ELSEVIER

FEBS Letters 342 (1994) 159–164

FEBS 13852

FEBS LETTERS

Evidence for Rap1 in vascular smooth muscle cells

Regulation of their expression by platelet-derived growth factor BB

R. Quarck[a,*], M. Bryckaert[a], C. Magnier[a], E. Corvazier[a], R. Bredoux[a], J. de Gunzburg[b], M. Fontenay[a], G. Tobelem[a], J. Enouf[a]

[a]*INSERM U. 348, Hôpital Lariboisière, 6 rue Guy Patin, 75010 Paris, France*
[b]*INSERM U. 248, Faculté de Médecine Lariboisière-Saint Louis, 10 avenue de Verdun, 75010 Paris, France*

Received 25 February 1994

Abstract

The effect of platelet-derived growth factor (PDGF) on Rap1 expression was investigated in rat vascular smooth muscle cells (SMC). First, evidence for Rap1 proteins was shown by their: (i) detection in membranes using a specific anti-Rap1 antibody, (ii) typical shift in electrophoretic mobility as a consequence of reduction, and (iii) cAMP-induced phosphorylation and immunoprecipitation. Then, the mitogenic activity of 10 ng/ml PDGF AA and BB for 48 h, resulting in a 2- and 5-fold increase in [^{3}H]thymidine incorporation, was correlated with that of total Rap1 protein expression which was found to be 99% ± 36% and 260% ± 70%, respectively. Further time-course studies established that this up-regulation of Rap1 proteins was only observed after 48 h of PDGF BB treatment. Lastly, comparative RT-PCR of both *rap1a* and *rap1b* mRNAs showed that PDGF BB also up-regulated the *rap1a* mRNA species, which was 1.5-fold increased in contrast with the *rap1b* mRNA species. It is concluded that the PDGF BB-induced SMC proliferation is associated with an up-regulation of Rap1a protein.

Key words: Platelet-derived growth factor; Rap1; Vascular smooth muscle cell

1. Introduction

The cell proliferation induced by growth factors involves a cascade of intracellular signal transduction mechanisms leading to cell mitosis. The existence of multiple signalling pathways is supported by the cooperation between oncoproteins like cytoplasmic protein kinases, GTP-binding proteins, or nuclear transcription factors. Among these is the small molecular weight GTP binding Ras-p21 oncoprotein which has been shown to play a key role in the signal transduction induced by growth factors leading to cell proliferation [1,2].

In contrast, little is known concerning the down-regulation of cell proliferation. Interestingly, a cDNA was initially characterized in NIH-3T3 cells transformed by the Kirsten-*ras* sarcoma virus as capable of reverting their transformed phenotype [3]. This cDNA was isolated, called *Krev-1* (Kirsten-ras revertant) and was suggested to encode for a transformation suppressor antigen. At the same time, another group isolated 2 cDNAs coding for the Ras-related GTP-binding Rap1 proteins: *rap1a* and *rap1b*, and the *rap1a* cDNA was identified as the *Krev-1* cDNA [4,5]. However, apart from this putative role of Rap1 proteins in suppressing transformation, their precise action mechanism remains under investigation, contrary to their structural characteristics which have been extensively described. Thus, like Ras-p21 proteins, Rap1 proteins have been shown to display an active form that binds GTP, and an inactive form that binds GDP [6]. Rap1 proteins can be phosphorylated by a cAMP-dependent serine/threonine protein kinase (PKA) [7] in the serine 180 and 179 position for, respectively, Rap1a [8] and Rap1b [9].

The aim of the present study was to improve the understanding of the role of Rap1 proteins, by exploring the regulation of their expression in cell proliferation induced by PDGF. For this purpose, we used a well-established proliferating model consisting of PDGF-in-

* Corresponding author. Fax: (33) (1) 49 95 85 79.

Abbreviations: rap, Ras-proximate; PDGF, platelet-derived growth factor; SMC, smooth muscle cells; PKA, cAMP-dependent protein kinase; C. Sub., catalytic subunit of PKA; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; EGTA, [ethylene bis (oxyethylenenitrilo)]tetraacetic acid; DTT, dithiothreitol; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcription-PCR; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; MMLV, murine Moloney leukemia virus; SSC, sodium salt citrate; GAP, GTPase activating protein.

SSDI 0014-5793(94)00237-P

duced proliferation of SMC. We first explored untreated SMC for the presence of Rap1 proteins mainly by Western blotting and immunoprecipitation using a specific anti-Rap1 antibody. The effect of PDGF on SMC proliferation and *rap1* expression was then assessed by testing (i) PDGF-induced mitogenic activity and (ii) PDGF-induced Rap1 protein and mRNA expressions. The results of these studies showed an up-regulation of both Rap1 proteins and *rap1a* mRNA species in PDGF BB-induced SMC proliferation.

2. Materials and methods

2.1. Cell culture

SMC were isolated from rat thoracic aorta and cultured according to Ross [10]. Briefly, the medium was cut into 2 mm long explants which were placed in culture flasks containing Ham's F10 medium supplemented with 20% foetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. SMC were cultured in an atmosphere of 5% CO_2 in air at 37°C. All the experiments were performed with cells which had undergone less than 6 passages.

