (Fig. 4e). Furthermore, a green fluorescent protein (GFP)-epitope-tagged Vav2 protein was also strongly tyrosine phosphorylated upon stimulation with PDGF or when cotransfected with the constitutively active form of Src, Src YF (Fig. 4f). Surprisingly, PDGF-induced Vav2 tyrosine phosphorylation was dependent on the activity of Src kinases, as judged by the ability of PP1 to inhibit this effect even at the lowest concentration tested (1 μM) (Fig. 4g), and upon cotransfection with a dominant-negative interfering Src protein, Src 251, which prevented the PDGF-induced tyrosine phosphorylation of Vav2 in a concentration-dependent manner (Fig. 4h). Together, these findings strongly indicate that Vav2 can participate in a signal transduction pathway connecting PDGF receptors and Src to nuclear events regulating c-*myc* expression through the stimulation of the activity of Rho-like GTPases.

Discussion

Mitogenic stimulation by PDGF is strikingly dependent on the activity of members of the Src family of NRTKs[5,7,8]. This need for functional Src kinases can be relieved by the expression of the simian-virus-40 large T antigen, which facilitated the establishment of mouse cell lines that can proliferate in response to PDGF even if they lack three of the most widely expressed NRTKs[7,36,37], or by the expression of c-*myc*[8]. The latter provided the first evidence of the strict requirement of Src kinases for the induction of c-*myc* by PDGF[8]. The activation of other transcriptional events, such as those controlling the expression of c-*fos*, does not seem to require Src[8], indicating a specific role for Src in the regulation of c-*myc*. However, the nature of the Src-dependent pathway by which PDGF receptors stimulate the expression of c-*myc* is still not known.

A reasonable candidate for mediation of the activation of c-*myc* is the Ras proto-oncogene, because stimulation of the Ras–Raf–MEK–MAPK pathway can enhance c-*myc* expression[38]. In fact, under our experimental conditions the constitutively active forms of Ras and MEK1 stimulated the c-*myc* promoter effectively, but an activated form of Src provoked a much greater response, which did not correlate with its ability to induce the MAPK pathway. Furthermore, the use of chemical inhibitors of MEK prevented the activation of MAPK in response to PDGF and activated Src but had only a limited effect on the ability of PDGF to stimulate the c-*myc* promoter and c-*myc* expression. Together, these results indicate that PDGF receptors can use biochemical routes distinct from the Ras–MAPK pathway to regulate the expression of the c-*myc* proto-oncogene.

In this regard, it is becoming clear that small GTP-binding proteins of the Rho family control signal transduction pathways that are essential for cell proliferation[10,11,30]. Thus, we next examined whether Rho proteins might have a role in signalling from PDGF receptors and Src to c-*myc*. We first found that activated forms of Rho, Rac and Cdc42 can stimulate the c-*myc* promoter potently, with Rac1 being the most active. In line with these results, we observed that Rac increases the steady-state levels of c-*myc* mRNA when expressed in transient as well as in stable cell lines, and that the direct activation of endogenous Rho GTPases by the use of bacterial toxins and overexpression of specific GEFs promotes c-*myc* expression. These results supported the possibility that small GTPases of the Rho family can initiate the activation of a biochemical route controlling c-*myc* expression. Furthermore, the blockade of endogenous Rho GTPases by bacterial toxins, or more specifically Rac and Cdc42 by their dominant-interfering molecules and by the overexpression of the CRIB domain of PAK, prevented the stimulation of c-*myc* expression by PDGF and Src. Together, these findings indicate that Rac, and to a smaller extent other Rho family members, might be sufficient to promote c-*myc* expression, and that these GTPases are a necessary component of the pathway linking PDGF receptors to c-*myc*.

