Characterization of the tyrosine phosphorylation of calpactin I (annexin II) induced by platelet-derived growth factor

Riccardo BRAMBILEA, Renata ZIPPEL,* Emmapaola STURANI, Laura MORELLO, Antonio PERES and Lilia ALBERGHINA

Department of General Physiology and Biochemistry, University of Milan, Via Celoria 26, 20133 Milan, Italy

Stimulation *in vivo* of Swiss 3T3 fibroblasts with platelet-derived growth factor (PDGF) in the presence of orthovanadate induces the tyrosine phosphorylation of a 39 kDa protein, identified as the phosphorylated slow-migrating form of calpactin I (annexin II) heavy chain, p36. In fact, in PDGF-stimulated cells, anti-(calpactin I) antibodies recognize a doublet of bands, p36 and p39, and the latter disappears upon treatment with phosphatase. In many regards phosphorylation of p39 differs from the rapid and transient phosphorylation of the PDGF receptor and of other substrates: (a) it has slower kinetics but is then stable for longer periods of time; (b) it occurs at 37 °C but not at 4 °C; and (c) whereas most of the tyrosine-phosphorylated proteins are associated with membrane-enriched preparations, membrane association of p39 only occurs in the presence of Ca^{2+} . Moreover, calpactin I leaks out of permeabilized cells at 0.1 μM free Ca^{2+} , whereas it remains associated with the cells at concentrations of $\text{Ca}^{2+} \geq 1 \mu\text{M}$. PDGF does not stimulate phosphoinositide turnover (and thus Ca^{2+} mobilization) at 4 °C; thus it can be suggested that the Ca^{2+} -dependent translocation of the protein to membrane/cytoskeletal structures is a necessary condition for its phosphorylation. In addition, calpactin I may not be a direct substrate for the PDGF receptor kinase, but rather the substrate of another tyrosine kinase activated by the receptor.

INTRODUCTION

Calpactin I (annexin II; p36) is a Ca^{2+} -, phospholipid- and actin-binding protein. Several studies have established that it interacts with membranes and cytoskeletal structures in a Ca^{2+} -dependent manner [1–4]. In addition, the association of p36 with the cytoplasmic side of the plasma membrane suggests that it may be involved in the organization of the network underlying the plasma membrane [1]. It has recently been shown that calpactin plays a role in Ca^{2+} -dependent membrane fusion and exocytosis [5,6].

Both calpactin I and calpactin II contain increased levels of phosphotyrosine in cells either expressing transforming tyrosine kinases or treated with growth factors which interact with tyrosine kinase receptors [7–9]. Calpactin II has been shown to become tyrosine-phosphorylated in A431 cells stimulated by epidermal growth factor (EGF) [10], whereas calpactin I, or more precisely its heavy chain, is phosphorylated in response to platelet-derived growth factor (PDGF) [11]. The site of tyrosine phosphorylation of calpactin I has been mapped at the N-terminal domain (Tyr-23) [12]. In addition, calpactin I is also phosphorylated at Ser-25, by protein kinase C (PKC) [13,14]. The extent of these phosphorylations is in general small, and apparently only a subpopulation of p36 is accessible to the kinase.

The tyrosine phosphorylation of p36 may alter its function, and it has been reported that tyrosine phosphorylation of p36 by *v-src* decreases its affinity for liposomes [7]. A certain number of physiological substrates of the PDGF receptor have been recently identified: they include phospholipase C- γ (PLC- γ), the GTPase-activating protein of p21^{ras} (GAP), the phosphatidylinositol 3-kinase and the proto-oncogene *raf* [15,16]. All of these proteins appear to be involved in the signal transduction pathway. Although clear evidence has not been obtained so far, it is likely

that tyrosine phosphorylation of calpactin has some physiological significance.

In the present work, we have investigated the tyrosine phosphorylation of the heavy chain of calpactin I in response to PDGF, utilizing orthovanadate as an inhibitor of phosphotyrosyl phosphatases, and we have shown that the response of calpactin I to PDGF is quite different from that exhibited by other substrate proteins. In the presence of vanadate, the tyrosine phosphorylation of calpactin I is much more stable than that of the PDGF receptor and of other substrates. In addition, we found that phosphorylation of calpactin I does not occur at low temperature, and that at such temperatures stimulation of phosphoinositide turnover by PDGF is prevented. Using permeabilized cells we have demonstrated that association between particulate cell structures and calpactin I, both tyrosine-phosphorylated and not phosphorylated, is dependent on the micromolar free Ca^{2+} concentration.

