Cell-cycle-dependent association of protein phosphatase 1 and focal adhesion kinase

Marco FRESU^{*1}, Mariarita BIANCHI^{*}, J. Thomas PARSONS[†] and Emma VILLA-MORUZZI^{*}

^{*}Department of Experimental Pathology, University of Pisa, Via Roma 55 56126 Pisa, Italy, and [†]Department of Microbiology and Cancer Center, University of Virginia Medical School, Hospital West Charlottesville, VA 22908, U.S.A.

Immunofluorescence studies with protein phosphatase-1 (PP1) isoforms-specific antibodies detected PP1 δ , but not α or γ 1, at focal adhesions. PP1 δ also co-immunoprecipitated with the focal adhesion kinase (FAK) and the α v-integrin. In the present study glutathione S-transferase (GST)–PP1 δ pulled-down FAK from fibroblasts extract and the interaction domain localized between residues 159 and 295 of δ . The association was confirmed by the ability to GST–FAK-related non-kinase (FRNK) to pull-down PP1 δ from fibroblasts extract. GST–FRNK also pulled-down purified muscle PP1 catalytic subunit, thus indicating direct interaction between FAK and PP1. FAK displays consensus sequences for phosphorylation by cell division cycle kinase-2–cyclin B, and might be a PP1 substrate. In fact, FAK immunoprecipitated from metabolically-labelled mitotic HeLa cells without tyrosine phosphatase inhibitors was phosphorylated on Ser only and was dephosphorylated *in vitro* by purified muscle PP1, with loss of phospho-Ser. No PP1 was associated with FAK immunoprecipitated from mitotic HeLa cells. However, pro-

gressively more PP1 activity was assayed in FAK-immunoprecipitates obtained from cells released from mitosis. The associated activity was maximal at 2 h from the mitotic release (when 85–90 % of the cells remained round) and decreased to basal level by 8 h (when cells were all polygonal). At the same time FAK underwent dephosphorylation, which was completed by 4 h. FAK obtained from cells at 1.5 h was Ser-phosphorylated, and underwent dephosphorylation during *in vitro* incubation, with loss of phospho-Ser, indicating the presence of active FAK-bound phosphatase. The only FAK-associated PP1 isoform between 1 and 8 h was PP1 δ . The results suggest that FAK dephosphorylation by PP1 δ occurs in cells released from mitosis, and confirmed the specific association of PP1 δ , as detected previously in adherent cells.

Key words: adhesion-associated protein tyrosine kinase, cell adhesion, mitosis, protein phosphorylation, serine/threonine phosphatase type 1.

INTRODUCTION

Protein phosphatase-1 (PP1) is, together with PP2A, the major protein Ser/Thr phosphatase in mammalian cells (reviewed in [1–2]). PP1 is found in membranes and in specific cell structures, including the nucleus [3–6]. The PP1 holoenzyme consists of a catalytic subunit bound to one among various unrelated regulatory subunits, which target the enzyme to specific functions and confer substrate specificity. In mammalian cells three isoforms of the PP1 catalytic subunit are found: α , γ 1 and δ (also called β). Though products of different genes [1–2,7,8], the isoforms are over 90 % identical and display significant sequence differences only at the C-termini. In fact, isoform-specific antibodies can be obtained only using the C-terminal peptides [6,9,10]. Studies with such antibodies indicated differential tissue and subcellular localization of the isoforms [6,11–16], suggesting association with specific regulatory subunits and differential functions. The C-terminal regions also contain regulatory sites that are phosphorylated by cell division cycle kinase-2 (cdc2) [17,18] and by Tyr kinases, such as src and abl [19].

In immunofluorescence studies we found that PP1 δ , but not PP1 α , or PP1 γ 1, localized to focal adhesions (FAs) [16,20]. Moreover, PP1 δ was found also in anti-FAK and anti-(α v-integrin) immunocomplexes, thus confirming the association with FAs components [20]. FAs are sites of contact between the

cytoskeleton and the extracellular matrix via transmembrane integrins. In these multimolecular structures the extracellular information is integrated and transduced, to eventually control cell shape and motility, as well as cell proliferation (reviewed in [21,22]). The focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that is recruited to FAs and activated upon cell attachment and spreading. FAK may be also involved in the positive regulation of cell motility, and high levels of FAK mRNA have been related to tumour invasiveness and metastasis (see [23–25] for review). Though most of its phosphorylation is on Tyr, FAK is also phosphorylated on Ser/Thr [23] and displays consensus sequences for cdc2–cyclin B at Ser⁷²² and Ser⁹¹¹ [26]. Though phosphorylation of Ser⁹¹¹ has been related to FAK degradation during apoptosis [26], the role of serine phosphorylation in FAK regulation is still mostly unclear.

