Abl tyrosine kinase regulates a Rac/JNK and a Rac/Nox pathway for DNA synthesis and *Myc* expression induced by growth factors

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Accepted 13 May 2005

Journal of Cell Science 118, 3717-3726 Published by The Company of Biologists 2005

doi:10.1242/jcs.02491

Summary

The cytoplasmic tyrosine kinase Abl is a Src substrate required for platelet-derived growth factor (PDGF) receptor signaling leading to *Myc* expression and DNA synthesis. Abl targets are, however, ill defined. Here we report that the small GTPase Rac is an important effector of its mitogenic function. PDGF-induced Rac activation was impaired in cells with inactive Abl and active Rac overcame the mitogenic defects found in these cells. Rac function required both a Jun N-terminal kinase (JNK) and a NADPH oxidase (Nox) pathway. Furthermore, co-activation of JNK and Nox were sufficient to mimic the Rac mitogenic rescue. Abl also regulated PDGF-induced JNK and Nox activation. Finally, we found that *Myc* is an important target of this signaling cascade: *Myc* induction was sensitive to small inhibitors of JNK and Nox activities and forced expression of *Myc* overcame the G1 block induced by dominant interfering mutants of mitogen-activated protein kinase kinase 4 (MKK4) and Nox2 activating subunit. We concluded that cytoplasmic Abl operates on a Rac/JNK and a Rac/Nox pathway for PDGF-induced *Myc* induction and DNA synthesis.

Key words: Src, Abl, PDGF Receptor, Rac, Mitogenesis, Signaling

Introduction

The cytoplasmic tyrosine kinases of the Src family (SFK) play important roles in mitogenic responses induced by growth factors. For example, activation of SFK by platelet-derived growth factor (PDGF) receptor allows phosphorylation of specific substrates involved in the expression of *Myc* for cell-cycle progression (Bromann et al., 2004). Several of these substrates have been identified including the adaptor Shc (Gotoh et al., 1997; Blake et al., 2000), the transcription factor Stat3 (Bowman et al., 2001) and the guanine nucleotide exchange factor (GEF) Vav2 (Bromann et al., 2004). The mechanism by which they impact on mitogenesis is still ill defined. Chiariello et al. reported that the small GTPases of the Rho family are mediators of Src-induced *Myc* expression suggesting that Vav2 participates in GTPase activation for the PDGF mitogenic response (Chiariello et al., 2001). However, Vav2 inactivation did not have a major effect in the *Myc* induction, indicating that additional GEF must be involved in this process (Chiariello et al., 2001).

Recently, we and others have identified the cytoplasmic tyrosine kinase Abl as an additional Src mitogenic substrate for PDGF-induced *Myc* expression and DNA synthesis (Plattner et al., 1999; Furstoss et al., 2002a). Abl is a non-receptor tyrosine kinase distinct from the Src family, with a dual nuclear and cytoplasmic localization. Nuclear Abl has been implicated in DNA damage response and cell growth inhibition. By contrast, the cytoplasmic kinase has a role in cytoskeletal rearrangement and the promotion of DNA synthesis (Pendergast, 2002). Abl function implicates Src-induced phosphorylation of Y245 and Y412 for both dorsal ruffles formation and mitogenesis (Furstoss et al., 2002a). Furthermore, Abl can be regulated by phosphatidylinositol 4,5-bisphosphate (PI4,5P_2) for PDGF-induced chemotaxis (Plattner et al., 2003). The molecular mechanism by which PI4,5P_2 modulates catalytic activity in vivo is still obscure. Abl has a related kinase, Arg, that is only present in the cytoplasm and that is also activated by PDGF in a Src-dependent manner (Plattner et al., 2004). Although not yet unidentified, Arg may have a specific function distinct to Abl (Plattner et al., 2004).

Downstream targets of Abl during mitogenesis are largely unknown. Recently Pendergast et al. identified the phospholipase Cγ1 as a novel Abl substrate (Plattner et al., 2003). Although PLCγ was shown as an important mediator of PDGF-induced DNA synthesis (Valius and Kazlauskas, 1993; Roche et al., 1996), it is not known whether its phosphorylation by Abl plays a role in this biological response. Similarly, Sini et al. identified the GEF SOS1 as an Abl substrate for Rac activation in vitro, but the biological significance of this phosphorylation was not provided (Sini et al., 2004). Again, whether SOS1 phosphorylation and Rac activation are required for Abl mitogenic function are not known. In this report, we addressed the role of small GTPases of the Rho family in the Abl mitogenic function and we show that Rac is an important mediator of cytoplasmic Abl for PDGF-induced *Myc* expression and DNA synthesis. Furthermore we identified Jun N-terminal kinases (JNK) and NADPH oxidase (Nox) activities as two important elements of the Abl-Rac signaling cascade for mitogenesis.

Materials and Methods

Reagents

Vectors used included those expressing Abl-PP-K$^-$ (AblP242E/P249E/K290M), Abl-NLS$^-$ and Myc (Furstoss et al., 2002a); p47phox-GFP, p47SD (p47phoxS303D/S304D/S328D), p67phoxV204A (Wu et al., 2003); pLXSN encoding RhoV14, RacV12, RacL61, Cdc42V12, RacL61/F37A and RacL61/Y40C (from P. Roux, CRBM, Montpellier, France); and RacL61/N52L, RacL61/D38N, RacL61/K132E and Shc2Y2F/R394F (ShcY239F/Y240F/R394F) vectors (generated by site directed mutagenesis). Vectors expressing Stat3YF (Stat3Y705F) was provided by T. Hirano (Osaka University Japan); DN-MKK4 (MKK4S220A/T224A) and DA-MKK4 (MKK4 S220E/T224E) were from Atfi (Inserm U482, Paris); DA-MKK6 (MKK6S207E/T211E) was from J. Raingeaud (Inserm U641, Chatenay-Malabry); and DA-MKK1 (MKK1S218E/S222D), DA-MKK5 (MKK5S222D/T226D) and DA-MKK7 (MKK7β1T275A/S277A) were from R. Hipskind (IGMM, Montpellier, France). Gst-Jun was a generous gift of R. Hipskind; antibodies specific to pJNK, JNK1, pMAPK, MAPK1 and 2, pS63-Jun, Jun were from Cell Signaling Technology; Ab-3 form Calbiochem, αPR4 and Abl (24-21) were described previously (Roche et al., 1998; Furstoss et al., 2002a). αPRC was generated against the GST fusion protein containing the 891-1106 sequence of human PDGFRβ; anti-Ras, -Rac, -Cdc42 and -Rho were from P. Roux; anti-p47phox, -p67phox and -p91phox were from M. Pucéat (CRBM, Montpellier, France) and described previously (Puceat et al., 2003). STI 571 inhibitor was a generous gift form B. Willi (Novartis). DPI, NAC, BrdU and catalase were from Sigma; SP600125 and SB203580 were from Calbiochem. [γ^{32}P]ATP was from Amersham. Fluorescent reagents, including secondary antibodies and rhodamine phalloidin, were from Interchim.

Cell culture, transfection, infection and DNA synthesis.