EXPERIMENTAL

Cell culture

Swiss 3T3 mouse fibroblasts were maintained as reported [17]. Cells were plated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). After 3 days the medium was replaced with fresh medium containing 1% FCS. Monolayers were used 24 h later.

Western blotting

Confluent monolayers in 35 or 60 mm Petri dishes were incubated in binding medium [18] containing 0.5% BSA and stimulated as indicated. Total cell proteins were extracted in SDS sample buffer, separated by SDS/PAGE using a 5–15% acrylamide gradient, and analysed by immunoblotting with antibodies against calpactins [4] or phosphotyrosine (P-Tyr) [19]

Abbreviations used: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; PKC, protein kinase C; PLC- γ , phospholipase C- γ ; FCS, fetal calf serum; PAP, potato acid phosphatase; LDH, lactate dehydrogenase.

* To whom correspondence should be addressed.

and ^{125}I -Protein A as reported [17]. In some cases, after autoradiography the relative band intensity was determined either by counting the ^{125}I radioactivity associated with it or by densitometric scanning.

Acid wash

Cells previously stimulated were placed on ice and incubated for 10 min with a solution containing 20 mM-acetic acid, 150 mM-NaCl and 0.25% BSA, pH 3.7, before extraction [20].

Measurement of phosphoinositide turnover

Confluent monolayers in 35 mm dishes were incubated with $\text{myo-}^3\text{H}$ inositol (2 $\mu\text{Ci}/\text{ml}$) for 24 h in an inositol-free basal Eagle's medium containing 2% dialysed FCS. Cells were then incubated in a Krebs-Ringer-Hepes solution containing 0.5% BSA and 10 mM-LiCl for 10 min, and finally stimulated as indicated. Cells were extracted with trichloroacetic acid and ^3H inositol phosphates were separated by anion-exchange chromatography [21].

Membrane preparation

Quiescent cells from 100 mm dishes were washed twice and then collected by scraping into solubilization buffer (10 mM-Tris/150 mM-NaCl, pH 7.4) containing either 1 mM-EGTA or 1 mM- CaCl_2 and various inhibitors of proteases and phosphatases (100 kallikrein-inhibitory units of aprotinin/ml, 1 mM-phenylmethanesulphonyl fluoride, 10 μg of leupeptin/ml and 100 μM -sodium orthovanadate). Suspensions were homogenized in ice with a ground glass tissue grinder (30 strokes) and then centrifuged at 500 g to remove nuclei and unbroken cells. Supernatants were then centrifuged for 45 min at 43 000 g at 4 $^\circ\text{C}$. Pellets, representing membranes and cytoskeleton-enriched preparations, were either extracted in SDS sample buffer or resuspended for phosphatase treatment (see below).

Phosphatase treatment

Membrane pellets prepared as described above with the addition of 1 mM- CaCl_2 were resuspended in a buffer containing 40 mM-Pipes, 1 mM-dithiothreitol, pH 6.0, and the same inhibitors indicated above. Aliquots of 200 μg of protein were treated with 12 μg of potato acid phosphatase (PAP) [22] or with buffer alone at 30 $^\circ\text{C}$ for 30 min, in either the presence or the absence of 20 mM-sodium phosphate. Reactions were stopped by adding SDS to a final concentration of 2.5%.