2.2. Thymidine incorporation assays

PDGF AA and BB-induced mitogenic activity was assessed by measuring incorporation of [^{3}H]thymidine into newly synthetized DNA. SMC were seeded in 12-well plates for 48 h, and then serum-deprived for 48 h. Next, recombinant PDGF AA (Genzyme, Cambridge, MA, USA) or BB (Serva, Heidelberg, Germany) and 1 μCi/ml of [^{3}H]thymidine (74 GBq/mmol, Amersham, UK) were added simultaneously for 48 h at 37°C. After precipitation with 10% ice-cold trichloracetic acid for 10 min, cells were solubilized at room temperature with 200 mM NaOH. Radioactivity was quantified in a β scintillation liquid counter (Beckman, Fullerton, CA, USA).

2.3. Cell fractionation

Cells were washed in 17 mM HEPES pH 7.0, 160 mM KCl and 0.05 mM EGTA. They were resuspended in an ice-cold buffer containing 10 mM HEPES pH 7.0, 10 mM KCl, 0.05 mM EGTA, 0.5 mM DTT, 10 μg/ml leupeptin, 50 μg/ml aprotinin and 100 μg/ml SBTI. The homogenates were sonicated and centrifuged for 10 min at 1,000 × *g* and 4°C. An aliquot of cell lysate was taken and remaining fraction was ultracentrifuged for 1 h at 100,000 × *g* and 4°C. The resulting pellet (membranes), the supernatant (cytosol) and the lysate were stored frozen at −80°C. Proteins were quantified by the Bradford method, using rabbit gammaglobulins as standard [11].

2.4. Western blotting

After reduction, proteins were separated by 11% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [12] and electroblotted onto nitrocellulose. The sheets were blocked for 2 h at room temperature by incubation in a buffer containing 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20 (v/v), 0.5% BSA (w/v) and 0.5% gelatin (w/v), incubated for 2 h at room temperature with a 1:1000 dilution of anti-Rap1 antibody in the same buffer. The specific anti-Rap1 antibody, which recognizes both Rap1a and Rap1b proteins was obtained as previously described [13] and affinity-purified on an agarose column coupled to the immunizing peptide. The antibody binding was revealed by further incubation with [^{125}I]protein A (0.1 μCi/ml, 1110 MBq/mg, Amersham) and autoradiography was performed at −80°C for 72 h. Rap1 protein expression was quantified by densitometric measurement of the radioactive bands using an LKB Ultroscan XL laser densitometer and results were analysed by a *t*-paired test.

2.5. Rap1 protein immunoprecipitation

The membrane fractions (300 μg) were phosphorylated in the presence of 20 μg/ml of the catalytic subunit of PKA (C. Sub., Sigma, St Louis, MO, USA) and 6 mM [γ-^{32}P]ATP (460 MBq/mmol, Amersham) for 10 min at 30°C, as previously described [14]. Phosphorylated proteins were then treated for immunoprecipitation using the anti-Rap1 antibody, as previously described [15], separated by SDS-PAGE, electroblotted onto nitrocellulose and autoradiographed at −80°C, for 1–5 days.

2.6. Polymerase chain reaction

Total RNAs were isolated from SMC according to Chomczynski et Sacchi [16]. 500 ng of total RNA sample was denaturated by heating for 3 min at 75°C and annealed to the 3′ primers corresponding to *rap1a*, *rap1b* [17] and SERCA 2b (sarcoendoplasmic reticulum Ca^{2+} ATPase) [18], by slow cooling to 30°C. Reverse transcription was carried out by adding 100 units of murine Moloney leukemia virus reverse transcriptase (MMLV-RT, Gibco-BRL, Paisley, Scotland) for 1 h at 42°C in PCR buffer containing 10 mM Tris-HCl pH 8.3, 50 mM KCL, 1.5 mM $MgCl_2$, 0.001% gelatin and 2.5 mM each of dATP, dCTP, dGTP and dTTP, in a total volume of 10 μl. Reverse transcription was terminated by inactivating the MMLV-RT for 10 min at 95°C. The PCR was performed in the same reaction mixture, adjusted to 50 μl with the PCR buffer containing the 5′ primers (*rap1a*-1b and SERCA 2b). PCR was initiated by adding 1.25 units of Taq polymerase (*Thermus aquaticus*, Eurogentec, Seraing, Belgium) and conducted in an automated thermocycler (Techne OSI, Cambridge, UK) for 30 cycles each consisting of denaturation at 95°C for 45 s, annealing at 55°C for 1 min and extension at 70°C for 2 min.