Abl.Arg$^{+/+}$ 3T3, Abl/Arg$^{-/-}$3T3, Abl/Arg$^{-/-}$3T3 expressing Abl-NLS$^-$, NIH3T3, cell culture, transfection and retroviral infections were described previously (Furstoss et al., 2002a; Furstoss et al., 2002b). For biochemistry, quiescent cells were treated or not with 20 ng/ml of PDGF-BB (UBI) at the indicated times before lysis. DNA synthesis was analysed by BrdU incorporation assays (Furstoss et al., 2002a). Actinic structure was vizualized using rhodamine phaloidine (Furstoss et al., 2002a). For cell treatment, inhibitors (or vehicule) were added to the medium 2 hours before stimulation. The percentage of transfected cells that incorporated BrdU was calculated by the following formula:

$$\%\ \text{BrdU-positive cells} = \frac{[\text{number of BrdU-positive transfected cells}]}{[\text{number of transfected cells}] \times 100}.$$

For each coverslip, about 150-200 cells were counted and the results of 3-5 independent experiments have been averaged and the mean±s.d. are shown.

Biochemistry and *Myc* expression

Cells lysis, immunoprecipitation and western blotting were described previously (Furstoss et al., 2002a). In vitro kinase assays for PDGFR and MKK have been described (Roche et al., 1998; Raingeaud et al., 1996). GTPase activities were measured as described (Charrasse et al., 2002) and precipitated using GST fusion proteins containing Ras-binding domain of RalGDS (Ras) (Herrmann et al., 1996), CRIB domain of PAK (Rac), Cdc42-binding domain of WASP (Cdc42) and RhoA-binding domain of Rhotekin (Rho) (C. Gauthier-Rouviere, CRBM, Montpellier, France). MAPK1 and 2 and JNK activities were assessed by western blotting of the whole cell-lysates using antibodies specific to the phosphorylated kinases.

Myc mRNA level was measured by both northern blotting (Furstoss et al., 2002a) and by real-time quantitative PCR. The following primers were used: Myc forward 5′-CGGAGGAAAACGACAAGAGG-3′ and reverse 5′-GTGCTCGTCTGCTTGAATGG-3′; β-tubulin forward 5′-CGGACAGTGTGGCAACCAGATCGG-3′ and reverse 5′-TGGCCAAAAGGACCTGAGCGAACGG-3′. Data were normalized using RT-PCR of the β-tubulin mRNA as an index of cDNA content after reverse transcription. The results of 2-5 independent experiments have been averaged and the mean±s.d. are shown.

Results

Abl regulates PDGF-induced Rac activation for mitogenesis

We first addressed the role of Abl in the regulation of Rho GTPases by PDGF. For this purpose, we used spontaneously immortalized fibroblasts issued from wild-type or Abl, Arg double-knockout mice (Koleske et al., 1998). As shown in Fig. 1, no clear difference in PDGF receptor β activation was observed. Therefore, the inability of cells to respond to PDGF-BB may not be attributed to a loss of receptor activation. The activities of Rho, Rac and Cdc42 were next measured in PDGF-stimulated cells. As shown in Fig. 1B, no clear Cdc42 activation was observed, although basal activity was downregulated for longer. PDGF also induced a transient and modest increase in Rho activity, but this was independent of Abl activity. By contrast, PDGF induced a rapid and sustained Rac activation that was inhibited in Abl/Arg-deficient cells as recently reported (Sini et al., 2004). Such a defect was attributed to a lack of Abl function because low expression of the cytoplasmic allele of the kinase Abl-NLS$^-$ substantially rescued the Rac response (Fig. 1C). Therefore, Abl is an important mediator of PDGF-induced Rac activation. Surprisingly, Rho was found downregulated for longer in control, but not in Abl/Arg-deficient, cells. We believe that this is due to the capacity of active Rac to inactivate Rho (Sander et al., 1999), probably through the activation of GTPase activating protein p190RhoGAP (Nimnual et al., 2003). The absence of Rho downregulation in Abl/Arg$^{-/-}$3T3 cells may therefore reflect a defect in Rac activation. Finally, we found that Abl did not affect PDGF-induced Ras activation (Fig. 1B), in agreement with the suspected Ras-independent nature of the Abl mitogenic pathway (Furstoss et al., 2002a).

The role of Rac in Abl mitogenic signaling was next addressed. NIH3T3 were transfected with the dominant interfering mutant Abl-PP-K$^-$, which has been shown to specifically target Abl signaling in the cytoplasm (Furstoss et al., 2002a). This kinase-dead allele of AblK290M includes a double proline mutation in the regulatory SH2-CD linker (P242E/P249E, Abl-PP) that stabilizes the kinase in an opened and ‘active’ conformation (Barila and Superti-Furga, 1998). In contrast to the regulated AblK290M, Abl-PP-K$^-$ is excluded from the nucleus (Furstoss et al., 2002a) in fibroblasts, allowing specific inhibition of Abl function in the cytoplasm. Transfected cells were then made quiescent by serum starvation and stimulated with growth factors for cell cycle re-entry. De novo DNA synthesis was monitored by recording bromodeoxyuridine (BrdU) incorporation into the nucleus. Abl-PP-K$^-$ inhibited PDGF-induced DNA synthesis, as previously reported (Furstoss et al., 2002a). We then addressed the capacity of Rho members to alleviate this inhibition. To this end, we co-expressed constitutively active alleles of these GTPases under the control of a retroviral promoter that allows

a low ectopic protein expression. In these conditions, we found that constitutive active RacV12 largely rescued mitogenesis (70%) (Fig. 2A). This effect was dependent upon growth factor stimulation as it did not induce DNA synthesis on its own (not shown). Other members of the Rho family, including Cdc42 and Rho itself, were much less effective (15-20%), showing a specificity towards Rac. Similar results were obtained with serum indicating that the Abl-Rac connection is not specific to PDGF (Fig. 2A). This suggests that Rac is a downstream element of the Abl mitogenic pathway. The mitogenic Rac rescue was next confirmed by other means. We first used Abl/Arg$^{-/-}$ immortalized fibroblasts. These cells exhibit a 4 hour delay for S phase entry (Furstoss et al., 2002a). Consequently, only 10-15% of cells have made DNA synthesis after 16 hours of PDGF stimulation instead of 50% in control cells. Retroviral transduction of RacV12 completely rescued mitogenesis (Fig. 3A). Similar results were obtained with 10% serum. Again, Cdc42V12 was less efficient. This gain of function was dependent upon a defect in Abl signaling as it was not observed in control cells: mitogenesis was not affected in Abl/Arg$^{+/+}$ cells infected with RacV12 retrovirus. We next confirmed these data with the use of STI 571 (imatinib mesylate), a pharmacogical inhibitor of Abl activity (Druker et al., 1996). This drug was not used in the case of PDGF since it also affects receptor kinase activity (Buchdunger et al., 2000). Nevertheless, this approach was still valuable for investigating the serum response. As shown in Fig. 3B, STI 571 largely reduced serum-induced DNA synthesis in control cells. However, no such inhibition was observed in cells expressing RacV12, even at a maximal concentration (e.g. 10 μM). Inhibition was specific for Abl as cell treatment with