In addition to FAK, other FAs proteins (such as paxillin and p130^{cas}) are also phosphorylated on Ser/Thr. The kinases involved include protein kinase C [22], Rho-associated kinases [27] and the integrin-linked kinase [28]. All the substrates of these kinases also represent potential substrates for PP1 or for PP2A, the other Ser/Thr phosphatase that has been related to adhesion [29].

In the present study we confirmed and characterized the association of PP1 with FAK, and indicated a possible role for PP1 δ in the dephosphorylation of FAK at mitotic exit. In fact,

Abbreviations used: cdc2, cell division control kinase-2; DMEM, Dulbecco's modified Eagle's medium; FA, focal adhesion; FAK, focal adhesion kinase; FRNK, FAK-related non-kinase; GST, glutathione S-transferase; IPTG, isopropyl β -D-thiogalactoside; PP1, protein phosphatase-1; Tos-Phe-CH₂Cl, tosylphenylalanylchloromethane.

¹ To whom correspondence should be addressed (villa@biomed.unipi.it).

previous work demonstrated that PP1 is inactivated at M-phase and reactivated at M-exit [17,18,30]. Active PP1 is required for the cell to exit from mitosis [31], and a major substrate dephosphorylated by PP1 at the M/G1 transition is the product of the retinoblastoma gene [32,33]. Consequently we investigated whether PP1 may be involved also in the dephosphorylation of FAK at the exit from mitosis.

MATERIALS AND METHODS

Materials

PMSF, benzamidine, tosylphenylalanylchloromethane (Tos-Phe-CH₂Cl) ('TPCK'), leupeptin, soya bean trypsin inhibitor, Protein A-Sepharose, Protein A-peroxidase, nocodazole, media and additives for cell culture were purchased from Sigma. Glutathione-Sepharose 4B, the pGEX vectors, anti-(glutathione S-transferase) (GST) antibodies, [³²P]H₃PO₄, [³²P]ATP, Hyperfilms-ECL[®] and -MP and the ECL[®] reagents were from Amersham Pharmacia Biotech. The restriction enzymes were from New England Biolabs. *Taq* DNA polymerase and the rapid DNA ligation kit were from Boehringer Mannheim. The chemicals for electrophoresis were from Bio-Rad. Immobilon-P was from Millipore. The PP1 isoform-specific antibodies were raised in rabbits injected with the C-terminal peptides of the isoforms [6,10]. The affinity-purified anti-FAK polyclonal antibodies (C-20) were from Santa Cruz. The PP1 catalytic subunit (37 kDa protein by electrophoresis) used in Figures 2(B) and 3 was purified from rabbit muscle as a mixture of the three isoforms and was enzymically active [34]. The recombinant PP1 α , γ 1 and δ used in Figures 2(A) and 6 were produced in bacteria as 37 kDa proteins and kindly supplied by Dr E.Y.C. Lee (Department of Biochemistry/Molecular Biology New York Medical College, Valhalla, NY, U.S.A.).

Plasmid preparation, bacterial growth and extracts

For the GST-fusion proteins of residues 159–295 (construct 2) and 295–327 (C-terminal tail construct 3) of PP1 δ , amplimers were produced by PCR, using oligonucleotides derived from the rat PP1 δ cDNA sequence: 5'-CTAGAATTCCCTATAGCTGCTAT-3' and 5'-ATCCTCGAGCAATATCTGGAATG-3' (antisense) for construct 2 and 5'-CTAGAATTCTTGAAACCATCTGA-3' and 5'-ATCCTCGAGATTCTGTCTTCAC-3' (antisense) for construct 3. The template DNA for amplification was full-length rat PP1 δ cDNA in pBluescript SK, kindly supplied by Dr M. Nagao (Biochemistry Division, National Cancer Center, Tokyo, Japan). The fragments were cloned into the *Eco*RI and *Xho*I sites of the pGEX4T-1 vector. BL-21 protease-minus bacteria were used for GST-fusion proteins production. The bacteria were grown at 37 °C in Luria-Bertani broth plus ampicillin, induced with 0.1 mM isopropyl β -D-thiogalactoside (IPTG) for 2 h, collected by centrifugation and resuspended in 25 mM Tris/HCl (pH 7.5), 150 mM NaCl, 2 mM 2-mercaptoethanol (bacterial lysis buffer) and protease inhibitors (0.02 % benzamidine, 0.02 % PMSF, 0.02 %, TPCK and 4 μ g/ml leupeptin). After quick freezing and thawing, 0.25 % Triton X-100, 50 units/ml DNase, 10 mM MnCl₂ and 10 mM MgCl₂ were added, followed by 30 min rotation at 4 °C and 10000 *g* centrifugation for 20 min. The extract obtained was either used immediately in pull-down assays or stored at -20 °C for subsequent use. The same procedures were used for the bacteria expressing the other GST-fusion proteins: GST-full-length rabbit PP1 α (supplied by Dr N. Berndt, Division of Hematology

