FEBS Letters 526 (2002) 82–86 FEBS 26378

Cyclin E and cyclin A are likely targets of Src for PDGF-induced DNA synthesis in fibroblasts

Olivia Furstoss[1], Gaël Manes[1], Serge Roche*

CRBM, CNRS UPR-1086, 1919 route de Mende, 34293 Montpellier, France

Received 11 July 2002; revised 22 July 2002; accepted 22 July 2002

First published online 30 July 2002

Edited by Giulio Superti-Furga

Abstract How tyrosine kinases of the Src family regulate platelet-derived growth factor (PDGF)-induced DNA synthesis remains elusive. Here we show that the E1A antigen of adenovirus 5 overrides the G1 block elicited by the kinase-inactive mutant SrcK$^-$. This was dependent upon the CR2 region of E1A that upregulated cyclin E and cyclin A and inactivated the pocket protein pRb. E1A rescue was independent of pRb. Expression of SrcK$^-$ in fibroblasts prevented PDGF-induced expression of cyclins E and A. This effect was overcome by E1A. Constitutive expression of cyclins E and A, but not D1, restored mitogenesis that was inhibited by SrcK$^-$. We conclude that both cyclin E and cyclin A are likely targets of Src mediating PDGF-induced DNA synthesis.

Key words: Src; PDGF; E1A; Cyclin A; Cyclin E; DNA synthesis

1. Introduction

The tyrosine kinases of the Src family are transiently activated by a number of growth factors and play important roles in DNA synthesis [1]. Inhibition of their function in vivo, either by microinjection of a neutralising antibody common to Src, Fyn and Yes, by overexpression of kinase-inactive form of the enzymes (SrcK$^-$, FynK$^-$), or by treatment of cells with a specific inhibitor, prevented induction of DNA synthesis [2–5]. Little is known about the components of the signalling cascade initiated by Src although specific substrates have been recently identified. These include the adapter Shc, the transcription factor STAT3 and the tyrosine kinase Abl [5–7]. Furthermore, several reports indicated that expression of c-*myc* is an important downstream target of Src [1].

Growth factors turn on signalling cascades that induce cell cycle progression through the restriction point at late G1, where cells are committed to S phase entry. One critical event is the phosphorylation and inactivation of the pocket protein pRb by cyclin-dependent kinases (cdk). Cdks are regulated by various mechanisms including binding of cyclins (D for cdk4/6 and E and A for cdk2) and association of small inhibitors. pRb inactivation is, however, not sufficient for S phase entry and additional events including those regulated by cdk2 are also required [8]. The signalling molecules activated by platelet-derived growth factor (PDGF) and responsible for late G1 progression are not known. Interestingly, microinjection of blocking antibody into cycling cells indicated that Src kinases were required for at least 8–10 h of mitogen stimulation [2,3]. Recently, we [7] and others [9] have shown that some viral antigens, which are known to perturb the cell cycle machinery, bypass the need of Src kinases for mitogenesis. This included the large T antigen of SV40 and the E1B protein of human adenovirus. Furthermore, this approach uncovered the tumour suppressor p53 as a critical target of Src. As a consequence, Src kinases are no longer required for mitogenic signalling in cells with inactivated p53. This accounts for the lack of requirement for Src kinases in *src*−/−, *fyn*−/−, *yes*−/− embryonic fibroblasts that were previously immortalised with the SV40 large T antigen [10]. Here we report that the E1A antigen of human adenovirus 5 can also bypass the requirement of Src kinases for mitogenesis. In addition, the use of viral proteins deficient in interacting with specific cell cycle regulators allowed the identification of cyclin E and cyclin A as putative effectors of Src during late G1.

2. Materials and methods

2.1. Antibodies and DNA constructs

Cst1 antibody was described previously [3] and affinity-purified and concentrated as in [3]. E1A monoclonal antibody was a gift of Dr Galas (CRBM, Montpellier, France). Cyclin D1, D2, E and A antibodies were from Santa Cruz and Sigma respectively and anti-FLAG from Sigma. pSGT constructs expressing SrcK$^-$ and Src-like adapter protein (Slap) have been described in [3,11], pcDNA3 constructs expressing 12S and 13S isoforms of E1A were from Dr Sardet (IGM, Montpellier, France), E1A mutants [12] from Dr Amati (ISREC, Epalinges, Switzerland), p16ink4a, p27Kip1 and pPSM.7LP [13] from Dr Blanchard (IGM, Montpellier, France), and human cyclin D1, E1 and A from Drs Baldin (CNRS, Toulouse, France), Sardet (IGM, Montpellier, France) and Superti-Furga (EMBL, Heidelberg, Germany) respectively. Vector expressing SrcK$^-$/Y527F was described in [14] and was subcloned in pBabe.