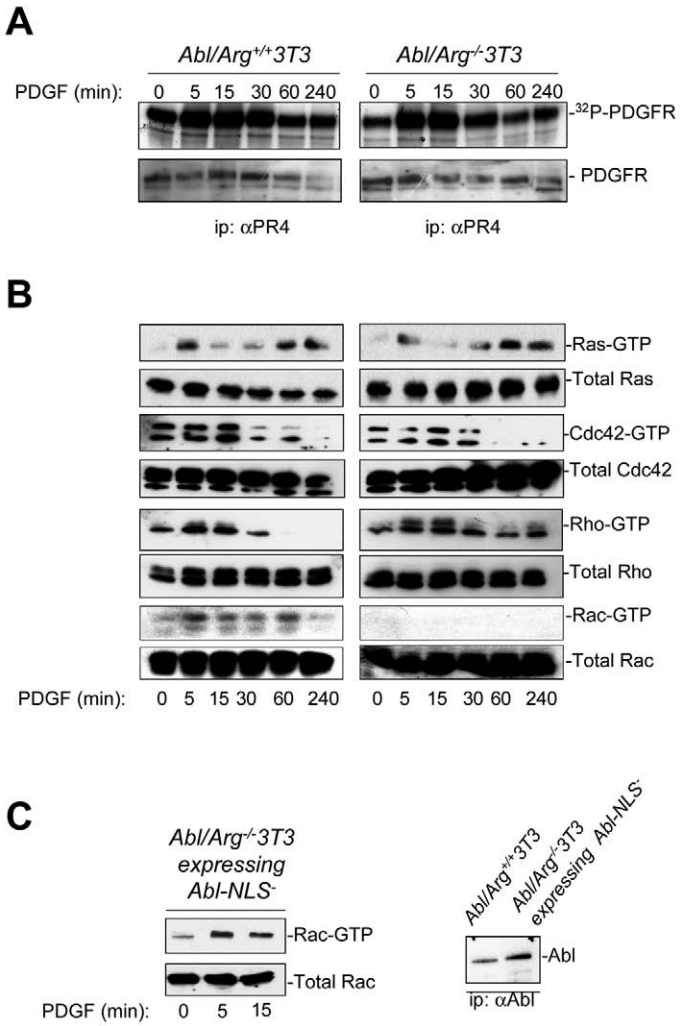

Fig. 1. Abl regulates PDGF-induced Rac activation. (A) Activation of PDGFRβ in Abl/Arg$^{+/+}$3T3 and Abl/Arg$^{-/-}$3T3 cells. In vitro kinase assay of the immunoprecipitated PDGFRβ from indicated cell lysates as described in Materials and Methods. Autophosphorylated PDGFR (^{32}P-PDGFR) is shown. The level of the immunoprecipitated receptor is also shown and was assessed by western blotting with the αPRC antibody. (B) PDGF-induced Rac activation is impaired in Abl/Arg$^{-/-}$3T3 cells. (C) Abl-NLS^{-} expression restored PDGF-induced Rac activation in Abl/Arg$^{-/-}$3T3 cells (left panel). Cells were stimulated with PDGF for 0-240 minutes. GTP-bound Ras, Rho Rac and Cdc42 (shown as GTPase-GTP) were precipitated as described in Materials and Methods and detected with specific antibody. Total GTPase level is also shown. (C, right panel) Western blotting of immunoprecipitated Abl from indicated cell-lysates using Ab-3 antibody. The level of Abl-NLS^{-} expressed in Abl/Arg$^{-/-}$3T3 was comparable with the endogenous level of Abl in Abl/Arg$^{+/+}$3T3 cells.

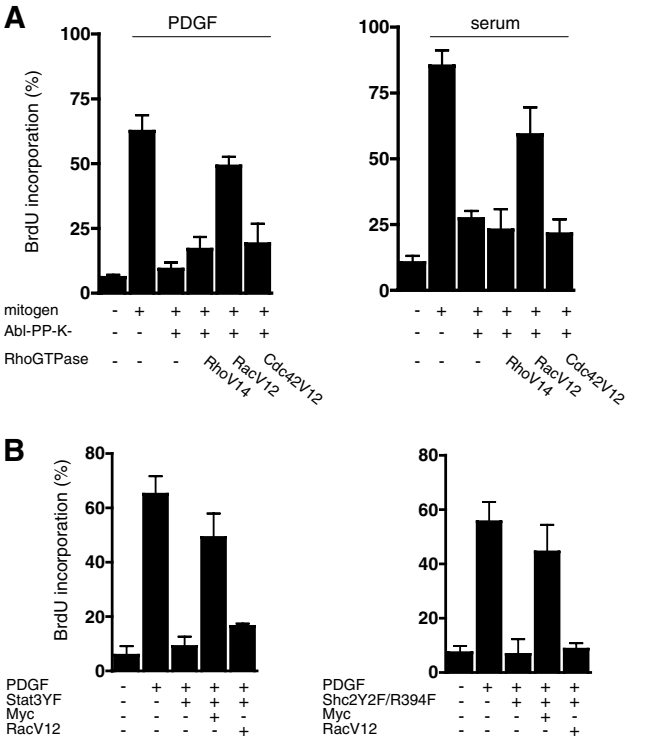

Fig. 2. RacV12 alleviates the G1 block induced by Abl-PP-K^{-}. (A) RacV12 rescues the G1 block induced by Abl-PP-K^{-}. Quiescent NIH3T3 transfected with the indicated constructs were stimulated or not with PDGF or 10% fetal calf serum as indicated for 18 hours in the presence of BrdU. (B) RacV12 does not rescue the G1 block induced by dominant interfering mutants of Shc and Stat3. Quiescent NIH3T3 transfected with the indicated constructs were stimulated or not with PDGF and proceeded as in A. Cells were fixed and stained for BrdU incorporation and ectopic protein expression as described in Materials and Methods. The mean±s.d. of the percentage of BrdU-positive cells present in expressing and non-expressing cells under the specified conditions is shown.