2.7. Southern blotting

The PCR products were submitted to electrophoresis on a 2% ethidium bromide-stained agarose gel and transferred onto Biohylon Z+ membranes (Bioprobe, Montreuil sous Bois, France). Membranes were prehybridized for 4 h at 65°C in 5 × sodium salt citrate buffer (SSC), 10 × Denhart's solution (0.2% (w/v) BSA, 0.2% (w/v) Ficoll and 0.2% (w/v) polyvinylpyrrolidone), 0.1% SDS and 250 μg/ml denatured herring sperm DNA. Hybridization was performed by adding 2.10^5 cpm/ml random primed labelled cDNA probes in the same solution overnight at 65°C. The *rap1a* and *rap1b* probes were respectively a 760 and a 730 bp *Hin*dIII/*Eco*RI fragment totally covering human *rap1a* and *rap1b* cDNAs [4,5]. The SERCA2b probe was an 848 bp cDNA fragment, obtained from a human platelet cDNA library [19]. Membranes were washed twice in 1 × SSC, 0.1% SDS for 20 min at 65°C, and twice in 0.1 × SSC, 0.1% SDS for 20 min at 65°C and autoradiographed on X-Omat AR films, at −80°C. RT-PCR products were quantified and results analysed as described in section 2.4.

3. Results

3.1. Evidence for the presence of Rap1 proteins in SMC

To detect Rap1 proteins, we first proceeded to SMC fractionation and submitted the resulting fractions to Western blotting using the anti-Rap1 antibody (Fig. 1A). Whereas no Rap1 proteins were detectable in the lysate (lane 1), comparison of the cytosol (lane 2) and the membranes (lane 3) clearly demonstrated the presence of a 24 kDa protein only in the membranes. Consequently, subsequent experiments were carried out using this membrane fraction. To confirm that the 24 kDa protein was identical to Rap1 proteins, we took advantage of the evidence that in human platelets [14] these proteins displayed an electrophoretic mobility shift which varied, depending on reducing or non-reducing conditions. Accordingly, as shown in Fig. 1B, non-reduced or reduced SMC membrane proteins were studied. Under non-reducing conditions, anti-Rap1 antibody reacted with a 22 kDa protein (lane 1) and under reducing conditions, with a 24 kDa protein (lane 2). Lastly, we established that this 24 kDa protein and Rap1 proteins were identical, by performing their PKA-dependent phosphorylation fol-