2.2. Cell culture and microinjection

Rb$^{+/+}$ and *Rb*$^{-/-}$ mouse embryo fibroblast (MEF) cells were provided by Dr Blanchard (IGM, Montpellier, France). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with glutamine and antibiotics (penicillin and streptomycin) at 37°C in a humidified 5% CO_2 atmosphere. Cells were microinjected as in [3]. The medium was replaced with DMEM containing 0.5% serum for at least 30 h for NIH3T3 and *Rb*$^{+/+}$ MEF and 3 days in the absence of serum for *Rb*$^{-/-}$ MEFs. DNA plasmids were injected into the nucleus 4 h prior to PDGF (20 ng/ml) stimulation and further incubated or not for 6, 14 and 18 h in the case of cyclin D1 expression, cyclin A expression and DNA synthesis respectively, before fixation. DNA syn-

*Corresponding author. Fax: (33)-4-67 52 15 59.
E-mail address: roche@crbm.cnrs-mop.fr (S. Roche).

[1] These authors have contributed equally to this work.

PII: S0014-5793(02)03120-4

thesis was scored by performing bromodeoxyuridine (BrdU) incorporation assays as described in [3].

2.3. Retroviral infection and biochemistry

Retroviral infection was performed as in [15] with some modifications. 100-mm-diameter culture dishes of BOSC cells were transfected overnight with the retroviral pBabe-puro plasmid expressing SrcK$^-$/Y527F. The next day, transfection medium was replaced with 5 ml of fresh medium. Viral particles were then harvested 24, 48 and 72 h later by filtering supernatants of the cells, and stored at −20°C until used for infection. NIH3T3 cells seeded into 100-mm-diameter culture dishes were then infected with two additions of 5 ml of virus-containing supernatant at a 12-h interval in the presence of 10 μg/ml Polybrene (Sigma). Infected cells were selected 48 h after the beginning of the infection with 7 μg/ml puromycin and resistant clones were pooled. Cells were next plated onto plastic 60-mm-diameter culture dishes, starved in 0.5% serum for 40 h, then stimulated with 20 ng/ml of PDGF BB 0–16 h as indicated and lysed into RIPA buffer as described in [7]. Western blotting from the whole cell lysate was performed as in [7].

2.4. Immunofluorescence

Immunofluorescence was performed as described in [3]. To analyse DNA synthesis, cells were incubated for 10 min with 1.5 M HCl, washed three times with phosphate-buffered saline (PBS) and stained with monoclonal (1:50) anti-BrdU antibody (Pharmingen). Cyclin D1, A and E1A proteins were visualised by incubation with specific antibodies. Coverslips were finally stained with rhodamine-conjugated anti-mouse antibody (ICN), washed in PBS containing 10 μM Hoechst 33258 (final concentration 1 mg/ml; Sigma), rinsed in water, inverted and mounted in Moviol (Hoechst) on glass slides. Slides were viewed with an Axiophot fluorescence microscope.

The percentage of injected cells that incorporated BrdU and that synthesised cyclin A, for each coverslip, was calculated by the following formula: % of BrdU-positive cells = (number of BrdU-positive injected cells/number of injected cells) × 100, % of cyclin A-positive cells = (number of cyclin A-positive injected cells/number of injected cells) × 100.