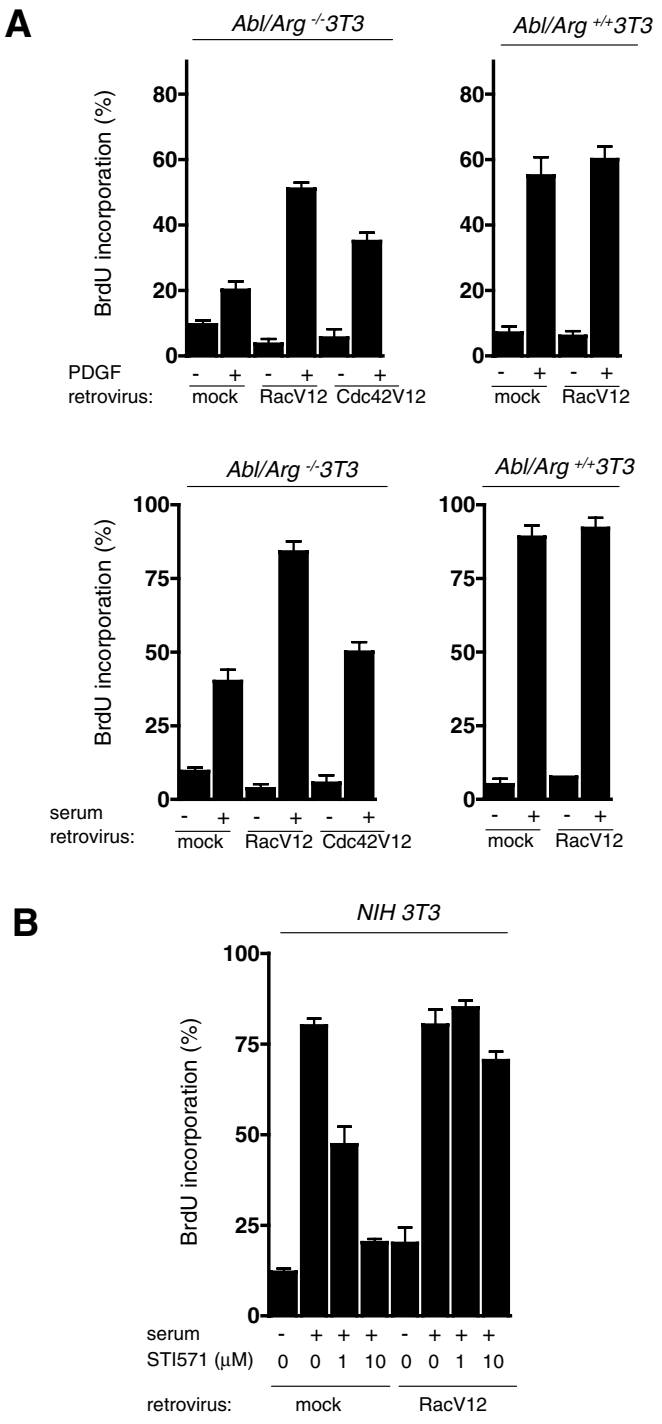

Fig. 3. RacV12 also restores mitogenesis in cells with loss of Abl kinase activity. (A) RacV12 rescues the mitogenic defect in Abl/Arg$^{-/-}$3T3 cells. Quiescent cells that were infected with wild-type (mock) or retrovirus as shown, were stimulated or not with indicated mitogen for 16 hours in the presence of BrdU. (Top panels) The PDGF responses; (bottom panels) the serum (10% fetal calf serum) responses. (B) RacV12 rescues the mitogenic inhibition induced by the Abl kinase inhibitor STI 571. NIH3T3 quiescent cells that were infected with wild-type (mock) or RacV12-expressing retrovirus were treated or not with various concentrations of STI 571 1 hour before stimulating cells with serum for 18 hours in the presence of BrdU as indicated. The mean±s.d. of the percentage of BrdU-positive cells under the specified conditions is shown.

the PDGF receptor inhibitor had no effect (Furstoss et al., 2002a), precluding PDGF as the major mitogen in the serum we used. Collectively, data obtained from these various approaches established a functional link between Abl and Rac for mitogenesis.

We next addressed whether Rac is a downstream effector to other Src mitogenic substrates, e.g. Stat3 and Shc. We first checked that Rac impinges on the Src mitogenic pathway. As expected, PDGF-induced Rac activation was dependent upon SFK activities (C. Bénistant and S.R., unpublished); furthermore, RacV12 gave a significant mitogenic rescue in cells with inactive SFK – although the rescuing effect was not as strong as the one obtained with Abl-PP-K^{-} (not shown). We next confirmed that expression of the non-phosphorylatable allele of Src substrates inhibited mitogenesis (Fig. 2B) (Blake et al., 2000; Bowman et al., 2001). Nevertheless, RacV12 could not overcome those inhibitions. By contrast, constitutive expression of *Myc* largely restored mitogenic signaling. Note that in our conditions, Myc did not induce mitogenesis in the absence of growth factors, furthermore this was specific to the Src pathway as Myc could not alleviate the G1 block induced by RasN17 (not shown) (Barone and Courtneidge, 1995). From this set of data we concluded that Rac is a specific effector of the Src substrate Abl for mitogenesis.

The Rac mitogenic rescue requires a JNK and a Nox pathway

We next searched the underlying mechanism for Rac mitogenic rescue. To this end, we took advantage of active RacL61 alleles with point mutation in the effector loop, known to interact with specific effectors (e.g. F37A, D38N, Y40C, N52L, K132E) (Bishop and Hall, 2000). Although a single mutation may affect several signaling cascades, we felt that this may bring some insight into the nature of downstream elements of the Abl-Rac pathway. Similarly to RacV12, all RacL61 alleles were expressed under a retroviral promoter for low expression. In these conditions they induced low Rac activity in cells (Fig. 4E) so that they did not induce DNA synthesis on their own (not shown). We then analyzed their capacity to alleviate the Abl-PP-K^{-} G1 block in PDGF-stimulated cells. First, we found that RacL61 was as active as RacV12 in restoring mitogenic signaling (70% of the PDGF response) (Fig. 4A). RacL61/F37A was also fully active. This mutant has been described to affect the capacity of Rac to activate Stat3 (Simon et al., 2000), indicating that this signaling protein may not impinge on that pathway. In agreement with this notion, we did not observe any reduction in PDGF-induced Stat3 activation in Abl-deficient cells (not shown). By contrast, mutants with reduced JNK (RacL61/Y40C and N52L) (Lambert et al., 2002) or NADPH oxidase (Nox) activation (RacL61/D38N and K132E) (Nisimoto et al., 1997; Puceat et al., 2003) were biologically defective. Again these rescuing effects were not specific to PDGF as similar data were obtained with serum (Fig. 4A). Such defects were not due to a lower enzymatic activity: all of these Rac alleles were as active as RacL61 on their ability to bind GTP in vivo (Fig. 4E). These data suggest that Rac mitogenic function implicates both a JNK and a Nox-dependent pathway. Accordingly, we found that cell treatment with low doses of a JNK inhibitor (SP600125) or compounds that inhibit superoxide accumulation (catalase and diphenylene

iodonium, DPI) strongly reduced the RacL61 mitogenic rescue (Fig. 4B). By contrast, SB203580 had no effect, precluding any involvement of p38 kinases α and β in that biological response (Fig. 4B). Since RacL61/N52L and RacL61/Y40C also exhibited reduced ability to activate p70 S6 kinase (Lambert et al., 2002), we checked whether the observed biological activity was due to an impairment in JNK activation. We found that co-expression of active JNK kinases MKK4 or MKK7 induced strong JNK activity in vivo (Fig. 4F) and corrected the biological defect observed with RacL61/N52L (Fig. 4C). Similarly, we found that RacL61/D38N rescued mitogenesis when co-expressed with an activator of Nox activity. For this purpose, we used the constitutively active allele p47SD (Wu et al., 2003) of the Nox2 organizer subunit Noxo2 (originally called p47phox). This allele consists of a replacement of three serines on Noxo2 known to be critically phosphorylated for Nox2 activation, with aspartic acid residues to mimic phosphoserines. This triple mutant has been shown to cause constitutive activation of the NADPH oxidase in a cell-free reconstitution system (Ago et al., 1999) and in endothelial cells (Wu et al., 2003). We also found that p47SD induced superoxide production in fibroblasts, although Nox2 was not detected in these cells (not shown). This may reflect the capacity of Noxo2 to activate additional members of the Nox family, including Nox1 and Nox3 (Banfi et al., 2003; Cheng et al., 2004). When analysed in our mitogenic assay, we found that p47SD compensated for the inability of RacL61/D38N by rescuing mitogenesis at a similar level to RacL61 (Fig. 4D). Collectively, these data indicate that the loss of mitogenic rescue induced by N52L and D38N mutations is probably due to an impairment in JNK and Nox activation, respectively.