and Oncology, Children's Hospital Los Angeles, Los Angeles, CA, U.S.A. and Dr J. W. Ludlow, Department of Biochemistry, University of Rochester, Rochester, NY, U.S.A.), GST-full-length chicken PP1 δ (supplied by Dr M. Ito, Department of Internal Medicine, Mie University, Mie, Japan) and GST-FAK-related non-kinase (FRNK) (residues 684–1054 corresponding to the C-terminal domain of chicken FAK, subcloned into pGEX-2TK) [35].

Cell culture and extracts

Fisher rat fibroblasts and HeLa cells were grown at 37 °C in water-saturated CO₂, in Dulbecco's modified Eagle's Medium (DMEM) added with 6 % or 8 % fetal calf serum respectively [30]. Following two washes in cold PBS, the cells were lysed in 50 mM Tris/HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 0.1 % Triton X-100 (cell-lysis buffer) and protease inhibitors as for bacterial lysis buffer.

Mitotic block and release into G1

HeLa cells were blocked in mitosis and released from the block as described previously [30]. In brief, HeLa cells were exposed to 50 ng/ml nocodazole for 16–18 h, the mitotic cells were collected by gentle pipetting, and either washed in PBS and lysed in cell lysis buffer (see above) or washed in DMEM at 37 °C, resuspended in DMEM/10 % fetal calf serum, re-plated and cultured for the specifically indicated time. The mitotic cells obtained from four 150-mm plates were plated on to one 100-mm plate or, proportionally, on to several 60-mm or 30-mm plates.

Metabolic labelling of mitotic cells and of cells released into G1

At 12 h after the addition of nocodazole, the medium was changed into H₃PO₄-free medium containing 50 ng/ml dazole and 10 % fetal calf serum (H₃PO₄-free). After 1 h the medium was changed into the same medium with the further addition of [³²P]H₃PO₄ (1 mCi/150 mm plate, in 1 ml of medium) and left for a further 5 h, when the mitotic cells were collected [30]. For further labelling of the cells released into G1, after washing in [³²P]H₃PO₄-free medium, the cells were released from mitosis in a medium that still contained [³²P]H₃PO₄ [0.2 or 0.5 mCi/ml of medium for the experiments of Figure 4 (inset) and 5 respectively]. In order to preserve Ser and Thr phosphorylations, the extraction buffer (see above) contained in addition 1 μ M okadaic acid.

Immunoprecipitation and immunoblotting

Purified anti-FAK polyclonal rabbit antibodies (20 μ l/ml of cell extract) or anti-PP1 α , -PP1 γ 1 or -PP1 δ hyperimmune rabbit sera (10 μ l/ml of cell extract) were used for immunoprecipitation with Protein A-Sepharose [6,10,30,33]. Following incubation at 4 °C for 90 min with shaking and three washes in cell lysis buffer containing 0.02 % PMSF and 0.02 % benzamidine, the immune complexes were boiled in Laemmli buffer. For further PP1 treatment (Figure 3), the immune complexes were washed once more with 25 mM imidazole, pH 7.5, resuspended in 25 μ l of the same buffer with 2 mM 2-mercaptoethanol, incubated with purified PP1 [34], as specifically indicated, and boiled in Laemmli buffer. Electrophoresis was on SDS/PAGE (10 % gel) and Immobilon-P was used for transblotting. The membrane was probed with the indicated antibodies (diluted either 1:1000 in PBS or as specifically indicated by the manufacturer), followed by Protein A-peroxidase and ECL[®], according to the manu-

facturer's instructions [6,30]. For reprobing with a second antibody, the membrane was incubated in 5 mM phosphate buffer, 2% SDS and 2 mM 2-mercaptoethanol at 60 °C for 30 min.

In vitro dephosphorylation of FAK by the associated PP1

FAK was immunoprecipitated from metabolically labelled cells released from mitosis for 1.5 h and extracted with okadaic acid-containing buffer (see above). Before washing, the anti-FAK complex obtained from 20 mg of cell extract was divided into six aliquots. Three aliquots were washed four times in cell lysis buffer containing 35 mM NaF, once in 25 mM imidazole, pH 7.5, also containing NaF, resuspended in phosphatase assay buffer (see below) containing 1 μ M okadaic acid and incubated as shown in Figure 5. The other three aliquots were washed and incubated in the same way, but without PP1 inhibitors (okadaic acid or NaF). At the end of the incubation the samples were boiled in Laemmli buffer, subjected to electrophoresis and transfer to Immobilon-P, for subsequent immunodetection and autoradiography.