3. Results

3.1. E1A overcomes the G1 block induced by SrcK$^-$

Quiescent NIH3T3 fibroblasts seeded onto glass coverslips were microinjected with a plasmid-encoding kinase-inactive SrcK$^-$ alone or together with a vector expressing the 12S E1A antigen of human adenovirus 5. Cells were then stimulated with PDGF for 18 h. DNA synthesis was visualised by adding BrdU in the medium that incorporates into the nucleus during the S phase of the cell cycle. An example of such an experiment is shown in Fig. 1A and statistical analysis is summarised in Fig. 1B: as previously reported, SrcK$^-$ strongly inhibited the mitogenic response induced by PDGF and inhibition was relieved by E1A coexpression. Similar results were obtained with the 13S E1A protein (not shown). This mitogenic rescue was still growth factor-dependent, as in our conditions E1A per se did not significantly induce DNA synthesis. Similar results were obtained in primary cultures of MEFs (see Fig. 2) indicating that this effect was not specific to the cell line used. The functional interaction between Src and E1A was reinforced by rescuing the PDGF receptor signalling in cells expressing Slap, which specifically inhibits the Src mitogenic function [11,16] (Fig. 1B).

3.2. CR2 domain of E1A is required to bypass the SrcK$^-$ G1 block

The mechanism by which E1A bypassed the Src requirement was next investigated with the use of E1A mutants. These mutants have been previously characterised in their ability to interact with various cell cycle regulators [17]. These included E1A 2R→G defective in binding the transcription coactivators p300/CBP, E1A Δ26–35 defective in binding the inhibitor p21CIP1/Waf1 of cdk2 plus the transcription coactivator p400, E1A 124,135A mutated in the CR2 region, defective in targeting pRb and upregulating cyclins E and A

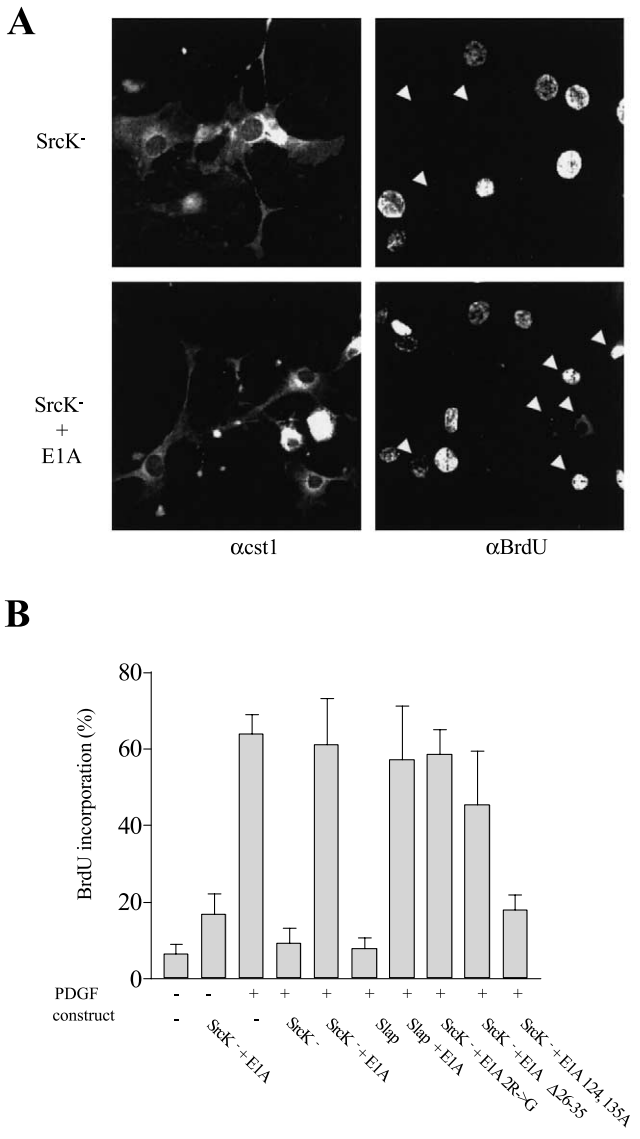

Fig. 1. Microinjection of E1A in NIH3T3 cells overcomes the G1 block induced by SrcK$^-$ and Slap. A: E1A overrides the SrcK$^-$ G1 block. NIH3T3 cells seeded onto coverslips were microinjected with SrcK$^-$-encoding construct (50 ng/ml), in the presence or the absence of E1A construct (50 ng/ml) as indicated, and stimulated or not with PDGF. Cells were fixed and processed for immunofluorescence as described in Section 2. Left panels show immunostaining of microinjected cells expressing SrcK$^-$ (αcst1); right panels show immunostaining of cells that have incorporated BrdU as indicated (αBrdU) B: E1A overrides the inhibitory effect induced by SrcK$^-$ and Slap. Cells were injected with indicated constructs (50 ng/ml), and stimulated or not with PDGF as indicated. Cells were processed for immunofluorescence as described in Section 2. The percentage of injected cells that incorporated BrdU for each coverslip was calculated by the following formula: % of BrdU-positive cells = (number of BrdU-positive injected cells/number of injected cells) × 100. The mean of several independent experiments and standard deviation are shown.