Co-activation of JNK and Nox pathways mimic the Rac mitogenic rescue

We next looked at whether there is a functional interaction between Abl and JNK during mitogenesis. To this end, we analysed the capacity of constitutively active MAP kinase activators to overcome the Abl-PP-K$^-$ mitogenic inhibition

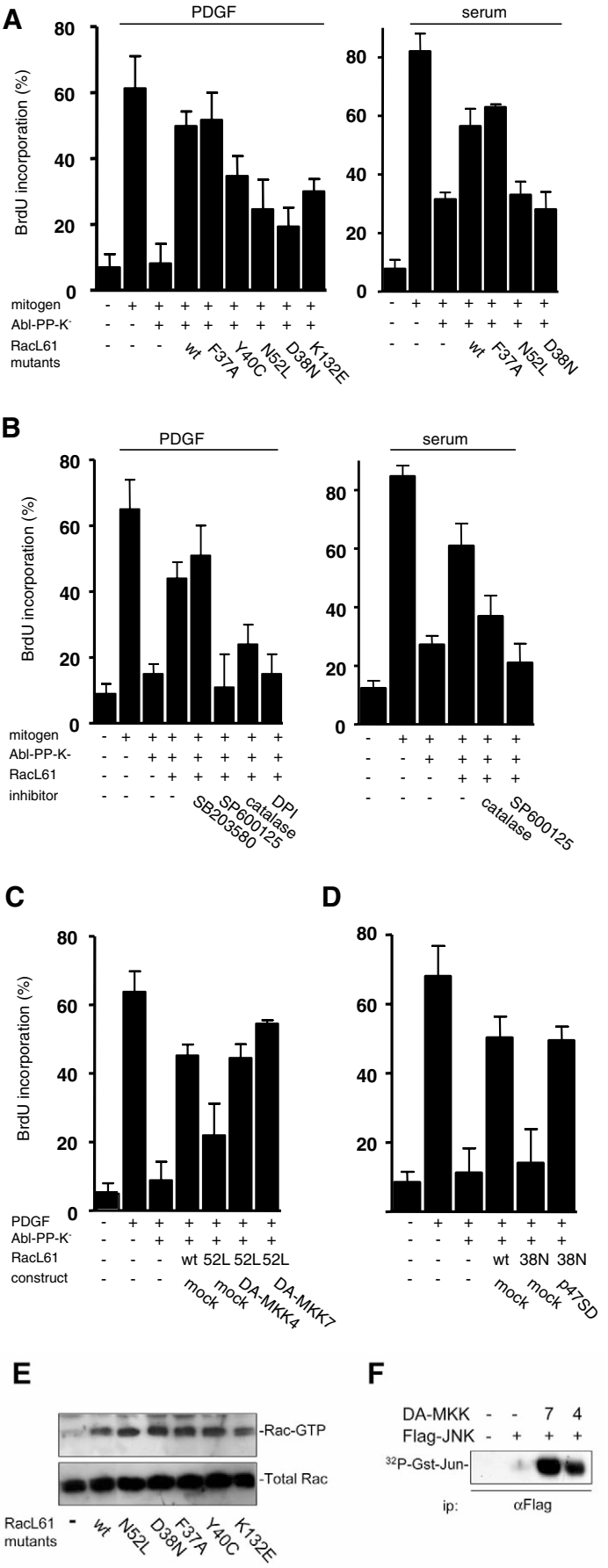

Fig. 4. The Rac mitogenic rescue implicates both a JNK and a Nox activity. (A) Structure-function analysis of the Rac mitogenic rescue. (B) The Rac mitogenic rescue is sensitive to inhibitors of JNK and Nox activities. (C) The biological defect of RacL61/N52L is compensated by activators of the JNK pathway. (D) The biological defect of the RacL61/D38N is compensated by an activator of the Nox pathway. Quiescent NIH3T3 transfected with Abl-PP-K$^-$ together or not with indicated constructs were stimulated or not with mitogen (20 ng/ml PDGF or 10% serum as shown) in the presence of BrdU and proceeded as in Fig. 2. In panel B, cells were treated with 0.5 μM SP600125, 20 μM SB203580, 0.5 μM DPI or 10,000 U catalase as indicated before stimulation. The mean±s.d. of the percentage of BrdU-positive cells present in expressing and non-expressing cells under the specified conditions is shown. (E) Activity of various RacL61 mutants used in this study. NIH3T3 infected with control or indicated RacL61 retroviruses were incubated overnight with 0.5% serum and assayed for Rac activity as described in Fig. 1. Total Rac level and Rac activity (Rac-GTP) is shown. (F) In vivo activity of DA-MKK4 and 7 used in this study. Cells were transiently transfected with indicated DA-MKK and Flag-tagged JNK1 constructs as indicated. Immunoprecipitated Flag-JNK was assayed for in vitro activity as described in Materials and Methods. The level of phosphorylated Jun (^{32}P-Gst-Jun) is shown.

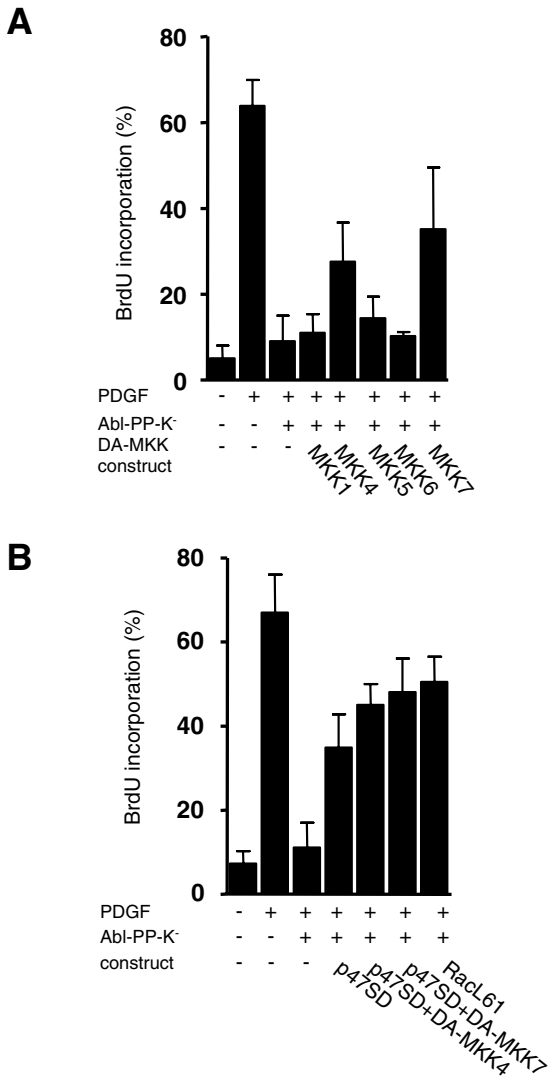

Fig. 5. Co-activation of a JNK and a Nox pathway mimic the Rac mitogenic rescue. Active DA-MKK4, DA-MKK7 (A) and p47SD (B) partially rescue the Abl-PP-K$^-$ G1 block. Quiescent NIH3T3 transfected with Abl-PP-K$^-$ together or not with the indicated constructs were stimulated or not with PDGF in the presence of BrdU and proceeded as in Fig. 2. The mean±s.d. of the percentage of BrdU-positive cells present in expressing and non-expressing cells under the specified conditions is shown.