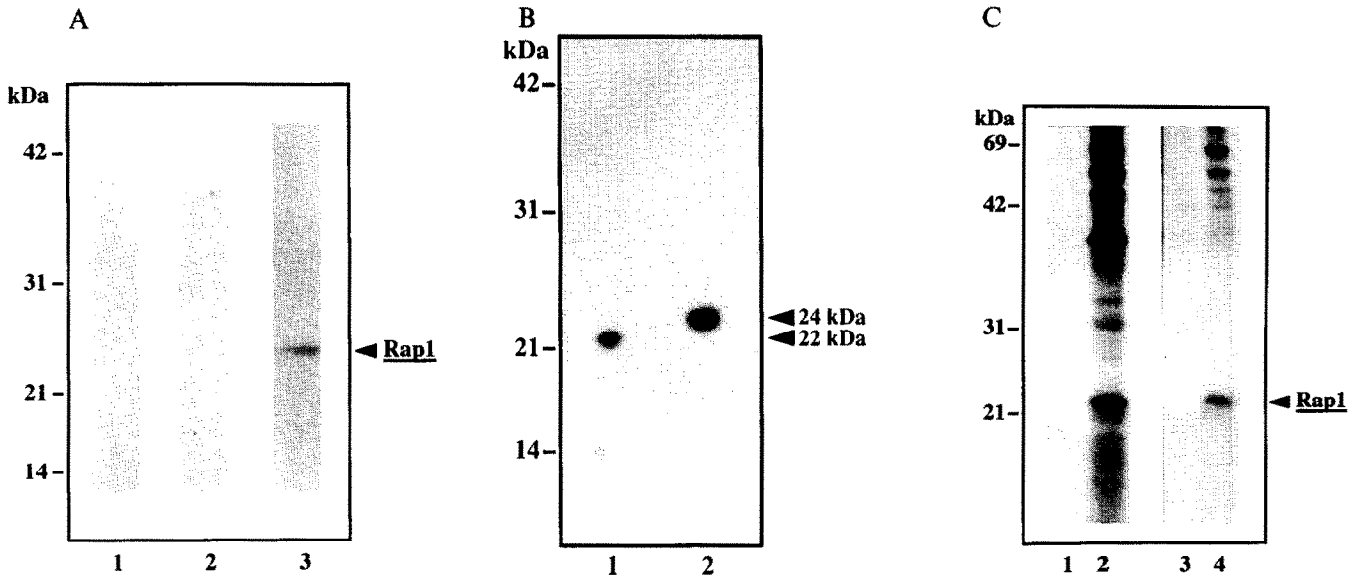

Fig. 1. Evidence for the presence of Rap1 proteins in SMC membranes. (A) Subcellular distribution of Rap1 proteins. SMC were fractionated as described in section 2 and each protein sample (100 μg) was separated by 11% SDS-PAGE followed by Western blotting, performed using anti-Rap1 antibody. Lane 1, SMC lysate fraction; lane 2, SMC cytosol fraction; lane 3, SMC membrane fraction. Rap1 protein molecular masses were estimated in kDa using standard markers (69, BSA; 42, ovalbumin; 31, bovine carbonic anhydrase; 21, soybean trypsin inhibitor; and 14, lysozyme). This figure is typical of 3 experiments. (B) Rap1 protein electrophoretic mobility shift. Membrane proteins (100 μg) were incubated for 5 min at 100°C with or without 5% β-mercaptoethanol and treated as described in A. Lane 1, non reduced SMC membrane proteins; lane 2, reduced SMC membrane proteins. Rap1 protein molecular masses were estimated in kDa using the same standard markers as in A. This figure is typical of 4 experiments. (C) Immunoprecipitation by anti-Rap1 antibody of the 24 kDa membrane cAMP-induced phosphoprotein. SMC membrane proteins (300 μg) were phosphorylated and then immunoprecipitated using the anti-Rap1 antibody, as described in section 2. Lane 1, cAMP-induced phosphorylation of SMC membrane fraction in the absence of C. Sub.; lane 2, cAMP-induced phosphorylation of SMC membrane fraction in the presence of C. Sub.; lane 3, immunoprecipitation of non-phosphorylated proteins (control), using the anti-Rap1 antibody; lane 4, immunoprecipitation of the cAMP-induced phosphorylated proteins, using the anti-Rap1 antibody. Rap1 protein molecular masses were estimated in kDa using the same standard markers as in A. This figure is typical of 2 experiments.

lowed by immunoprecipitation of the resulting phosphoproteins, using the anti-Rap1 antibody (Fig. 1C). Lanes 1 and 2 show the phosphorylation of SMC membrane proteins in the absence of C. Sub. (lane 1) or in its presence (lane 2), whereas lanes 3 and 4 show the corresponding immunoprecipitates. Comparison of lanes 2 and 4 established beyond doubt that the 24 kDa cAMP-induced phosphoprotein is immunoprecipitated by the anti-Rap1 antibody (lane 4). Consequently, the results of these experiments taken together clearly established the expression and identity of Rap1 proteins in SMC membranes.

3.2. PDGF-induced SMC mitogenic activity

Before exploring the effect of PDGF on Rap1 protein expression, we tested both PDGF AA and BB-induced mitogenic activity, by measuring [^{3}H]thymidine incorporation into newly synthetized DNA (Fig. 2). PDGF AA induced very low [^{3}H]thymidine incorporation, which reached a plateau at 235% of the control value for a concentration of 10 ng/ml, thus demonstrating very weak mitogenic activity. In contrast with PDGF BB, [^{3}H]thymidine incorporation rose in a dose-dependent manner for concentrations of 0 to 10 ng/ml and this rise reached a plateau at 520% of the control value, for 10 ng/ml. Consequently, these conditions, i.e. SMC treated with 10 ng/ml of PDGF AA or BB for 48 h, were used for subsequent studies.

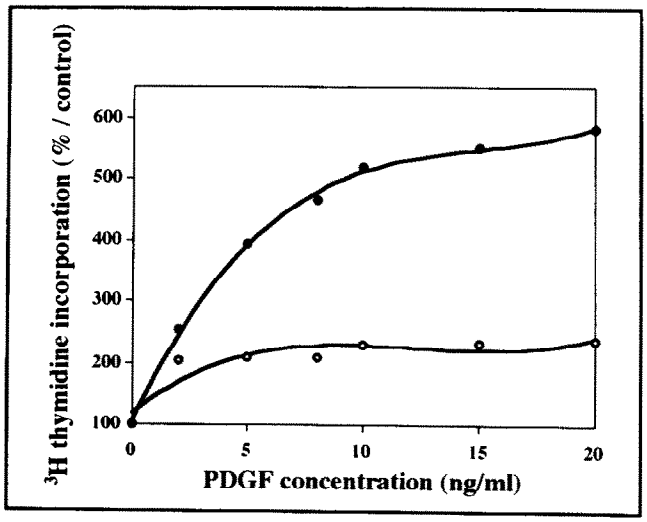

Fig. 2. PDGF AA and PDGF BB-induced SMC mitogenic activity. After 48 h of serum deprivation, SMC were incubated with rising concentrations (0 to 20 ng/ml) of either PDGF AA (○) or PDGF BB (●), and with 1 μCi/ml of [^{3}H]thymidine. Results are expressed as percentage of [^{3}H]thymidine incorporation relative to a control without growth factor (100%) and are representative of 3 experiments conducted in duplicate.