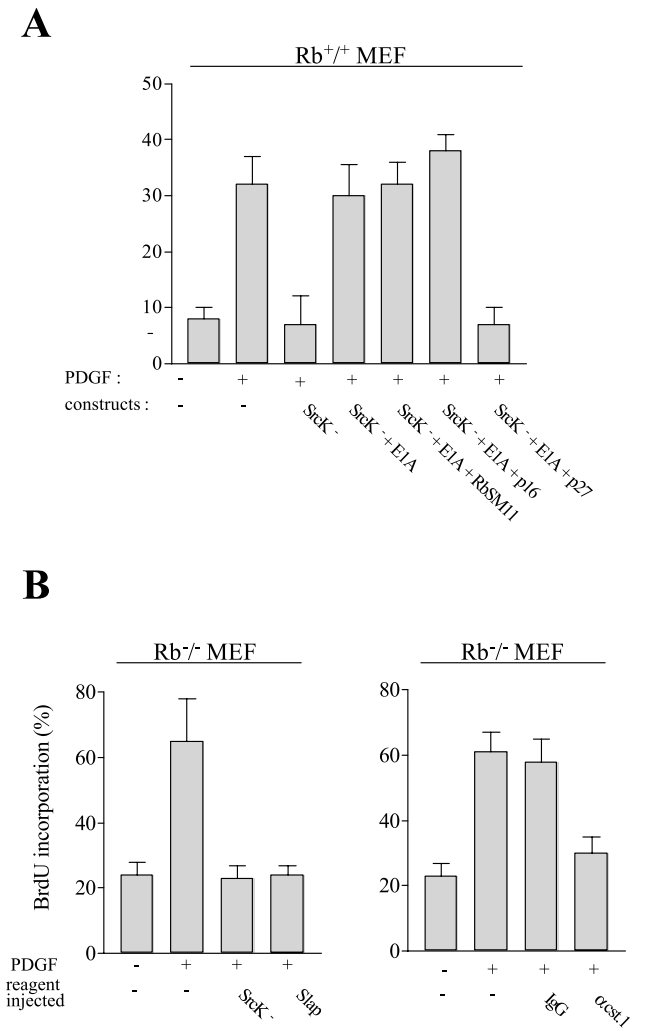

Fig. 2. Inactivation of pRb is not sufficient to override the requirement of Src kinases for mitogenesis. A: Inactivation of pRb is not sufficient to override the G1 block induced by SrcK$^-$. *Rb*$^{+/+}$ MEFs seeded onto coverslips were microinjected with SrcK$^-$ (50 ng/ml) in the presence or not of E1A (50 ng/ml) constructs plus indicated constructs (100 ng/ml) and stimulated or not with PDGF. B. Src kinases are still required for PDGF mitogenesis in pRb-deficient cells. *Rb*$^{-/-}$ MEFs seeded onto coverslips were microinjected with SrcK$^-$- or Slap-encoding constructs (left panel), non-immune (IgG) or cst1-neutralising antibody (right panel) as indicated and stimulated or not with PDGF. Cells were fixed and processed for immunofluorescence as described in Section 2. The percentage of injected cells that incorporated BrdU for each coverslip was scored as in Fig. 1. The mean of several independent experiments and standard deviation are shown.