(Fig. 5A). This included MAPK1 and 2 activator MKK1, p38 activator MKK6, MAPK5 activator MKK5, JNK activator MKK7 and the dual p38 and JNK activator MKK4. All these MKK alleles were active in vivo (Brunet et al., 1994; Raingeaud et al., 1996; Kato et al., 1997 and Fig. 4E). Nevertheless, we found that only kinases that activate JNK gave a partial but significant rescue: 30% for MKK4 and 45% for MKK7. The absence of effect obtained with other kinases indicates that this biological process is specific to this type of MAPK. Furthermore, the partial effect suggests that additional pathways are required for maximal cell response. We then considered Nox as a separate effector for Abl mitogenic signaling. We found that when co-expressed with Abl-PP-K$^-$, the Noxo2 dominant active p47SD significantly restored mitogenesis (40%) (Fig. 5C). This supports the notion that a Nox pathway lies in Abl mitogenic signaling. Interestingly, co-expression of active DA-MKK4 or DA-MKK7, together with p47SD, overcame the Abl-PP-K$^-$ G1 block at a similar level (70%) to that obtained with RacL61 (Fig. 5C). Therefore, co-activation of JNK and Nox activities are sufficient to mimic the Rac mitogenic rescue.

Abl regulates PDGF-induced JNK and Nox activation

We next addressed whether Abl regulates JNK activity in PDGF-stimulated cells. As shown in Fig. 6A, PDGF-induced a modest but significant JNK activation. This enzymatic response was largely reduced in Abl/Arg-deficient cells. Accordingly, JNK regulates PDGF-induced Ser63 phosphorylation of Jun (not shown) and this was also reduced in Abl/Arg$^{-/-}$ cells. Reintroduction of the cytoplasmic Abl allele Abl-NLS$^-$ significantly rescued JNK activation and Jun phosphorylation in agreement with a role of Abl in this signaling cascade (Fig. 6A). By contrast, Abl deficiency did not affect PDGF-induced early activation of MAPK 1 and 2, showing specificity.

The role of Abl on PDGF-induced Nox activation was also investigated. Activation of Nox requires membrane translocation of a Noxo and a Nox activating regulatory subunit (Noxa) (Lambeth, 2004). Although both Noxo2 and Noxa2 (originally called p67phox) were expressed in our cells, we could not detect a clear recruitment to the membrane probably due to inappropriate biological tools (not shown). Nevertheless, we found that ectopic p47phox-GFP (green fluorescent protein) that was diffusely localized in resting cells rapidly translocated to actin-enriched dorsal ruffles upon PDGF stimulation (Fig. 6B). This was confirmed by colocalization of the GFP fusion protein with these actinic structures. P47 membrane recruitment was largely impaired in Abl/Arg$^{-/-}$ cells stimulated with PDGF but totally rescued in cells expressing Abl-NLS$^-$. These data are consistent with an important role for Abl in PDGF-induced Nox activation in fibroblasts.

Myc is an important target for JNK and Nox mitogenic functions

The impact of Rac on Abl-induced *Myc* expression was next addressed in PDGF-stimulated cells. This growth factor induced a large increase in *Myc* mRNA levels within 1 hour of stimulation. As previously reported, this response was largely impaired in Abl/Arg-deficient cells and this was largely compensated by Abl expression in the cytoplasm (Furstoss et al., 2002a). Here we found that *Myc* induction was also restored by retroviral expression of RacV12 (Fig. 7A). This gain of expression was dependent upon a defect in Abl signaling as it was not observed in control cells. We also found that the *Myc* induction was dependent upon JNK and Nox activities as it was sensitive to specific pharmacological inhibitors (SP600125 for JNK, DPI and *N*-acetylcysteine NAC for Nox) (Fig. 7B). Interestingly, cell treatment with both types of inhibitors prevented the *Myc* response, which suggests that JNK and Nox activities are important regulators of *Myc* mRNA induction. Finally, we evaluated the impact of