Cell permeabilization

Experiments with permeabilized cells were performed as described [23] with some modifications. Quiescent cells were detached from the plates, resuspended in conditioned medium and incubated with shaking for 3 h at 37 $^\circ\text{C}$ [24]. Portions corresponding to 5×10^5 cells were washed once in the following buffer: 120 mM-KCl, 30 mM-NaCl, 1 mM- MgCl_2 , 1 mM-ATP, 1 mM- K_2HPO_4 , 1 mM-EGTA, 10 mM-Pipes, at final pH 7.0. Each aliquot was then resuspended in 0.2 ml of the same buffer containing different concentrations of CaCl_2 , calculated according to [25], in order to obtain the desired concentration of free Ca^{2+} : 0.026 mM- CaCl_2 (10 nM free Ca^{2+}), 0.21 mM- CaCl_2 (100 nM free Ca^{2+}), 0.73 mM- CaCl_2 (1 μM free Ca^{2+}), 0.99 mM- CaCl_2 (10 μM - Ca^{2+}). Permeabilization was started by addition of 40 μM -digitonin (stock solution 4 mM, prepared in absolute ethanol), at 37 $^\circ\text{C}$. After 10 min, cells were centrifuged, washed once in the same buffer without digitonin, extracted in SDS sample buffer and immediately boiled for 5 min. Supernatants also received 2.5% SDS. Permeabilization was monitored by positive Trypan Blue staining and release of lactic dehydrogenase (LDH) activity [26].

Materials

Highly purified human PDGF was prepared as previously described [20]. The ED_{50} (dose having half-maximal effect) measured for ^3H thymidine incorporation in quiescent Swiss 3T3 fibroblasts was 1 ng/ml. A stock solution of sodium orthovanadate (100 mM) was prepared as described [11].

Antibodies against *P*-Tyr were kindly supplied by Dr. P. M. Comoglio [19]. Antibodies raised against calpactin I heavy chain and calpactin II were generously provided by Dr. J. Glenney, and were used as described [4].

RESULTS

Detection of a tyrosine-phosphorylated protein of 39 kDa in PDGF-stimulated cells

PDGF stimulation of quiescent fibroblasts brings about the tyrosine phosphorylation of the PDGF receptor, which is detectable by Western blot analysis with antibodies against *P*-Tyr as a 170 kDa band (Fig. 1a, lanes a-e). The kinase activity of the receptor is directed also towards cellular substrates but, as reported before [11], it is difficult to identify other tyrosine-

Fig. 1. Effect of orthovanadate on PDGF-dependent tyrosine phosphorylation of cellular proteins

(a) Quiescent cells were stimulated with human PDGF (100 ng/ml) for the times indicated (lanes b-e) or were treated (lanes f-j) with 1 mM-orthovanadate for 15 min and then with PDGF for the times indicated at 37 $^\circ\text{C}$. Proteins were separated by SDS/PAGE and then immunoblotted with antibodies against *P*-Tyr, as described in the Experimental section. (b) The ^{125}I -Protein A radioactivity associated with the bands corresponding to the 170 kDa PDGF receptor (●) and the 39 kDa protein (○) from lanes a and f-i is shown.

Fig. 2. p39 is the phosphorylated form of calpactin I heavy chain

(a) Vanadate-pretreated cells were stimulated with human PDGF for 45 min at 37 °C. Aliquots of the extracts were fractionated by SDS/PAGE and immunodecorated with antibodies against calpactin I (lane a), calpactin II (lane b) or *P*-Tyr (lane c), as indicated. (b) Crude membrane preparations were prepared in the presence of 1 mM-CaCl₂ (as described in the Experimental section) from cells treated with PDGF and vanadate. Protein (200 µg) was incubated with no additions (lanes a and d) or treated (lanes b, c, e and f) with 12 µg of PAP in the absence (lanes b, e) or in the presence (lanes c, f) of 20 mM-sodium phosphate for 30 min at 30 °C. Extracts were probed with antibodies against *P*-Tyr (lanes a–c) or against calpactin I (lanes d–f). Only the part of the blot containing calpactins is shown.

phosphorylated proteins on the blot in response to PDGF, probably due to their scarcity in the cells and to the transience of the response. As shown in Fig. 1(a) (lanes f–i), a short preincubation with orthovanadate, a specific inhibitor of phosphotyrosyl phosphatases which by itself has little effect on tyrosine phosphorylation of cellular proteins (lane j), increases the tyrosine phosphorylation of the PDGF receptors. In addition, it allows the detection of several other proteins that are phosphorylated in response to PDGF, and in particular a 39 kDa band (p39; in the specific case of Fig. 1a resolved better as a doublet).