[12]. While the first two mutants showed some rescue effect, E1A 124,135A could not reverse the G1 block induced by SrcK$^-$ (Fig. 1B)

3.3. Inactivation of pRb is not sufficient to override the SrcK- G1 block

We next tested whether E1A acts by inactivating pRb. The rescue effect was analysed in the presence of a constitutively active form of pRb (pPSM.7LP) which cannot be inhibited by phosphorylation and the cdk4/6 inhibitor p16ink4a which prevents endogenous pRb inactivation [13]. As shown in Fig. 2A, E1A overrode the G1 block induced by SrcK$^-$ in MEF cells. However, signalling rescue was still observed when coexpressing p16ink4a or PSM.7LP. In contrast, E1A function was abolished by constitutive expression of the cdk2 inhibitor p27Kip1. Similar results were obtained in NIH3T3 (not shown) indicating that this effect was not specific to our primary culture of MEFs. We concluded that the signalling rescue observed with E1A does not involve pRb downregulation but rather the stimulation of cdk2 activity. This also suggests that besides the pRb pathway, Src may regulate important cell cycle events for S phase entry. Indeed, we observed that PDGF mitogenic response still required the Src pathway in cells that do not express pRb: inactivation of the Src signalling cascade either by SrcK$^-$ or Slap constructs, or by injection of the Src-neutralising antibody cst1, all inhibited mitogenesis in *Rb*$^{-/-}$cells (Fig. 2B).

3.4. Src kinases regulate cyclin E and cyclin A expression in cells stimulated by PDGF

We next tested the possibility that E1A overrides the SrcK$^-$ G1 block by inducing constitutive expression of cyclins. For this purpose, we first investigated whether Src regulates cyclins expression. As shown in Fig. 3A, PDGF induced synthesis of cyclin A, as assessed by immunofluorescence, which was not observed in SrcK$^-$-microinjected cells. In contrast, cyclin D1 was detected in both injected and non-injected cells, showing specificity of inhibition. This also indicated that Src kinases might not be mandatory for expression of all cyclins such as cyclin D1. Statistical analysis of these experiments indicated that cyclin A was detected in less than 5% of quiescent cells, whereas up to 40% of cells had synthesised cyclin A after PDGF stimulation. This response was abrogated by SrcK$^-$ (2%) but rescued by E1A coinjection (45%). The effect of SrcK$^-$ on cyclin E level could not be investigated by this approach due to the absence of appropriate reagent.

The incidence of Src kinases on cyclins was next analysed biochemically. The kinase-inactive SrcK$^-$/Y527F was expressed in NIH3T3 cells using a retroviral infection approach [15]. This allows a high efficiency (90–100%) of ectopic protein expression with a five-fold overexpression relative to endogenous Src kinases (as assessed by Western blotting with the cst1 antibody that recognises Src, Fyn and Yes). Infected cells were then made quiescent by withdrawing serum from the medium, and further stimulated with PDGF for various time periods. As shown in Fig. 3B, PDGF induced a strong increase in cyclin A and cyclin E levels in mock-infected cells. In cells expressing SrcK$^-$/Y527F, both responses were largely reduced (60 and 80% respectively) confirming our immunofluorescence analysis. Cyclin D1 and D2 levels were also increased during PDGF stimulation in control cells. While the cyclin D2 response was attenuated to some extent by SrcK$^-$/Y527F, no significant inhibition of cyclin D1 expression was observed.

3.5. Cyclin E and cyclin A override the SrcK$^-$ G1 block

Finally, the functional consequence of cyclin inhibition was analysed by performing microinjection rescue experiments. As shown in Fig. 4, constitutive expression of cyclin A largely restored PDGF receptor signalling in cells injected with SrcK$^-$. Observed DNA synthesis was dependent on the presence of growth factors as none of the cyclins induced any response per se. A similar effect was obtained with cyclin E. Rescues were specific to this type of cyclins as it was not observed with cyclin D1. We conclude that both cyclin E