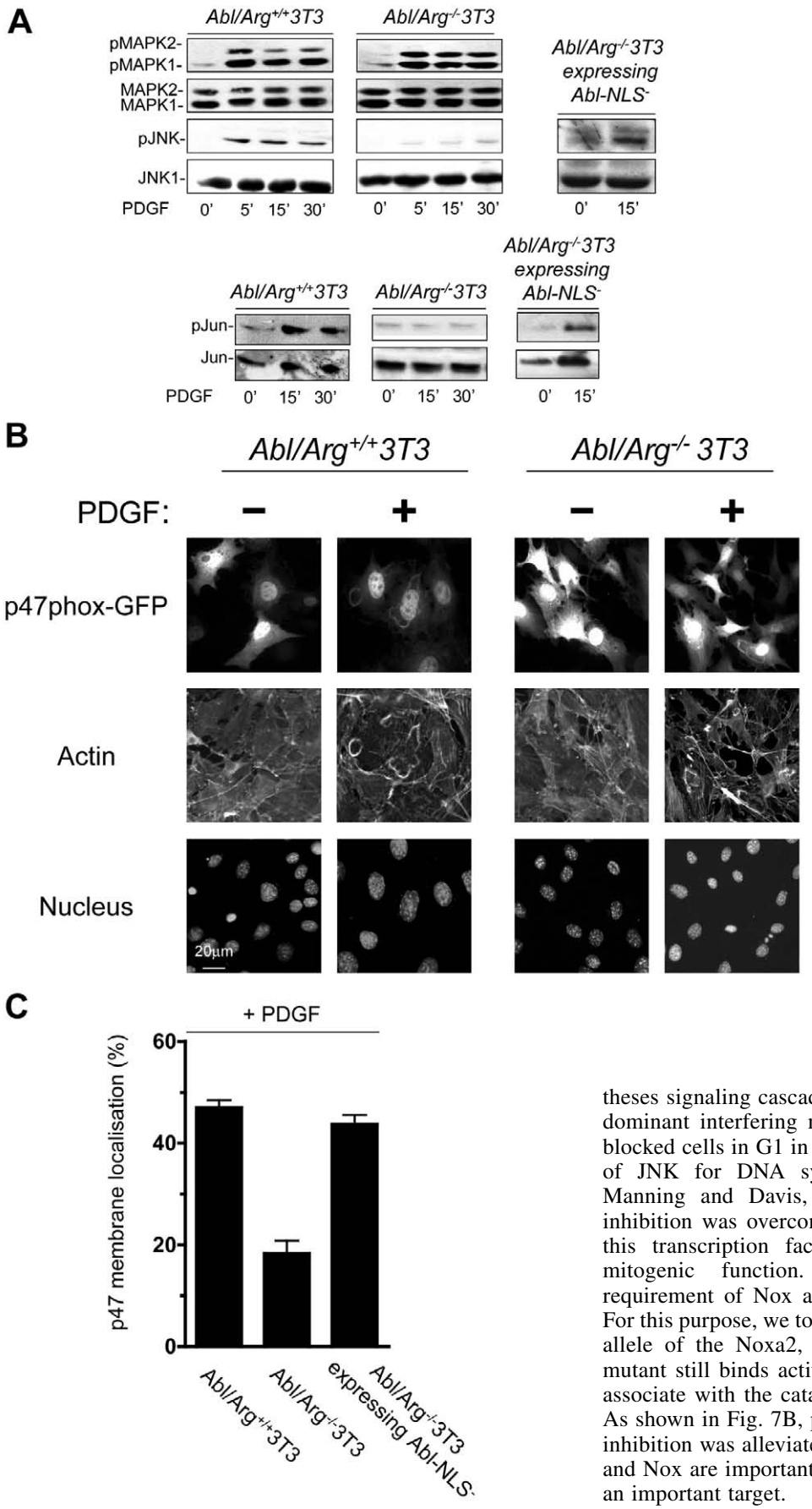

Fig. 6. Abl regulates PDGF-induced JNK and Nox activation. (A) Abl regulates PDGF-induced JNK activation. (Top panels) The level of active MAPK (pMAPK) and JNK (pJNK) from cells stimulated with PDGF (0-30 minutes). The level of MAPKs and JNK1 is also shown. (Bottom panels) The level of Jun and pS63-Jun (pJun) from indicated cell-lysates. (B,C) Abl regulates PDGF-induced Noxo2 (p47phox) membrane translocation. (B) Immunofluorescence localization of p47phox-GFP, actin structure and nuclei from indicated cells stimulated or not with PDGF as shown. (C) Statistic analysis of p47 membrane recruitment upon PDGF stimulation. Indicated quiescent cells transfected with p47phox-GFP were stimulated with PDGF for 5 minutes and the percentage of cells with p47 membrane localization was counted. The mean±s.d. (n=5) of the percentage of cells that exhibited p47-GFP at the membrane is shown.

theses signaling cascades on mitogenesis. We found that the dominant interfering mutant DN-MKK4 (Wu et al., 2003) blocked cells in G1 in agreement with an important function of JNK for DNA synthesis (Weston and Davis, 2002; Manning and Davis, 2003) (Fig. 7C). Importantly, this inhibition was overcome by Myc co-expression confirming this transcription factor as an important target for its mitogenic function. Similarly, we investigated the requirement of Nox activity during mitogenesis (Fig. 7D). For this purpose, we took advantage of the dominant negative allele of the Noxa2, p67V204A (Han et al., 1998). This mutant still binds active Rac but it has lost the capacity to associate with the catalytic subunit (Nisimoto et al., 1997). As shown in Fig. 7B, p67V204 induced a G1 block and this inhibition was alleviated by Myc expression. Therefore JNK and Nox are important regulators of mitogenesis and *Myc* is an important target.

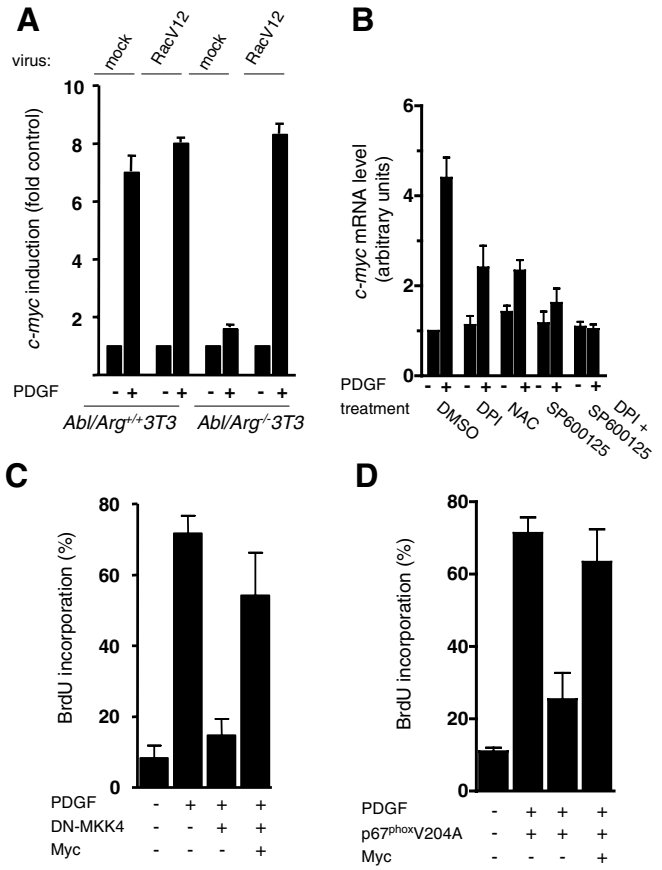

Fig. 7. JNK and Nox signal to *Myc* for the induction of DNA synthesis. (A) RacV12 rescues the *Myc* induction defect in Abl/Arg$^{-/-}$ cells. Indicated quiescent cells infected with wild-type (mock) or indicated retroviruses were stimulated or not with PDGF for 1 hour. The level of *Myc* mRNA was quantified by real-time quantitative RT-PCR as described in Materials and Methods. The ratio of *Myc* mRNA in stimulated and non-stimulated cells is shown (*Myc* induction). (B) *Myc* induction is sensitive to inhibitors of JNK and Nox activities. Quiescent NIH3T3 treated with DMSO (control) or indicated inhibitors (10 μM or 10 mM for NAC) were stimulated or not for 1 hour with PDGF. *Myc* mRNA was assessed by northern blotting as described in Materials and Methods. % Myc mRNA level=[Myc mRNA level]/[Myc RNA level of control quiescent cells]. Myc rescues the G1 block induced by DN-MKK4 (C) and p67V204A (D). Quiescent NIH3T3 transfected with indicated constructs were stimulated or not with PDGF in the presence of BrdU and proceeded as in Fig. 1B. The mean±s.d. of the percentage of BrdU-positive cells present in expressing and non-expressing cells under the specified conditions is shown.

Discussion

Rac has been implicated in mitogenesis induced by v-Abl (Renshaw et al., 1996) and we now show that it also plays a role in Abl mitogenic signaling as induced by growth factors. The mechanism by which Abl regulates PDGF-induced Rac activation is currently unknown. One obvious mechanism involves phosphorylation and/or activation of a GEF as suggested for Trio, Vav and SOS1 (Liebl et al., 2000; Bassermann et al., 2002; Sini et al., 2004). Alternatively it may also inhibit a RhoGDI or a GAP. In addition to mitogenesis, Rac has been largely implicated in PDGF-induced cytoskeletal rearrangement including dorsal ruffles (Burridge and Wennerberg, 2004). We and others have shown that this process is also regulated by cytoplasmic Abl (Furstoss et al., 2002a; Plattner et al., 1999; Sini et al., 2004). Therefore Rac may also be a target of Abl for dorsal ruffle formation. How Abl regulates Rac for both mitogenic and morphological responses is not known. One hypothesis implicates a single pool of Rac. However, several observations suggest the existence of a specific Rac signaling cascade for DNA synthesis: several active RacL61 alleles used in this study induced morphological changes while they were inefficient in rescuing the Abl mitogenic defect. Similarly, constitutively active alleles of the Rac GEF Vav2 and Trio poorly rescued the G1 block induced by Abl-PP-K^{-} while strongly inducing lamelipodia formation (not shown). Finally, this report also established a link between mitogenic Abl and Rac in cells stimulated with serum, which does not induce Rac-dependent cytoskeletal rearrangement. The identification of Rac regulators involved in these morphological and mitogenic responses will be highly informative in this regard.