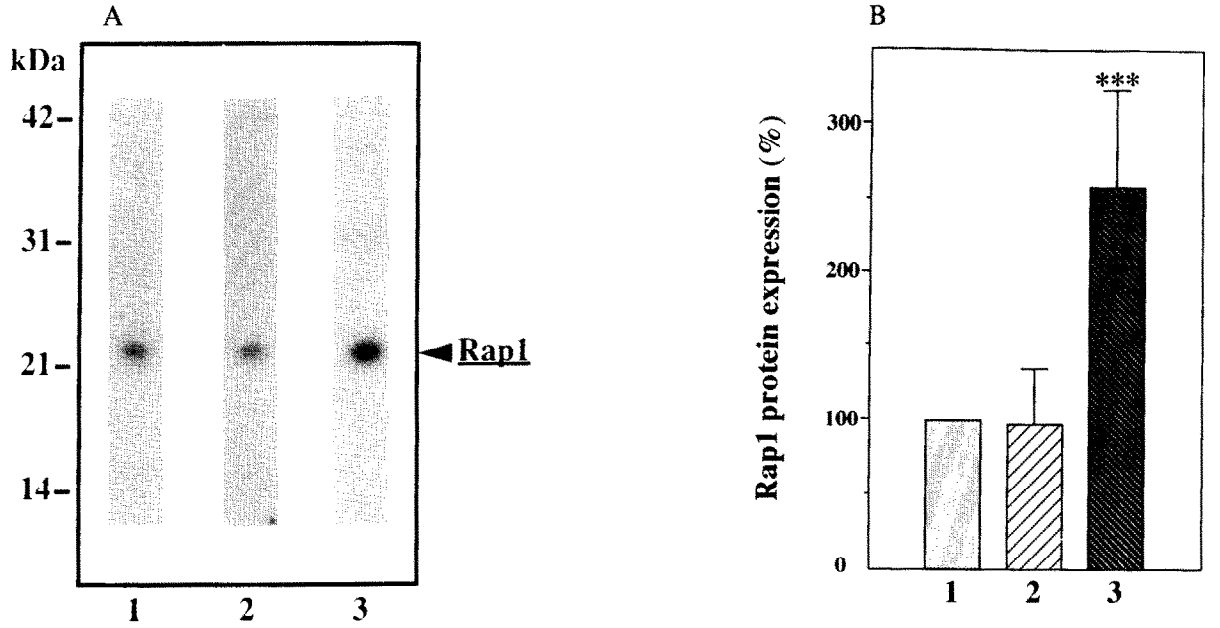

Fig. 3. Effect of PDGF AA and BB on Rap1 protein expression. After 48 h serum deprivation, SMC were grown for 48 h in the presence of 10 ng/ml of PDGF AA, or PDGF BB, or in the absence of PDGF. Membrane proteins (100 μg) were treated as described in Fig. 1A. (A) Western blotting. Lane 1, membranes isolated from untreated SMC; lane 2, membranes isolated from SMC stimulated by PDGF AA; lane 3, membranes isolated from SMC stimulated by PDGF BB. Rap1 protein molecular masses were estimated in kDa using the same standard markers as in Fig. 1A. This figure is typical of 4 experiments. (B) Western blot densitometry. Autoradiograms were scanned using a laser densitometer as described in section 2. For each experiment, the control values obtained without growth factor (bar 1) were arbitrarily taken as 100%. The results (bars 2 and 3) are expressed as percentage of Rap1 protein expression versus the control (100%) and are the means ± S.E.M. of 4 experiments. (***$P \leq 0.005$).

3.3. *Effect of PDGF AA and BB on Rap1 protein expression in SMC*

Then, we explored the regulation of Rap1 protein expression upon stimulation with PDGF AA or BB. For this purpose, membrane fraction proteins were isolated from PDGF AA or BB-treated or untreated SMC and analysed by Western blotting using the anti-Rap1 antibody (Fig. 3A). Whereas PDGF AA had no detectable effect on Rap1 protein expression in SMC (lane 2), compared to control untreated SMC (lane 1), this expression increased upon stimulation with PDGF BB (lane 3). Rap1 protein expression was then quantified by densitometric scanning (Fig. 3B). PDGF AA (bar 2) had no effect on Rap1 protein expression (99% ± 36%), as compared to control (bar 1), whereas PDGF BB significantly increased this expression (bar 3), which reached 260% ± 70% of the control value.

3.4. *Time-dependent effect of PDGF BB on Rap1 protein expression*

In order to define whether this regulation was related to proliferation, membrane fraction proteins were isolated from SMC treated with PDGF BB for 6 h, 24 h and 48 h or from untreated SMC and analysed as above (Fig. 4A). No apparent change in Rap1 protein expression was observed after 6 h (lane 2) or 24 h (lane 3) compared to control untreated SMC (lane 1). In contrast, after 48 h of PDGF BB stimulation, this expression increased (lane 4). Rap1 protein expression was then quantified by densitometric scanning (Fig. 4B). PDGF BB had no effect on Rap1 protein expression after 6 h (bar 2, 94% ± 11%) or 24 h of PDGF BB stimulation (bar 3, 103% ± 6%). However, 48 h of PDGF BB stimulation significantly rose Rap1 protein expression, which reached 191% ± 22% of the control value (bar 4).