Two-dimensional phosphoaminoacid analysis

Phosphoproteins, transferred to Immobilon-P, were hydrolysed in 6 M HCl at 110 °C for 1 h and analysed as described previously [36] (see also [30]).

Phosphatase assay on the FAK-immunocomplexes

FAK was immunoprecipitated and washed as indicated above. This was followed by one additional wash in 25 mM imidazole, pH 7.5, resuspension of the immunocomplexes in 25 μ l of 25 mM imidazole/2 mM 2-mercaptoethanol (phosphatase assay buffer) and addition of 25 μ l of 3 mg/ml 32 P-labelled phosphorylase *a* for a 20 min phosphatase assay at 30 °C (as described further in [6,10,30]). Okadaic acid (5 nM) was added to some of the assays in order to inhibit any PP2A if present. The phosphatase activity was calculated after subtracting the radioactivity of control immune complexes, prepared with pre-immune instead of hyperimmune serum, and run in parallel. The phosphatase activity is given as (fmol of P_i released from the substrate/min) $\times$ mg of cell extract used for the immunoprecipitation.

Pull-down assays

GST-fusion proteins from bacterial extracts were bound to 25–50 μ l of glutathione-Sepharose beads, pre-treated with lipid-free BSA. After 1.5 h of rotation at 4 °C and three washes with bacterial lysis buffer (containing Triton X-100, benzamidine and PMSF, as above) the beads were mixed either with cell extract or with purified proteins (diluted in cell extraction buffer). This was followed by incubation and processing as described for immunoprecipitation and immunoblotting.

RESULTS

Association of cellular FAK with GST–PP1 δ

The finding of PP1 δ in FAs and in anti-FAK immune complexes suggested the interaction between PP1 δ and FAK [16,20]. In order to confirm this association, various GST–PP1 forms were produced in bacteria and used in pull-down assays. We found that GST–PP1 δ (full-length protein) pulled-down FAK from fibroblasts extract (Figure 1; FAK blot, which also shows FAK immunoprecipitated from the same extract). In order to further

Figure 1 Pull-down of cellular FAK with PP1 δ

GST–PP1 δ fusion proteins with either full-length (δ) or residues 159–295 (construct 2) or residues 295–327 (construct 3), or GST alone (G), were bound to glutathione-Sepharose beads and used to pull-down FAK from Fisher rat fibroblasts extract (1.5 mg/pull-down). Following electrophoresis by SDS/PAGE (10% gel) and transblotting onto Immobilon-P, immunodetection used first the anti-FAK antibodies to detect the pulled-down FAK (FAK blot). This was followed by removal of the first antibody and re-probing with the anti-GST antibodies to detect δ , 2, 3 or GST alone (GST blot). Lane F shows FAK immunoprecipitated from 0.3 mg of fibroblasts extract. Molecular mass standards (k refers to kDa): 116 k, β -galactosidase; 68 k, BSA; 45 k, ovalbumin; 29 k, carbonic anhydrase.

characterize this interaction, we prepared the GST-fusion proteins of two deletion mutants of PP1 δ : construct 2 (residues 159–295; this sequence is over 95% identical in the three PP1 isoforms), and construct 3 (residues 295–327), reproducing the C-terminus, which is specific to PP1 δ . Construct 2 was able to pull-down FAK, though the FAK signal was on average lower than that obtained with GST–PP1 δ (as in the experiment shown in Figure 1). On the other hand the C-terminus and GST alone did not pull-down FAK, (Figure 1; GST blot). The proteins additionally detected in the FAK blot were bacterial proteins, which were revealed by Amido Black staining and were most likely not related to FAK. Since the PP1 δ domain apparently responsible for the interaction with FAK is almost identical in the three PP1 isoforms (see the Introduction section), we performed pull-down assays also with full-length GST–PP1 α and GST–PP1 γ 1 and found that both of them pulled-down FAK (results not shown).

Altogether, the results indicated that PP1 δ was able to associate with cellular FAK *in vitro*; and that the interaction domain localized to the half of the PP1 molecule towards the C-terminus, but did not include C-terminus.