Our report identified JNK as a downstream effector of the Abl mitogenic pathway. Indeed various elements of the JNK cascade, including MKK4, JNK-1 and Jun, play important functions for the promotion of cell growth (Weston and Davis, 2002). While JNK has been originally described as a mediator of a stress response (Weston and Davis, 2002), we believe that a modest activation is used by growth factors for DNA synthesis. Previous reports argued against an important role for JNK in Rac transforming activity (Joneson et al., 1996; Lamarche et al., 1996). These data were obtained by expressing a high level of RacL61 so that DNA synthesis was observed in the absence of any growth factors. Therefore JNK requirement may be bypassed by a compensatory signaling or the low JNK activity induced by those Rac alleles was sufficient to ensure the cell response. More recently, the group of Der implicated JNK for mitogenic signaling including cyclin D expression (Westwick et al., 1997). This is consistent with our data implicating JNK in the Abl-Rac signaling cascade initiated by growth factors. Therefore JNK may be required for Rac signaling at least during mitogenesis. Our report also suggests that *Myc* is an important target of JNK in this pathway. *Myc* induction primarily involves mRNA stabilization during early stimulation of growth factors (Blanchard et al., 1985). This raises the idea that JNK may impact on *Myc* messenger stability. Additionally, JNK may also affect the rate *Myc* transcription. This notion would be consistent with a role of JNK in phosphorylation and activation of the AP-1 complex Jun:JunD for expression of this gene (Iavarone et al., 2003). Therefore Abl may also impact on *Myc* through activation of an AP-1 complex.

Nox is the other effector identified in the Abl-Rac mitogenic signaling. Accordingly, reactive oxygen species (ROS) are important transducers of the proliferative signals induced by growth factors (Lambeth, 2004) or by active Rac (Joneson and Bar-Sagi, 1998). Although the Nox involved in fibroblast mitogenic signaling has not been identified, it may probably involve Nox1. Indeed, Nox1 is expressed in fibroblasts and it has been implicated in cell growth promotion induced by growth factors (Kwon et al., 2004) and oncoproteins (Mitsushita et al., 2004). Furthermore, Nox1-3 can be activated

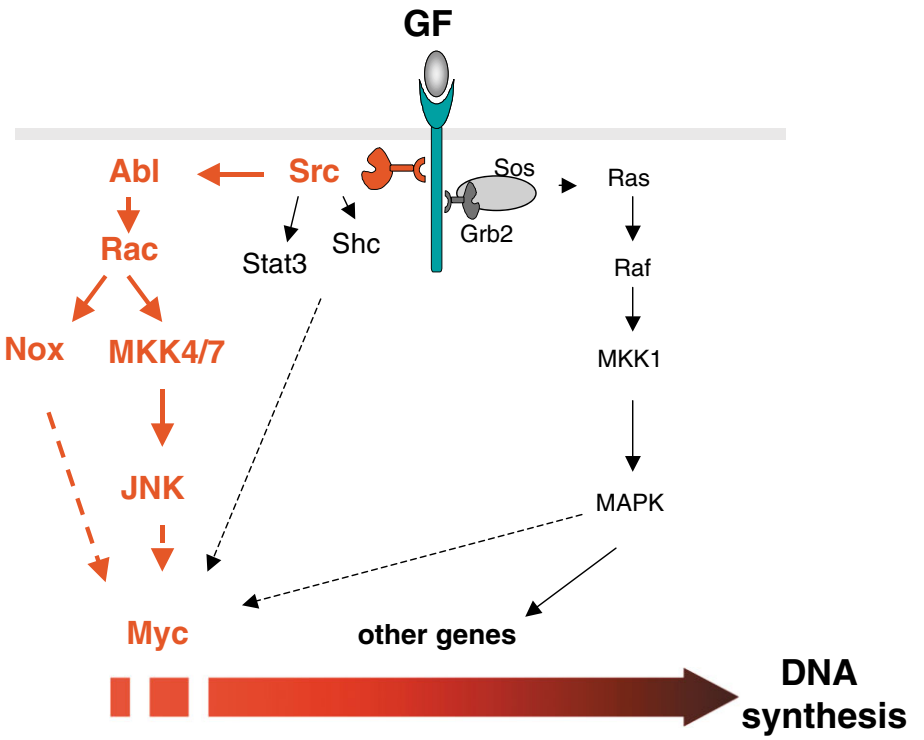

Fig. 8. A model of how Abl mediates mitogenesis in response to growth factors. Growth factor (GF)-induced SFK activation allows phosphorylation of Abl in the cytoplasm for increased catalytic activity. Abl then operates on a Rac/JNK and Rac/Nox pathway for Myc expression, a transcription factor required for induction of DNA synthesis. JNK and Nox may participate in *Myc* induction through mRNA stabilization and/or gene transcription. Note that Abl substrates for Rac activation are currently unknown. SFK-induced *Myc* implicates additional substrates, including Stat3 and Shc. The Ras signaling cascade may participate in mitogenesis through Myc protein stabilization and expression of other genes.

by Noxo2 and Noxa2 subunits (Banfi et al., 2003; Cheng et al., 2004). Whatever the nature of the NADPH oxidase involved in this pathway, our rescue experiments indicate that *Myc* is an important effector of ROS mitogenic function. The molecular mechanism by which Nox impacts on *Myc* is, however, not known. It is generally assumed that ROS reversibly inactivates phosphatases for sustained signaling. For example ROS generated by Rac inhibits low molecular weight protein tyrosine phosphatase for sustained p190RhoGAP phosphorylation and Rho downregulation (Nimnual et al., 2003). Similarly, overexpressed Nox1 also inactivates the lipid phosphatase PTEN, allowing growth factors to induce robust PI3K and Akt activation (Kwon et al., 2004). Therefore one may surmise that ROS generated upon growth factor stimulation inactivates a specific phosphatase for *Myc* expression. Finally, the partial rescue (70%) obtained with a constitutively active Rac suggest that Abl may also signal outside Rac. Therefore additional Abl mitogenic effectors must be expected.

The work of several labs including ours indicates that, in addition to a Ras/MAP kinase pathway, growth factors initiate a tyrosine kinase signaling cascade (the receptor itself >Src>Abl), which is also required for DNA synthesis (Fig. 8). *Myc* induction turned out to be an important downstream event of this signaling pathway (Browman et al., 2004). Our report now indicates that Abl operates on a Rac/JNK and Rac/Nox pathway for Myc expression and mitogenesis. *Myc* is also required for cell transformation induced by various oncogenes. This raises the possibility that Abl plays a role in cell-transformation induced by tyrosine kinases. Therefore, unraveling the impact of Abl in cell transformation may give important insights into contribution of this signaling pathway in cancer cell growth.

We thank A. Atfi, I. Behourmann, C. Gauthier-Rouviere, J. Guiramand, R. Hipskind, T. Hirano, M. Pucéat, J. Raingeaut, P. Roux and B. Willi for reagents used in this study. We thank C. Bénistant and colleagues for discussions, sharing unpublished data and critically reading the manuscript. This work was supported by the AICR, ARC, CNRS and INSERM. A.B. was supported by the AICR, O.F. by the ARC and FRM, and S.R. by INSERM.

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