3.5. *Effect of PDGF BB on rap1a and rap1b mRNA expression in SMC*

Lastly, we tried to define how the amount of Rap1 was regulated by studying the effect of PDGF BB on *rap1* mRNA species. Alternatively, this study gave the opportunity to differenciate between the two *rap1* mRNAs (*rap1a* and *rap1b*) because of the availability of specific cDNA probes, in contrast with the anti-Rap1 antibody. For this analysis, we devised co-amplification experiments by RT-PCR of either *rap1a* and *rap1b* mRNAs, with an internal control mRNA, which was previously demonstrated unmodified upon PDGF BB-induced SMC proliferation [20] and termed SERCA2b. Fig. 5 shows the Southern blot of *rap1a* and SERCA2b co-amplification, conducted on total RNAs extracted from untreated SMC (lane 1) and SMC treated by PDGF BB for 48 h (lane 2) in the presence of MMLV-RT. No amplification product was found in the absence of MMLV-RT (lane 3). Two DNA fragments of 362 bp and 270 bp, respectively, corresponding to *rap1a* and SERCA2b were visible in untreated (lane 1) and in PDGF BB-stimulated SMC (lane 2). As regards the effect of PDGF BB, whether it did not modify the SERCA2b mRNA expression, as expected, Fig. 5 clearly

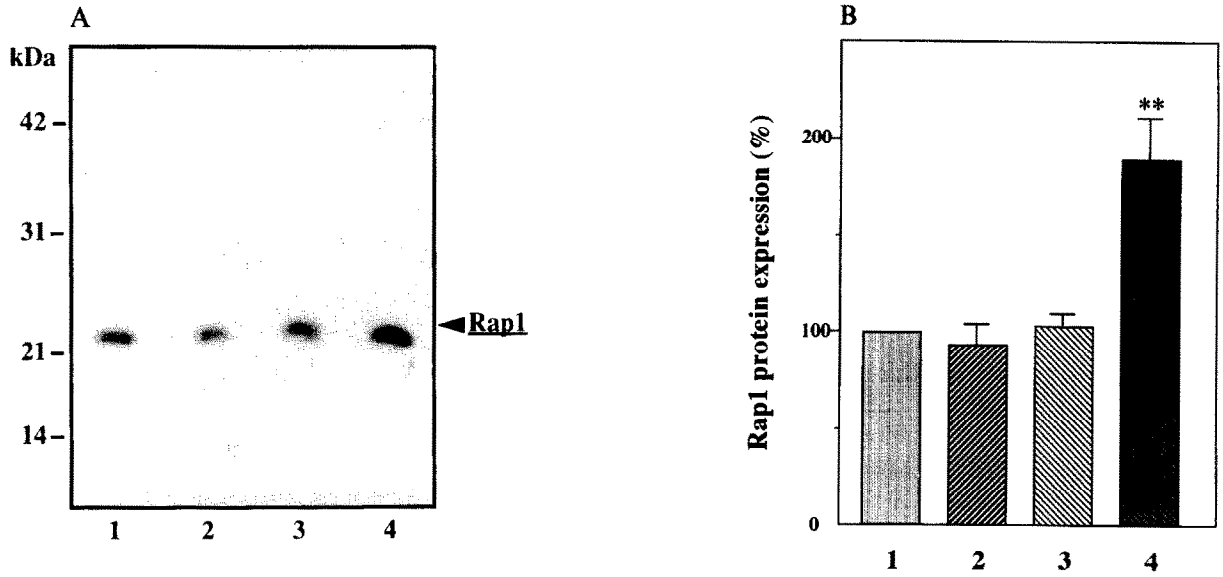

Fig. 4. Time-dependent effect of PDGF BB on Rap1 protein expression. After 48 h serum deprivation, SMC were grown for 6 h, 24 h and 48 h in the presence of 10 ng/ml of PDGF BB, or in the absence of PDGF. Membrane proteins (100 μg) were treated as described in Fig. 1A. (A) Western blotting. Lane 1, membranes isolated from untreated SMC; lane 2, membranes isolated from SMC stimulated by PDGF BB for 6 h; lane 3, membranes isolated from SMC stimulated by PDGF BB for 24 h; lane 4, membranes isolated from SMC stimulated by PDGF BB for 48 h. Rap1 protein molecular masses were estimated in kDa using the same standard markers as in Fig. 1A. This figure is typical of 4 experiments. (B) Western blot densitometry. Autoradiograms were scanned using a laser densitometer as described in section 2. For each experiment, the control values obtained without growth factor (bar 1) were arbitrarily taken as 100%. The results (bars 2, 3 and 4) are expressed as percentage of Rap1 protein expression versus the control (100%) and are the means ± S.E.M. of 4 experiments. (**$P \leq 0.01$).

shows a significant increase in *rap1a* mRNA species (compare lanes 1 and 2). Similar experiments carried out on *rap1b* did not demonstrate any effect on this mRNA species expression (data not shown).

These *rap1a*, *rap1b* and SERCA2b mRNA expressions were further quantified by densitometric scanning and the relative expressions of *rap1a* and *rap1b* were estimated (Table 1). It appeared that: (i) the *rap1a* and *rap1b* mRNA expressions were identical in untreated SMC (0.97 ± 0.03; 0.99 ± 0.13), and (ii) PDGF BB specifically induced a 1.5-fold increase in *rap1a* mRNA expression (0.97 ± 0.03 versus 1.42 ± 0.19), as *rap1b* mRNA expression was not modified (0.99 ± 0.13 versus 0.94 ± 0.03).