Association of cellular and purified muscle PP1 with GST–FRNK

The association between PP1 and FAK was also demonstrated by the opposite experiment, namely pulling-down PP1 with FAK. Substituting for FAK, which is difficult to obtain in soluble form, we used its C-terminal, non-kinase domain, known as FRNK [37]. This binds most of the interactors of FAK, and can be obtained abundantly as a GST-fusion protein. The specific construct used encoded residues 686–1054 of chicken FAK. GST–FRNK pulled-down PP1 δ from fibroblasts extract (Figure 2A; PP1 δ blot). In addition, minor amounts of PP1 α and PP1 γ 1 were also detected in parallel pull-down assays (Figure 2A, PP1 α and PP1 γ 1 blots). The identification of the isoforms was con-

Figure 2 Pull-down of the cellular PP1 isoforms with FRNK (A) and direct association of purified PP1 with FRNK (B)

(A) GST–FRNK (F) or GST alone (G) were bound to glutathione–Sepharose beads and used to pull-down PP1 from Fisher rat fibroblasts extract (1.5 mg/pull-down). Three blots were run in parallel and probed with the isoform-specific antibodies to detect PP1 α , γ 1 and δ (α , γ 1 and δ blots respectively). This was followed by removal of the first antibody and re-probing with the anti-GST antibodies, to detect GST–FRNK or GST. Only one of the three films obtained is shown (GST blot). Recombinant PP1 isoforms (30 ng of each 37 kDa protein; see the Materials and methods section) were added to the electrophoresis as positive controls (shown in the α , γ 1 and δ lanes). (B) Either GST–FRNK (F) or GST alone (G) were bound to glutathione–Sepharose beads and used to pull-down 10 μ g of PP1 catalytic subunit (37 kDa protein, mixture of the three isoforms purified from rabbit muscle). The blot obtained was first probed with the anti-PP1 antibodies (mixture of the three isoform-specific antibodies, PP1 blot) to detect the pulled-down PP1, then re-probed with the anti-GST antibodies to detect GST–FRNK or GST (GST blot). Purified PP1 was also added to the electrophoresis as positive control (PP1 lane). Molecular mass standards are as in Figure 1.

firmed by the use of recombinant PP1 α , γ 1 and δ as positive controls in the immunoblot (in this case 37 kDa proteins were used, i.e. of the same mass as cellular PP1; see the Materials and methods section). The results indicated differential pull-down of the isoforms, since in the extract used (likewise for other cell extracts) the isoforms were present in amounts that looked similar, within the limits of comparing signals from different antibodies (results not shown, but see [6,10,30,38] for the analysis of the PP1 isoforms in various cell extracts with these same antibodies). The apparent preferential association of PP1 δ was in line with our previous finding of only PP1 δ at FAs [20], and was further confirmed by the results shown in Figure 6.

Figure 3 Phosphorylation of FAK in metabolically labelled mitotic cells and *in vitro* dephosphorylation by muscle PP1

HeLa cells (on six 150-mm plates) were exposed to nocodazole for 17 h and to [32 P]H $_3$ PO $_4$ during the last 5 h of nocodazole-treatment (see the Materials and methods section). Two anti-FAK immunoprecipitates were prepared from the mitotic cell extract (FAK IP), one of which was additionally treated at the end of the immunoprecipitation with 2 units of purified muscle PP1 at 30 °C for 20 min (+PP1, same preparation as in Figure 2B). This was followed by electrophoresis, blotting, probing to identify FAK (FAK blot) and exposure for radioactivity detection (autorad.). The two protein bands corresponding to FAK were then cut from the Immobilon-P membrane, hydrolysed and subjected to two-dimensional phospho amino acid (PAA) analysis (–PP1 and +PP1; see the Materials and methods section). Unlabelled Phospho-Ser (S), Phospho-Thr (T) and Phospho-Tyr (Y) were added to each sample before the high-voltage electrophoresis, stained with ninhydrin at the end of the separation and used to identify the position of the phospho amino acids on the autoradiographs.

The above results suggested direct interaction between PP1 and FAK. However, the experiments always used cell extracts and the presence of an additional component mediating the interaction cannot be excluded. In order to clarify this point, the pull-down assay was performed with purified PP1 catalytic subunit. This was obtained from rabbit muscle by a procedure that removes all the regulatory subunits [34], yielding PP1 which migrates at 35 kDa by gel filtration and is a mixture of the three isoforms. PP1 was diluted in cell-lysis buffer and used in pull-down experiments with GST–FRNK, as described above. GST–FRNK, but not GST alone, pulled-down PP1 (Figure 2B; PP1 blot), thus indicating direct interaction between the two proteins. Muscle PP1 was also used as positive control in the immunoblot (Figure 2B).