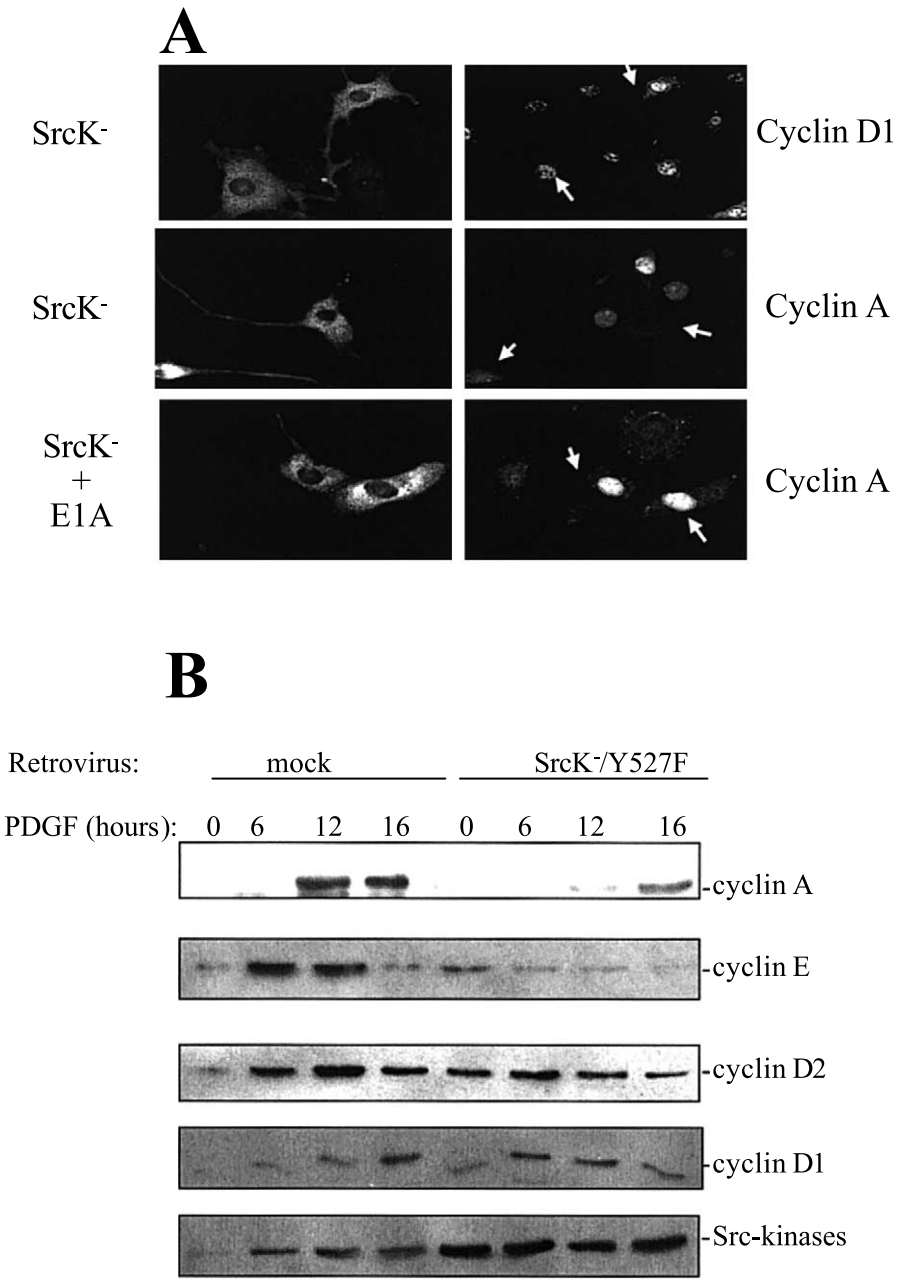

Fig. 3. Src kinases regulate PDGF-induced cyclin E and A expression. A: NIH3T3 cells seeded onto coverslips were microinjected with SrcK$^-$-encoding constructs, in the presence or the absence E1A as indicated, and stimulated or not with PDGF. Cells were fixed and processed for immunofluorescence as described in Section 2. Left panel shows microinjected cells expressing SrcK$^-$, right panel shows immunostaining of cyclin D1 or cyclin A as shown. B: NIH3T3 cells infected with wild type retroviruses (mock) or retroviruses expressing SrcK$^-$/Y527F as indicated were made quiescent and stimulated for the indicated hours with PDGF. Cell lysates were made as described in Section 2 and the level of Src kinases (Src, Fyn and Yes) and the cyclins D1, D2, E and A was assessed by Western blotting using specific antibody as indicated in Section 2.

and cyclin A are putative downstream effectors of the Src pathway during PDGF receptor signalling.