Recent studies indicate that Vav1 and Vav2 can act as a tyrosine-phosphophorylation-dependent GEF for Rac and Cdc42 (refs 32, 35, 39). Interestingly, our data indicate that PDGF induces the phosphorylation of Vav2 and that this is dependent on the activity of Src kinases. Together, these observations provide support for the existence of a 'Src pathway'[8,22], independent of Ras but dependent on small GTPases of the Rho family, that is initiated by the activation of Src by PDGF receptors and the consequent activation of Vav2 and Rac1. In turn, this signalling route culminates in the transcription of c-*myc* and, together with those pathways controlling the expression of other transcription factors such as c-*fos*, in DNA synthesis in response to PDGF (Fig. 5). These findings also raise the possibility of the existence of a novel mechanism by which GTP-binding proteins of the Rho family might exert their growth-promoting function by regulating the expression of the c-*myc* proto-oncogene. This 'Rho GTPase–Myc pathway' can also help to explain the ability of Rho, Rac and Cdc42 to stimulate G1 cell cycle progression and subsequent DNA synthesis when microinjected into quiescent fibroblasts, and the ability of their dominant-negative mutants to block serum-induced cell proliferation[40]. As the activation of c-*myc* is independent of Ras and MAPK, these observations could also provide a mechanism by which Rho proteins and their upstream GEFs might exert their oncogenic potential, even if they do not stimulate the MAPK pathway[11,41]. Instead, Rho GTPases might use a complex network of kinases controlling the activity of other members of the MAPK superfamily, such as JNK and p38, which might contribute to the activation of nuclear factor-κB, STATs and E2F[42–44] that can then stimulate the activity of the c-*myc* promoter[17,45,46]. Further work will be required to explain fully how Rac and other Rho GTPases regulate c-*myc* expression, thus contributing to the mitogenic response to PDGF and other polypeptide growth factors that require functional Src kinases to signal cell proliferation. □

Methods

Reagents.

The plasmids pcDNAIII-MEK EE, pcDNAIII-HA–MAPK, pcDNAIII-onco-Dbl, pcDNAIII-proto-Dbl, pcDNA-onco-Vav1 and pCEFL-GST–PAK-N have been previously described[25,32,41]. The c-*myc* promoter reporter plasmid pMyc-Luc was kindly provided by R. Pestell[47]. To construct this plasmid the c-*myc* P1/P2 promoter from position –157 to +500 was cloned in the pA3-Luc reporter plasmid. The integrity of the plasmid was confirmed by dideoxy DNA sequencing. Position +1 corresponds to the beginning of the first exon of the human c-*myc* gene. AU5-tagged forms of Ras V12, Rho wild-type (wt), Rho QL, Rho N19, Rac wt, Rac QL, Rac N17, Cdc42 wt, Cdc42 QL and Cdc42 N17 were generated by cloning the corresponding complementary DNAs (cDNAs) in the pCEFL AU5 vector. pcDNAIII/GS-Myc-V5 was purchased from Invitrogen. The pFos-SRE luciferase reporter plasmid carrying the SRE from the c-*fos* promoter was obtained from Stratagene. Expression vectors (pcDNAIII derivatives) for proto-Vav2 and onco-Vav2 were kindly provided by X. R. Bustelo. The plasmid pCEFL EGFP-Vav2 wt was generated by cloning Vav2 wt in pCEFL enhanced green fluorescent protein (EGFP) in frame with the carboxy-terminal EGFP coding sequence. The plasmid pCEFL-Vav2 ΔDH was generated by polymerase chain reaction, deleting the Vav2 DH domain (from base 600 to 1195 of the coding sequence) from the wild-type molecule. The expression vectors pSM-Src YF (constitutively active) and pSM-Src YF-KM (dominant-negative) were kindly provided by H. Varmus. The bacterial expression vector pGEX2TGL-GST–CNF-1 was kindly provided by K. Aktories[28]. The expression vector for dominant-negative, C-terminal-truncated Src, Src 251, was kindly provided by P. Schwartzberg. pRSV Sos myristoylated was kindly provided by A. Aronheim. The pGal4-Elk1 plasmid, which expresses the transactivation domain of the Elk1 (amino acid residues 307–428) transcription factor fused to the DNA-binding domain of the yeast transcription factor Gal4 and the TATA-Gal4-driven luciferase reporter plasmid, pGal4 Luc, were described previously[48]. Additional information on each plasmid is available on request. Human recombinant PDGF-BB (Intergen, Purchase, New York) was used at a final concentration of 12.5 ng ml^{-1}. The PP1 (Biomol) and PD98059 (Calbiochem, La Jolla, California) inhibitors were added 20 min before stimulation with PDGF, at the indicated concentrations. *Clostridium difficile* toxin B was purchased from List Biological Laboratories (Campbell, California).