These experiments demonstrated the interaction between PP1 and FAK or FRNK, and suggested that the interaction is direct. The remainder of this study addressed the issue of whether FAK might be a PP1 substrate. Since FAK displays consensus

sequences for the mitotic kinase cdc2-cyclin B at Ser⁷²² and Ser⁹¹¹ (see the Introduction section), we investigated the phosphorylation of FAK in mitotic cells and whether PP1 might be involved in its dephosphorylation.

Phosphorylation of FAK in mitotic cells and its *in vitro* dephosphorylation by PP1

HeLa cells were blocked in pseudo-mitosis with nocodazole and metabolically labelled by exposing them to [³²P]H₃PO₄ during the last 5 h of nocodazole treatment. The round mitotic cells (representing 50–60 % of the total cells) were collected and lysed in the presence of 1 μ M okadaic acid, to preserve Ser/Thr phosphorylations, and in the absence of Tyr-phosphatase inhibitors, to eliminate FAK Tyr-phosphorylation. The immunoprecipitated FAK was found phosphorylated (Figure 3, –PP1 autorad.) and the only phospho amino acid detected following FAK hydrolysis was phospho-Ser (Figure 3, –PP1 PAA; note the absence of any phospho-Tyr, although protein hydrolysis was performed under conditions that would preserve phospho-Tyr, if present). A second immunoprecipitate, prepared in tandem, was treated before electrophoresis with active PP1 purified from skeletal muscle. Muscle PP1 was used instead of recombinant PP1 since the latter has very poor specific activity. PP1 treatment caused FAK dephosphorylation with loss of phospho-Ser (Figure 3, +PP1 autorad. and +PP1 PAA). The results showed that Ser-phosphorylated FAK was an *in vitro* substrate for PP1, hence potentially also an *in vivo* substrate. This might occur at the exit from M-phase, when PP1 is activated. In fact the PP1 activity decreases in mitotic cells, and increases again at mitotic exit, when different PP1 forms are reactivated in a complex way that is not yet fully understood (see e.g. [18,30,31]).

Activation of the FAK-associated PP1 and FAK dephosphorylation in cells released from the mitotic block

In order to investigate the association of PP1 with FAK in cells released from mitosis, mitotic HeLa cells were washed to remove nocodazole and allowed to enter G1 (see the Materials and methods section, and [30]). Mitotic cells as well as cells collected at 0.5, 1, 2, 4 and 8 h from the mitotic release were used to prepare the extracts. At 2 h all the cells were attached to the plate, but mostly still round, apart from 10–15 % of polygonal cells. The polygonal cells increased to up to 50 % at 4 h and to over 95 % at 8 h. FAK was immunoprecipitated and the immunocomplexes were assayed for the presence of associated PP1 activity. No activity was found in the FAK-complexes from mitotic cells (Figure 4, time 0 min). As the cells were released from mitosis, the FAK-associated PP1 activity increased, reaching a peak at 2 h and returning to basal level by 8 h (Figure 4). Some of the assays were run in the presence of 5 nM okadaic acid, in order to inhibit any PP2A. However, no difference was noticed with or without the PP2A inhibitor. In addition, an immunoblot indicated that no PP2A was present in the anti-FAK complexes (results not shown).

In a parallel experiment the mitotic cells were metabolically labelled (as in Figure 3) and released in the presence of [³²P]H₃PO₄ (see the Materials and methods section). The immunoprecipitated FAK was still phosphorylated at 30 min and 1 h, but was totally dephosphorylated by 4 h after mitosis (Figure 4, inset), indicating that FAK underwent dephosphorylation during this time. This, together with the activation of the FAK-associated PP1, suggested the involvement of PP1 in FAK dephosphorylation.

Figure 4 Association of PP1 activity with FAK in cells released from the mitotic block into G1

Mitotic HeLa cells were washed to remove nocodazole, re-plated and grown for up to 8 h (see the Materials and methods section). Aliquots (3 mg) of extract prepared from cells collected at the indicated time-points were used to immunoprecipitate FAK and assay the associated PP1 activity (using [³²P] phosphorylase *a* as a substrate; see the Materials and methods section). The activity of control complexes (prepared with the same amount of extract used for the FAK-immunoprecipitates and pre-immune serum) was subtracted from each FAK-associated activity during the PP1 activity calculations. The results are expressed as mean ($\pm$ S.E.M. of three experiments). Inset: phosphorylation of FAK in cells released from mitosis for up to 4 h. [³²P] H₃PO₄ metabolically labelled mitotic HeLa cells were released from the mitotic block as above and re-plated in the presence of [³²P]H₃PO₄ (see the Materials and methods section). FAK was immunoprecipitated from 2 mg of the cell extract obtained at the indicated time-points. The blot obtained was first probed to detect FAK (FAK blot) and then exposed for radioactivity detection (autorad.).