4. Discussion

Here we show that adenovirus antigen E1A bypasses the requirement of Src kinases for growth factor receptor signalling in fibroblasts. In addition our data show that cyclins E and A are likely targets of the Src pathway in late G1. This notion is in agreement with previous reports indicating that Src regulates induction of cyclin A by cell adhesion [18] or by expression of the viral protein HBx [19], or that cyclins E and A are upregulated during vSrc-induced cell transformation [20]. Src-induced cyclin A expression may involve pRb phosphorylation [21,22], as suggested by the partial requirement of Src for cyclin D expression. Additionally, Src may use an alternative mechanism that is independent of pRb and that implicates cyclin E [13,23]. How Src mediates cyclin E expression is not known. However, Myc has been involved in the expression of cyclin E [24] and several reports including ours

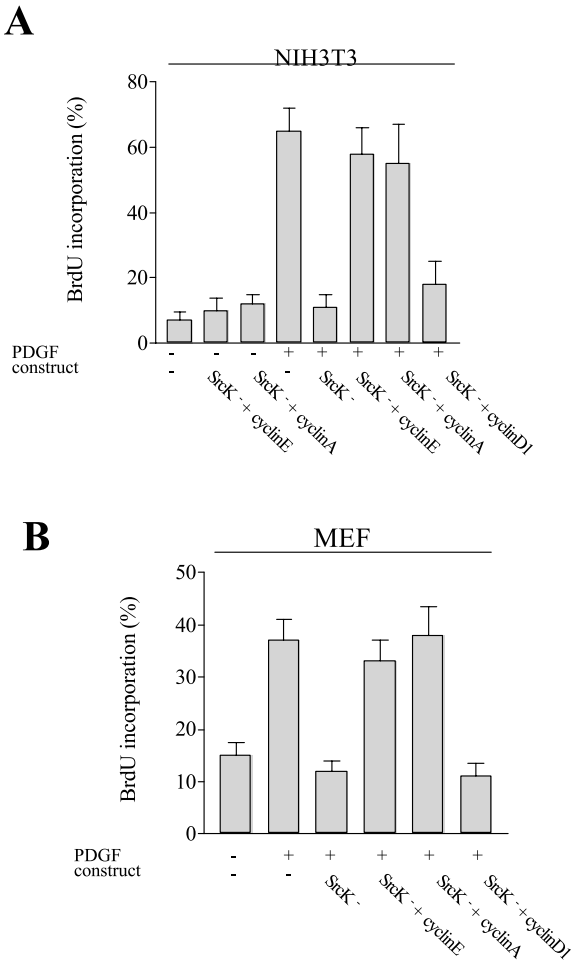

Fig. 4. Constitutive expression of cyclin A or cyclin E overrides the G1 block elicited by SrcK$^-$. NIH3T3 cells (A) or primary culture of MEFs (B) seeded onto coverslips were microinjected with SrcK$^-$-encoding construct (50 ng/ml), in the presence or the absence of constructs expressing cyclin A, E or D1 as indicated (100 ng/ml), and stimulated or not with PDGF. Cells were fixed and processed for immunofluorescence as described in Section 2. The percentage of injected cells that incorporated BrdU for each coverslip was scored as in Fig. 1. The mean of several independent experiments and standard deviation are shown.

have shown that Myc is an important target of the Src pathway [5,7,25]. Therefore one mechanism would be that Src kinases induce cyclin expression through Myc.

Finally our data strongly suggest that Src and Ras lie in separate pathways for PDGF-induced cell cycle progression at late G1. pRb is the main target of Ras as it is no longer required for mitogenesis in *Rb*$^{-/-}$ cells [26]. In contrast our data clearly show that Src also regulates cell cycle regulatory proteins independent of pRb, which are important for DNA synthesis and targeted by E1A. The best illustration is the requirement of Src for mitogenesis observed in pRb-deficient cells. In line with this, we also observed that ectopic expression of cyclin D1 does not overcome the G1 block induced by SrcK$^-$ (Fig. 4) while it does when induced by RasN17 (not shown). One of these important Src targets probably includes cyclin E. This notion is supported by the capacity of ectopic cyclin E to rescue mitogenesis that was inhibited by SrcK$^-$. However, the fact that the E1A rescue effect was blocked by the cdk2 inhibitor p27Kip1 suggests that Src may target additional regulators of cdk2. The identification of such regulators that drive cells in late G1 needs further investigation.