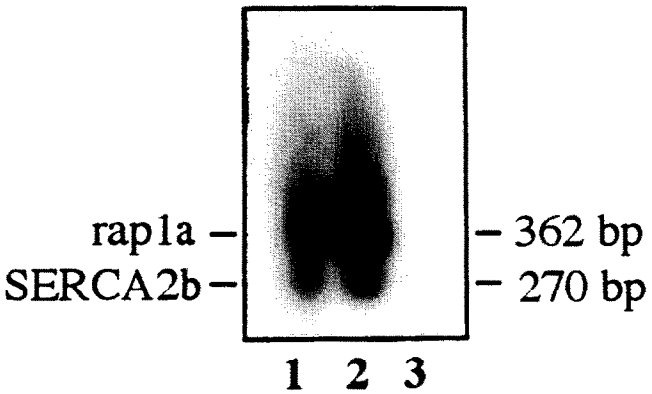

Fig. 5. Effect of PDGF BB on *rap1a* mRNA expression. After 48 h serum-deprivation, SMC were grown for 48 h in the presence of 10 ng/ml of PDGF BB, or in the absence of PDGF and total RNAs were extracted as previously described. 500 ng of total RNA was submitted to comparative co-amplification by RT-PCR of *rap1a* and SERCA 2b mRNA species, using a mixture of the different primers. The amplification products were analysed by Southern blotting, as described in section 2, using a mixture of the ^{32}P-labelled *rap1a* and SERCA2b cDNA probes. Lane 1, amplification products of total RNAs extracted from untreated SMC; lane 2, amplification products of total RNAs extracted from SMC stimulated by PDGF BB for 48 h; lane 3, control amplification products of total RNAs, in the absence of MMLV-RT. Numbers on the right side of the figure indicate the size of the amplification products in base pairs (bp). This figure is representative of 3 experiments.

4. Discussion

This work clearly shows that: (i) Rap1 proteins are expressed in rat vascular SMC membrane fractions, (ii) their expression is up-regulated by PDGF BB, and (iii) this up-regulation of Rap1 proteins was further correlated with an increase in *rap1a* mRNA species. As far as we know this is the first demonstration of the regulation of Rap1 proteins in a proliferating model.

Table 1
PDGF BB effect on the relative expressions of *rap1a* and *rap1b* mRNA species

	− PDGF BB	+ PDGF BB
rap1a/SERCA2b	0.97 ± 0.03	1.42 ± 0.19**
rap1b/SERCA2b	0.99 ± 0.13	0.94 ± 0.03

Southern blot autoradiograms were scanned using a laser densitometer, as described in section 2. The results are expressed as ratio of *rap1*/SERCA2b mRNA expressions and are the means ± S.E.M. of 3 experiments. **$P \leq 0.01$.

The identification of Rap1 proteins in rat SMC is based on (i) the use of a specific anti-Rap1 antibody, (ii) Rap1 protein PKA-dependent phosphorylation and their subsequent immunoprecipitation, and (iii) the specific electrophoretic shift in Rap1 protein migration depending on reducing conditions. As regards this shift in Rap1 protein mobility on SDS-PAGE, it is now known that the structure of these proteins was stabilized by several intrachain disulfide bridges (3 for Rap1a and 2 for Rap1b), which were cleaved under reducing conditions; hence we could believe that this cleavage probably involved a change in the tertiary conformation of the protein structure. Thus, Rap1 proteins are expressed in SMC, appeared associated to the membrane fraction, in a total agreement with the recent location of these proteins in several cell types [13,21,22].

In the second part of this study, we first investigated the effects of PDGF AA and BB on SMC proliferation and Rap1 protein expression. Thus, we showed that only PDGF BB exhibited significant mitogenic activity as well as an up-regulation of total Rap1 protein expression. The most plausible explanation for our observation that PDGF AA had no significant effect on mitogenic activity and did not affect Rap1 protein expression is that it only reflects the presence of an insufficient number of PDGF AA receptors. Indeed, in rat SMC, 5,000 PDGF AA and 31,000 PDGF BB binding sites per cell have been found [23]. Subsequent time-course studies showed that the increase in Rap1 protein expression only occurred after 48 h of stimulation by PDGF BB, suggesting that this up-regulation requires protein neosynthesis and is related to the state of cell proliferation. Lastly, through mRNA studies, we defined that (i) the Rap1 protein up-regulation was correlated with an increase in mRNA expression, suggesting that PDGF BB acts at the *rap1* transcriptional level, and (ii) that the Rap1 protein concerned by the regulation should be the Rap1a protein.

So, these results allowed us to suggest that Rap1a protein can effectively be involved in modulating cell proliferation mechanisms. Indeed, PDGF BB is known to induce a mitogenic pathway which involves the activation of Ras [24] and of its potential effector, the Ras-specific GTPase activating protein (Ras-GAP) [25,26]. In addition, Rap1 proteins exhibit a high affinity for the Ras-GAP [27] and the GTP-binding form of Rap1 protein was recently demonstrated to interfere with downstream signalling from Ras to the nucleus [28]. Therefore, it appears that Rap-GTP could be a potent antagonist of Ras by competing with Ras for its effector and then could prevent further signal transduction towards the nucleus. Hence, it is conceivable that in non-transformed cells, such as PDGF BB-induced proliferating SMC studied here, these Rap1 proteins might play a role in regulating cell proliferation by preventing cells from entering into anarchic division cycles.