The intensities of the bands corresponding to the PDGF receptors and p39 in cells treated with PDGF and vanadate have been quantified (Fig. 1b). The phosphorylation of the receptor as well as that of most other proteins appears to follow a rapid but quite transient time course. p39, however, exhibits different behaviour. It accumulates more slowly, but is then stable even during long periods of treatment (at least up to 3 h; results not shown), by which time the phosphorylation of the receptors and of other proteins has dropped almost to basal levels.

p39 is the phosphorylated form of calpactin I heavy chain

Calpactin I and calpactin II (respectively p36 and p35) have been previously reported to become tyrosine-phosphorylated in response to growth factors or in cells transformed by *v-src* [7–9]. Extracts of cells stimulated at 37 °C with PDGF and vanadate were probed in Western blots with antibodies raised against calpactin I, calpactin II [4] and *P*-Tyr [19]. Fig. 2(a) shows that antibodies against the heavy chain of calpactin I recognize two bands (p36 and p39), and that the slower form co-migrates with the p39 recognized by anti-*P*-Tyr antibodies. Antibodies against calpactin II recognize a 35 kDa protein, as expected, but slower-migrating forms were not observed.

Covalent modifications such as phosphorylation can alter the electrophoretic mobility of proteins. The data reported in Fig. 2(b) demonstrate that p39 is a phosphorylated form of calpactin I. Both p36 and p39 can be found associated with cellular membranes prepared from cells stimulated with PDGF and

Fig. 3. Calpactin I phosphorylation is slow and temperature-dependent

Quiescent cells were pretreated with vanadate for 15 min and then stimulated with PDGF for the times indicated at 37 °C (lanes a–c, e and f) or at 4 °C (lanes d and g). Immunoblotting was with antibodies against *P*-Tyr (lanes a–d) or against calpactin I (lanes e–g). Only the part of the blot containing calpactin is shown.

vanadate (see below). When these membranes were treated with PAP, the p39 band recognized by anti-(calpactin I) antibodies completely disappeared, whereas p36 was perfectly stable. As expected, PAP treatment also induced the disappearance of the p39 recognized by antibodies against *P*-Tyr. Sodium phosphate blocked the action of PAP. Thus the p39 observed in cells stimulated by PDGF in the presence of vanadate is a phosphorylated form of calpactin I.

Tyrosine phosphorylation of calpactin I heavy chain is temperature-dependent

Calpactin I is phosphorylated on tyrosine residues if cells are exposed to PDGF and vanadate at 37 °C, but it is not phosphorylated when stimulation is performed at 4 °C. Fig. 3 shows that p39 is detected by anti-*P*-Tyr and anti-(calpactin I) antibodies in cells stimulated for 45 min at 37 °C (lanes c and f). However, p39 is completely absent from cells stimulated at 4 °C for the same length of time (lanes d and g), and for times as long as 3 h (results not shown). For comparison, a 8 min stimulation at 37 °C is also shown (lanes b and e), in which the p39 is still almost undetectable. This behaviour is different from that of other substrates recognized by anti-*P*-Tyr antibodies. As shown in Fig. 4 (lanes b and d) the pattern of proteins which become tyrosine-phosphorylated in the presence of PDGF and vanadate is similar at 37 °C and at 4 °C, with the exception of p39 (Figs. 3 and 4). Thus, although full receptor activation and tyrosine phosphorylation of many proteins take place at 4 °C, the heavy chain of calpactin I is not phosphorylated at this temperature. Moreover, at 37 °C phosphorylation of calpactin I occurs with some delay with respect to the other proteins (see Figs. 1 and 3).

The tyrosine-phosphorylated form of calpactin I is not affected by a mild acid treatment of intact cells which, as shown before [20], dissociates surface-bound PDGF and causes dephosphorylation of the receptors. Monolayers previously treated with PDGF and vanadate for 45 min were washed for 10 min with an acidic solution in ice (Fig. 4). The acidic treatment induced the dephosphorylation of the PDGF receptor and of other proteins, whereas p39 was completely unaffected. This observation indicates that, in contrast with the receptor and other substrates, maintenance of the phosphorylation of calpactin I does not require persistent ligand activation of the receptor kinase. This finding, together with the fact that phosphorylation of calpactin I is temperature-dependent, suggests that calpactin may not be a direct substrate of the PDGF receptor. Moreover, some temperature-dependent events may be required to allow phosphorylation of calpactin I.