Acknowledgements: We thank Drs Amati, Baldin, Blanchard, Gallas, Roux, Sardet and Superti-Furga for providing reagents used in this study and our colleagues for discussion and critical reading of the manuscript. G.M. and O.F. were supported by the Fondation pour la Recherche Médicale, and S.R. by INSERM. This work was supported by the CNRS and the French charity ARC.

References

[1] Abram, C.L. and Courtneidge, S.A. (2000) Exp. Cell Res. 254, 1–13.
[2] Twamley-Stein, G.M., Pepperkok, R., Ansorge, W. and Courtneidge, S.A. (1993) Proc. Natl. Acad. Sci. USA 90, 7696–7700.
[3] Roche, S., Koegl, M., Barone, M.V., Roussel, M.F. and Courtneidge, S.A. (1995) Mol. Cell. Biol. 15, 1102–1109.
[4] Broome, M.A. and Hunter, T. (1996) J. Biol. Chem. 271, 16798–16806.
[5] Blake, R.A., Broome, M.A., Liu, X., Wu, J., Gishizky, M., Sun, L. and Courtneidge, S.A. (2000) Mol. Cell. Biol. 20, 9018–9027.
[6] Bowman, T. et al. (2001) Proc. Natl. Acad. Sci. USA 98, 7319–7324.
[7] Furstoss, O., Dorey, K., Simon, V., Barilla, D., Superti-Furga, G. and Roche, S. (2002) EMBO J. 27, 514–524.
[8] Sherr, C.J. and Roberts, J.M. (1999) Genes Dev. 13, 1501–1512.
[9] Broome, M.A. and Courtneidge, S.A. (2000) Oncogene 19, 2867–2869.
[10] Klinghoffer, R.A., Sachsenmaier, C., Cooper, J.A. and Soriano, P. (1999) EMBO J. 18, 2459–2471.
[11] Roche, S., Alonso, G., Kazlauskas, A., Dixit, V.M., Courtneidge, S.A. and Pandey, A. (1998) Curr. Biol. 8, 975–978.
[12] Alevizopoulos, K., Catarin, B., Vlach, J. and Amati, B. (1998) EMBO J. 17, 5987–5997.
[13] Knudsen, K.E., Fribourg, A.F., Strobeck, M.W., Blanchard, J.M. and Knudsen, E.S. (1999) J. Biol. Chem. 274, 27632–27641.
[14] Lara-Pezzi, E., Roche, S., Andrisani, O.M., Sanchez-Madrid, F. and Lopez-Cabrera, M. (2001) Oncogene 20, 3323–3331.
[15] Inoue, K., Roussel, M.F. and Sherr, C.J. (1999) Proc. Natl. Acad. Sci. USA 96, 3993–3998.
[16] Manes, G., Bello, P. and Roche, S. (2000) Mol. Cell. Biol. 20, 3396–3406.
[17] Frisch, S.M. and Mymryk, J.S. (2002) Nat. Rev. Mol. Cell. Biol. 3, 441–452.
[18] Guadagno, T.M., Ohtsubo, M., Roberts, J.M. and Assoian, R.K. (1993) Science 262, 1572–1575.
[19] Bouchard, M., Giannakopoulos, S., Wang, E.H., Tanese, N. and Schneider, R.J. (2001) J. Virol. 75, 4247–4257.
[20] Riley, D., Carragher, N.O., Frame, M.C. and Wyke, J.A. (2001) Oncogene 20, 5941–5950.
[21] Philips, A., Huet, X., Plet, A., Le Cam, L., Vie, A. and Blanchard, J.M. (1998) Oncogene 16, 1373–1381.
[22] Hurford Jr., R.K., Cobrinik, D., Lee, M.H. and Dyson, N. (1997) Genes Dev. 11, 1447–1463.
[23] Alevizopoulos, K., Vlach, J., Hennecke, S. and Amati, B. (1997) EMBO J. 16, 5322–5333.
[24] Amati, B., Alevizopoulos, K. and Vlach, J. (1998) Front. Biosci. 3, D250–268.
[25] Barone, M.V. and Courtneidge, S.A. (1995) Nature 378, 509–512.
[26] Leone, G., DeGregori, J., Sears, R., Jakoi, L. and Nevins, J.R. (1997) Nature 387, 422–426.