Acknowledgements: We thank Dr Eliane Berrou and Regis Bobe for technical assistance and helpful discussions. This work was supported by grants from the Association pour la Recherche contre le Cancer, and the Association Française contre les Myopathies.

References

[1] Mulcahy, L.S., Smith, M.R. and Stacey, D.W. (1985) Nature 313, 241–243.
[2] McCormick, F. (1993) Nature 363, 15–16.
[3] Kitayama, H., Sugimoto, Y., Matsuzaki, T., Ikawa, Y. and Noda, M. (1989) Cell 56, 77–84.
[4] Pizon, V., Chardin, P., Lerosey, I., Olofsson, B. and Tavitian, A. (1988) Oncogene 3, 201–204.
[5] Pizon, V., Lerosey, I., Chardin, P. and Tavitian, A. (1988) Nucleic Acids Res. 16, 7719.
[6] Kawata, M., Matsui, Y., Kondo, J., Hishida, T., Teranishi, Y. and Takai, Y. (1988) J. Biol. Chem. 263, 18965–18971.
[7] Fischer, T.H., Collins, J.H., Gatling, M.N. and White, G.C. II (1991) FEBS Lett. 283, 173–176.
[8] Quilliam, L.A., Mueller, H., Bohl, B.P., Prossnitz, V., Sklar, A., Der, C.J. and Bokoch, G.M. (1991) J. Immunol. 147, 1628–1635.
[9] Kawata, M., Kikuchi, A., Hoshijima, M., Yamamoto, K., Hashimoto, E., Yamamura, H. and Takai, Y. (1989) J. Biol. Chem. 264, 15688–15695.
[10] Ross, R. (1971) J. Cell. Biol. 50, 172–186.
[11] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[12] Laemmli, U.K. (1970) Nature 227, 680–685.
[13] Béranger, F., Goud, B., Tavitian, A. and de Gunzburg, J. (1991) Proc. Natl. Acad. Sci. USA 88, 1606–1610.
[14] Corvazier, E., Enouf, J., Papp, B., de Gunzburg, J., Tavitian, A. and Lévy-Toledano, S. (1992) Biochem. J. 281, 325–331.
[15] Enouf, J., Corvazier, E., Papp, B., Quarck, R., Magnier, C., Kovàcs, T., Bredoux, R., Lévy-Toledano, S., de Gunzburg, J., Wuytack, F. and Caen, J. (1994) Brit. J. Haematol. 286, 338–346.
[16] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[17] Magnier, C., Bredoux, R., Kovàcs, T., Quarck, R., Papp, B., Corvazier, E., de Gunzburg, J. and Enouf, J. (1994) Biochem. J. 297, 343–350.
[18] Bobe, R., Bredoux, R., Wuytack, F., Quarck, R., Kovàcs, T., Papp, B., Corvazier, E., Magnier, C. and Enouf, J. (1994) J. Biol. Chem. 269, 1417–1424.
[19] Enouf, J., Bredoux, R., Papp, B., Djaffar, I., Lompré, A.M., Kieffer, N., Gayet, O., Clemetson, K., Wuytack, F. and Rosa, J.P. (1992) Biochem. J. 286, 135–140.
[20] Magnier, C., Papp, B., Corvazier, E., Bredoux, R., Wuytack, F., Eggermont, J., Maclouf, J. and Enouf, J. (1992) J. Biol. Chem. 267, 15808–15815.
[21] Matsui, Y., Kikuchi, A., Kawata, M., Kundo, J., Teranishi, Y. and Takai, Y. (1990) Biochem. Biophys. Res. Commun. 166, 1010–1016.
[22] Maridonneau-Parini, I. and de Gunzburg, J. (1992) J. Biol. Chem. 267, 6396–6402.
[23] Sachinidis, A., Locher, R., Vitter, W., Tatje, D. and Hoppe, J. (1990) J. Biol. Chem. 265, 10238–10243.
[24] Satoh, S., Endo, M., Nakafuku, M., Nakamura, S. and Kaziro, Y. (1990) Proc. Natl. Acad. Sci. USA 87, 5993–5997.
[25] Kaplan, D.R., Morrison, D.K., Wong, G., Mc Cormick, F. and Williams, L.T. (1990) Cell 61, 125–133.
[26] Adari, H., Lowy, D., Willumsen, B.M., Der, C.J. and Mc Cormick, F. (1991) Science 240, 518–521.
[27] Hata, Y., Kikuchi, A., Sasaki, T., Schaber, M.D., Gibbs, J.B. and Takai, Y. (1990) J. Biol. Chem. 265, 7104–7107.
[28] Cook, S.J., Rubinfeld, B., Albert, I. and Mc Cormick, F. (1993) EMBO J. 12, 3475–3485.