Reporter gene assays.

NIH 3T3 cells were transfected with the 'FuGENE™ 6' Reagent (Roche), with different expression plasmids together with 1 μg of pcDNAIII-β-Gal and 1 μg of the reporter plasmid. After incubation for 24 h in serum-free medium, the cells were lysed with reporter lysis buffer (Promega). Luciferase activity present in cellular lysates was assayed with D-luciferin and ATP as substrates, and light emission was quantified with the Monolight 2010 luminometer as specified by the manufacturer (Analytical Luminescence Laboratory, San Diego, California).

Cell culture.

NIH 3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, New York) supplemented with 10% (v/v) bovine calf serum (Bio Whittaker). To establish NIH 3T3 cells stably expressing AU5-tagged versions of the different constitutively active Rho GTPases, cells were transfected by the 'LipofectAMINE Plus' Reagent (Gibco BRL), in accordance with the manufacturer's instructions. Cells were selected in G418 (0.75 mg ml^{-1}) for ~3 weeks. EGFP-expressing cells were also established and used as a negative control.

Northern blot analysis.

NIH 3T3 cells were serum-starved for 24 h and treated as indicated. Cells were then washed with cold PBS, and total RNA was extracted by homogenization with Trizol (Gibco BRL) in accordance with the manufacturer's specifications. Total RNA (10–20 μg) was fractionated in 2% formaldehyde–agarose gels, transferred to nitrocellulose membranes and hybridized with ^{32}P-labelled DNA probes prepared with the Prime-a-Gene Labeling System (Promega). As DNA template we used a 450-bp *Pst*I DNA fragment from the human *c-myc* gene (pCDNAIII/GS-Myc-V5). Accuracy of RNA loading and transfer was confirmed by fluorescence under ultraviolet after staining with ethidium bromide.

Western blot analysis and antibodies.

Lysates of total cellular proteins or immunoprecipitates were analysed by protein immunoblotting after SDS–PAGE with the specific rabbit antisera or mouse monoclonal antibodies. Immunocomplexes were detected by enhanced chemiluminescence (ECL) (Amersham) with the use of goat antiserum against rabbit or mouse IgG coupled to horseradish peroxidase (Cappel, West Chester, Pennsylvania). As primary antibodies we used rabbit polyclonal antisera against c-Myc (N-262), Erk1/2 (C-14), RhoA (119), Rac1 (C-14), Cdc42 (P1) (Santa Cruz Biotechnology, Santa Cruz, California), GFP (ClonTech) and Vav2 (gift from J. I. Lee); sheep polyclonal antisera against Vav2 (anti-SHD and DPH regions) (Calbiochem); mouse monoclonal antibodies against phosphotyrosine (Santa Cruz, and Upstate Biotechnology, Lake Placid, New York), PDGF receptor B, phospho-MAPK (Upstate Biotechnology) and haemagglutinin (HA) epitope (HA.11; Berkeley Antibody Company, Richmond, California); HRP-conjugated, mouse monoclonal antibodies against phosphotyrosine (Zymed, San Francisco, California).

Purification of GST–CNF-1.

The GST fusion protein was isolated by affinity chromatography with glutathione–Sepharose (Pharmacia)[28].

Kinase assays.

Methods to evaluate the phosphorylating activity of MAPK by kinase assays *in vitro* have been described previously[41].

Reproducibility of data.

Experiments were repeated at least three times with consistent results.

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