In vitro dephosphorylation of FAK by the FAK-associated phosphatase

In order to confirm the dephosphorylation of FAK by the PP1 present in the FAK immunocomplexes, metabolically labelled FAK was obtained from cells at 1.5 h after the mitotic release. Extraction and immunoprecipitation were performed in the presence of okadaic acid (in its absence FAK was totally dephosphorylated), followed by extensive washing to remove okadaic acid and reactivate PP1, and incubation at 30 °C. Control samples prepared in the presence of okadaic acid were processed in parallel (see the Materials and methods section). The results indicated FAK dephosphorylation by the associated PP1, which was minor at 45 min but became marked by 120 min incubation (Figure 5A). On the other hand okadaic acid preserved phosphorylation throughout the incubation (Figure 5A). Phospho amino acid analysis detected the presence of only phospho-Ser at all time-points (Figure 5B). This result, together with the results of Figure 3, indicated that phospho-Ser was the only phospho amino acid present in FAK from both mitotic and

Figure 5 *In vitro* dephosphorylation of FAK from cells at 1.5 h from the mitotic release by the FAK-bound PP1 (A) and phospho amino acid analysis (B)

(A) HeLa cells were metabolically labelled with [32 P]H $_3$ PO $_4$ during both mitosis and release (see Figure 4, and the Materials and methods section) and collected at 1.5 h after mitosis. FAK was immunoprecipitated from 20 mg of cell extract, divided into six aliquots and incubated at 30 °C for the indicated time in the presence (+) or in the absence (-) of the PP1 inhibitor okadaic acid (OA), as described in detail in the Materials and methods section. This was followed by electrophoresis, blotting, probing to identify FAK (FAK blot) and exposure for radioactivity detection (autorad.). (B) The FAK protein bands were cut from the Immobilon-P membrane, hydrolysed and subjected to two-dimensional phospho amino acid analysis as in Figure 3. In order to reduce the exposure time, equivalent material pooled from three independent experiments was used for each phospho amino acid. Experiments were carried out in the presence (+OA) or absence (-OA) of okadaic acid.

early G1 cells, when FAK was extracted and processed in the absence of tyrosine phosphatase inhibitors. On the other hand, the use of buffers containing 1 mM orthovanadate preserved phospho-Tyr in FAK, and tyrosine phosphorylation of FAK was higher in cells released from mitosis than in mitotic cells (results not shown).

Association of PP1 δ with FAK in cells released from the mitotic block

In order to find out which PP1 isoform was present in the FAK immunocomplexes, three sets of FAK-immunoprecipitates were

Figure 6 Association of PP1 δ with FAK in cells released from the mitotic block for up to 8 h

Aliquots (3 mg) of extract prepared from mitotic HeLa cells (M) or cells collected at 1, 2, 4 and 8 h after the mitotic release (see also Figure 4, and the Materials and methods section) were used to immunoprecipitate FAK (FAK IP). Two sets of samples were run in parallel and used to prepare two blots. The first blot was probed with the anti-PP1 δ antibodies (δ blot), the second with the anti-PP1 α antibodies (α -blot), followed by re-probing with the anti-FAK antibodies (FAK blot). Recombinant δ or α were added to the electrophoresis as positive controls (δ and α lanes; see also Figure 2A). Molecular mass standards are as in Figure 1.

prepared from mitotic cells and cells released from mitosis (two of them are shown in Figure 6) and probed with the anti-PP1 δ , anti-PP1 α and anti-PP1 γ 1 antibodies respectively. This detected the presence of PP1 δ in the anti-FAK complexes obtained from the cells released up to 8 h (a decrease was noticed at 8 h), whereas no associated PP1 δ was detected at M-phase (Figure 6; δ blot). The 45 kDa protein also visible on the blot, and not related to PP1, might be a FAK-associated protein which becomes abundant at 2 and 4 h, hence detected by ECL[®]. This, in turn, might reflect the complex changes in protein

interactions involved in FA formation. On the other hand, neither PP1 α (Figure 6; α blot) nor PP1 γ 1 (results not shown) were detected throughout the release. The blots were subsequently re-probed to detect FAK. Re-probing of the anti-PP1 α blot is shown in Figure 6 (FAK blot).

Altogether the immunoblot analysis indicated that the isoform most likely responsible for FAK dephosphorylation in cells released from mitosis was PP1 δ . Detection of no FAK-associated PP1 δ at mitosis was consistent with the assay of no associated PP1 activity in these same cells (Figure 4) and indicated that PP1 δ associated with FAK after mitosis. This was also consistent with the finding that in mitotic cells FAK was phosphorylated (Figure 3). Additionally, the δ protein level did not change significantly between 1 and 4 h, when the PP1 assay showed significant variation in the activity. This might also be compatible with further activation of the associated PP1.