Association of phosphorylated calpactin I to membranes and cytoskeleton depends on Ca²⁺

PDGF possibly induces the activation of phosphoinositide turnover through the tyrosine phosphorylation of PLC-γ

Fig. 4. An acid wash does not affect the phosphorylation of calpactin I

Cells were pretreated with vanadate (van) for 15 min and then stimulated with human PDGF for 45 min either at 4 °C (lanes b and c) or at 37 °C (lanes d and e). Cells in lanes c and e were then incubated in an acidic buffer (pH 3.7, see the Experimental section) for 10 min before extraction and immunoblotting with antibodies against *P*-Tyr.

Fig. 5. Membrane association of phosphorylated calpactin I

Quiescent cells either received no additions or were stimulated with vanadate (van; 15 min) and human PDGF (45 min) as indicated. Crude membranes were then prepared either in the presence of 1 mM- CaCl_2 or in its absence (1 mM-EGTA), as shown in the Figure. Corresponding portions of pellets (a–d) and supernatants (e–h) were analysed by Western blotting with antibodies against *P*-Tyr (a) or against calpactin I (b). In (b) only the part of the blots containing calpactin is shown. Lanes a–d, membranes; lanes e–h, supernatant.

[16,27–32]; as a consequence, inositol trisphosphate would trigger the release of Ca^{2+} from intracellular stores with a resultant increase in the cytosolic free Ca^{2+} concentration [33,34]. In addition, calpactin binds to actin and phospholipids in a Ca^{2+} -dependent manner. Thus we investigated the association of the phosphorylated form of calpactin I with membranes.

Crude membranes were prepared both from untreated cells and from cells previously stimulated with PDGF and vanadate. Isolation of membranes was performed either in the presence of 1 mM- Ca^{2+} or in its absence (1 mM-EGTA). After high-speed centrifugation, both particulate and soluble fractions were analysed by immunoblotting with anti-*P*-Tyr antibodies (Fig. 5a). Most of the proteins phosphorylated in response to PDGF and vanadate were membrane-associated, even when membranes

Fig. 6. Cell association of calpactin I during permeabilization requires micromolar free Ca^{2+}

(a) Cells either received no additions (lanes a–e) or were stimulated with vanadate (15 min) and human PDGF (45 min) at 37 °C (lanes f–j). Portions corresponding to 5×10^5 cells were then permeabilized (lanes b–e and g–j) with 40 μM -digitonin for 10 min at 37 °C in the presence of different concentrations of free Ca^{2+} . After centrifugation, cells and supernatants (SN) were analysed by Western blotting with antibodies against calpactin I. In lanes a and f, non-permeabilized cells were analysed. (b) The ^{125}I -Protein A radioactivity associated with calpactin I in the supernatants (●) and in the cells (○) was quantified from (a) (lanes b–e) and expressed as a percentage of the total (supernatants plus cells). The histogram on the left shows values from non-permeabilized cells (a, lane a): □, cells; ■, supernatant.

were prepared in the absence of Ca^{2+} . In contrast, association of p39 was Ca^{2+} -dependent. Membranes and supernatants were also probed with anti-(calpactin I) antibodies (Fig. 5b). Calpactin I was found to be totally membrane-associated in the presence of 1 mM- Ca^{2+} , but in its absence it was largely found in the supernatant. In accordance with the finding obtained with anti-*P*-Tyr antibodies (Fig. 5a), p39 recognized by anti-calpactin antibodies was part of the 'mobile' Ca^{2+} -sensitive fraction, since it was recovered in the supernatants in the absence of Ca^{2+} (Fig. 5b, lane g), but was associated with membranes in the presence of Ca^{2+} (lane d).

The Ca^{2+} -dependence of the association of calpactin I with cellular structures was further investigated in a different and possibly more physiological system. Cells were permeabilized with 40 μM -digitonin [23] in the presence of concentrations of free Ca^{2+} ranging between 0.04 and 10 μM . Determination of the LDH release into the supernatant allowed us to monitor permeabilization [26] and indicated that permeabilization was not affected by the different concentrations of Ca^{2+} present in the buffer (results not shown). After 10 min the cells were separated from the supernatants and both fractions were analysed using anti-(calpactin I) antibodies (Fig. 6a, lanes a–e). The results, quantified in Fig. 6(b), indicate that at Ca^{2+} concentrations $\leq 0.1 \mu\text{M}$ the heavy chain of calpactin I almost totally leaks out

Fig. 7. PDGF and vanadate do not stimulate polyphosphoinositide turnover at 4 °C

Cells labelled with *myo*-[³H]inositol were stimulated at 37 °C for 10 min or at 4 °C for 30 min as indicated. Total inositol phosphate accumulation was determined as described in the Experimental section.

of the permeabilized cells, whereas at Ca^{2+} concentrations higher than $0.1 \mu\text{M}$ it remains associated with cellular structures. When the same experiment was performed with cells previously stimulated for 45 min at 37 °C with PDGF and vanadate (Fig. 6a, lanes f–j), we found that the phosphorylated form of calpactin I exhibited the same Ca^{2+} -dependence. This experiment demonstrates in a non-artificial system that association of calpactin I with particulate cell structures depends on micromolar Ca^{2+} concentrations. It is possible that the rise in cytosolic calcium induced by PDGF is a condition for translocation of calpactin I to the membranes, and therefore for its phosphorylation on tyrosine in response to PDGF.

Since phosphorylation of calpactin does not occur at 4 °C, we investigated whether PDGF was able to stimulate the turnover of polyphosphoinositides at this temperature. The results reported in Fig. 7 indicate that, at 4 °C, stimulation of inositol phosphate accumulation is not observed in response to PDGF, to vanadate, or to both together. Thus it appears that, at 4 °C, PDGF and/or vanadate do not induce an increase in intracellular free Ca^{2+} . As a consequence, at this temperature calpactin I cannot associate with membrane/cytoskeletal structures.

Tyrosine phosphorylation of calpactin I following stimulation with PDGF and vanadate does not seem to be altered either by PKC activation or by its down-regulation (acute and chronic treatment with phorbol esters; results not shown). Therefore it appears that the phosphorylation of calpactin I by PKC which has been previously documented [13,14] does not affect the tyrosine phosphorylation of this protein induced by PDGF.

DISCUSSION

Tyrosine phosphorylation is one of the key events in signal transduction from PDGF. Many proteins involved in this process are targets of the ligand-activated receptor kinase, which is itself tyrosine-phosphorylated [15,16]. Nevertheless, how the phosphorylation affects the activity of these proteins is not always understood. Among the substrates of the PDGF receptor, the heavy chain of calpactin I was recognized quite early [4].

The molecular mass of calpactin I heavy chain is 36 kDa; however, its phosphorylated form migrates as a band of 39 kDa which is recognized by both anti-*P*-Tyr and anti-(calpactin I) antibodies. Thus, under conditions in which the phosphorylated

form accumulates in discrete amounts, anti-(calpactin I) antibodies recognize a doublet of bands (p36 and p39). In different experiments after 1 h of treatment with PDGF we have estimated that about 10–15% of the protein is in the 39 kDa form.

The tyrosine-phosphorylated form of calpactin I is barely detectable in fibroblasts stimulated with PDGF alone (Fig. 1, lanes a–e). However, orthovanadate appears to act synergistically with PDGF in inducing and maintaining the phosphorylation of calpactin I. Although vanadate has been reported to induce several cellular effects [35,36], we used it as a specific inhibitor of phosphotyrosyl phosphatases [37,38]. In effect, it allows a more intense phosphorylation of the receptor and of its substrates. In spite of the presence of vanadate, however, the phosphorylation of the receptor and of most of the substrates remains a transient event. On the contrary, the phosphorylation of calpactin I occurs with some delay and appears to be long-lasting.

Calpactin I is not phosphorylated at 4 °C, whereas the receptor and most of the other substrates are phosphorylated at low temperatures as well as at 37 °C. This finding may suggest that p36 is not a direct substrate of the PDGF receptor kinase. Another indication of this came from the acid wash experiment. Whereas the phosphorylated bands corresponding to the receptor and to other substrates were made very faint by this treatment, the p39 band was completely unaffected. As shown before [20], the acid treatment removes cell-surface-bound PDGF, causing inactivation of the kinase and dephosphorylation of the receptors. On this basis, one can expect that substrates which are directly phosphorylated by the receptor (whose phosphorylated state depends on the continuous activity of the kinase) are rapidly dephosphorylated as a consequence of the acid treatment. It may be recalled that PLC- γ , which is phosphorylated both at 37 °C and at 4 °C in response to EGF, and is no longer recognized by anti-*P*-Tyr antibodies if cells are treated with acid, is a direct substrate of the EGF receptor [16,39]. On the other hand, indirect substrates may be phosphorylated by intracellular kinase(s), which in turn are activated by the receptors. These substrates should be more stable upon removal of the ligand.

In the case of calpactin I phosphorylation, possible kinases are members of the *src* family of tyrosine kinases. The kinase activity of pp60^{c-src} is transiently increased following PDGF stimulation of quiescent fibroblasts [40,41], and this observation has been recently confirmed and extended to *c-fyn* and *c-yes* [42]. Moreover, these kinases have been found to become associated with the activated PDGF receptor [42]. On the other hand, calpactin I has been found to be phosphorylated by pp60^{v-src} *in vivo* and *in vitro* [7,8]. Thus the effect of PDGF on calpactin may be mediated by a *src*-like kinase.

Interaction of calpactin with membranes and phospholipids is largely conditioned by Ca^{2+} : on the one hand, phospholipids decrease the K_m of calpactin I for Ca^{2+} , and on the other, calpactin associates with phospholipid-containing liposomes in the presence of micromolar Ca^{2+} concentrations [3]. We have found that the phosphorylated form of calpactin behaves in the same way as the non-phosphorylated protein in its Ca^{2+} -dependent association with membranes. Both p36 and p39 are found in the supernatants if membranes are prepared in the presence of EGTA, whereas both of them remain associated with membranes if they are prepared in the presence of 1 mM- Ca^{2+} . Thus the phosphorylated form is part of the mobile calpactin. In addition, we have shown that calpactin leaks out of permeabilized cells at concentrations of free Ca^{2+} up to 100 nM, whereas it remains associated with cellular structures at 1–10 μM - Ca^{2+} .

Our group has recently shown that in quiescent fibroblasts the basal concentration of cytoplasmic free Ca^{2+} , measured by fura 2 microfluorimetry in single cells, is around 200 nM, and that it increases up to 1–2 μM upon stimulation with PDGF [34]. In

addition, the initial transient rise in free Ca^{2+} is often followed by repeated Ca^{2+} oscillations. It can be suggested that the rise in Ca^{2+} induced by PDGF through the increase in InsP_3 allows the translocation of calpactin from cytoplasm to membranes and cytoskeletal structures, and that this may be a condition for its phosphorylation. It is also possible that vanadate contributes to maintain an elevated Ca^{2+} concentration. We have obtained a direct evidence for this in single mouse oocytes [43]. In addition, as shown in Fig. 7 and already demonstrated by others [27], vanadate stimulates phosphoinositide turnover. Exposure to PDGF and vanadate at low temperature does not increase phosphoinositide turnover, and thus presumably does not alter the Ca^{2+} concentration. If the increase in the concentration of free Ca^{2+} is a condition for the phosphorylation of calpactin I, this could be a good explanation for the fact that calpactin is not phosphorylated at 4 °C.

Finally, another possibility that could explain the temperature-dependence of the phosphorylation of calpactin I and the lag observed after PDGF addition is that receptor internalization may be a prerequisite for the tyrosine phosphorylation of calpactin I. However, for calpactin II, which behaves in a similar manner in response to EGF, this has been shown not to be the case [44].

In summary, our analysis of the tyrosine phosphorylation of calpactin I induced by PDGF indicates that calpactin I heavy chain may not be a direct substrate of the PDGF receptor and that other events, which are part of the response to PDGF at 37 °C, have to take place to allow the phosphorylation. The rise in intracellular Ca^{2+} may be important in this respect.

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