DISCUSSION

In this study we reported the first demonstration of direct association of PP1 with FAK. PP1 δ was the only isoform detected in association with FAK in cells released from mitosis and the isoform preferentially pulled-down by GST-FRNK. These results were in agreement with the original observation about the presence of only PP1 δ in FAs and its association with FAK immunoprecipitated from fibroblasts [16,20]. On the other hand, FAK was pulled-down also by GST-PP1 α and GST-PP1 γ 1, explained by considering the fact that the FAK-binding region of PP1, as well as the overall PP1 sequence (excluding the C-terminus), are highly conserved among the isoforms. The isoform specificity observed *in vivo* (Figure 6 and [20]) cannot be explained by the present results. One possibility might be the presence of an isoform-specific PP1 regulatory subunit, modulating the interaction between δ and FAK and favouring the association of PP1 δ over the other isoforms. Based on this hypothesis, the search for this putative regulatory subunit is currently undertaken through gel filtration and affinity chromatography experiments. Future work will need also to clarify if the FAK-associated PP1 is in any way related to the myosin phosphatase. This enzyme is involved in the regulation of the cytoskeleton during cell migration, and displays also the PP1 δ isoform [16]. The myosin phosphatase is inactivated by Rho-kinase phosphorylation on its myosin-binding subunit [39], but does not seem to localize to FAs [16]. On the other hand our experiments excluded the involvement of PP2A, another Ser/Thr phosphate involved in cell adhesion (e.g. [29]). In fact, immunoblot analysis of the anti-FAK immunoprecipitates did not detect any PP2A, and some of the assays of the FAK-associated PP1 activity were run in the presence of 5 nM okadaic acid, which would inhibit any PP2A present.

The experiments also indicated that the domain of FAK that interacts with PP1 is located in its C-terminal, non-kinase domain, corresponding to the FRNK sequence [37], which is known to bind most of the FAK-interactors and also to compete with FAK *in vivo*. However, analysis of the complete FAK amino acid sequence also indicated the presence of the KRFVHR sequence (residues 540–545 of chicken FAK) that is outside of FRNK (residues 684–1054 of FAK). The KRFVHR sequence is similar, though not identical with the (R/K)(V/I)XF motif present in PP1 regulatory subunits [40]. Whether this motif might represent a second PP1-binding site will be investigated by preparing FAK-deletion mutants of this region and using them to pull-down PP1.

In order to investigate the role of the association of PP1 with FAK, we tested the hypothesis that FAK is a substrate for

PP1. FAK phosphorylation on Ser/Thr is low in adherent cells and it is high in mitotic cells. All the phosphorylation of mitotic FAK was found on Ser, when FAK was extracted in the absence of tyrosine phosphatase inhibitors, a condition that allows dephosphorylation of the Tyr residues of FAK by the endogenous tyrosine phosphatases. The finding of Ser-phosphorylation is in agreement with the presence of two Ser residues (Ser⁷²² and Ser⁹¹¹) with consensus sequences for the mitotic kinase cdc2–cyclin B [26]. We obtained both *in vivo* and *in vitro* evidence that FAK may be a PP1 substrate in cells released from mitosis. In fact, the phospho-Ser of mitotic FAK was removed *in vitro* by PP1. *In vivo* the FAK-associated PP1 was activated as cells were released into G1 and FAK underwent dephosphorylation. Moreover, this PP1 was able to dephosphorylate FAK during a subsequent *in vitro* incubation. The *in vivo* FAK-dephosphorylation preceded cell spreading and was completed by the time when not more than half of the cells were polygonal. The results also indicated that the FAK-bound isoform in cells released from mitosis was PP1 δ . We reported previously the activation of this isoform at mitotic exit [30] and its potential involvement in the dephosphorylation of hyperphosphorylated pRb (the retinoblastoma gene product which is dephosphorylated at mitotic exit, [32,33]). The present results indicated that FAK might represent another substrate for PP1 δ .

The role of FAK phosphorylation at M-phase is not known. One possibility is that Ser-phosphorylated FAK is stored in an inactive state for subsequent use, when FAs will be reassembled. Subsequent dephosphorylation might allow FAK localization to the FAs that are reassembled when cells spread on the substrate and become polygonal.

Cells released from mitosis are in some aspects similar to cells that go from suspension to attachment to a substrate. FAK is known to be recruited to FAs and activated as Tyr-kinase at this time. Little is known of the changes in the Ser-phosphorylation of FAK in such a process. Since PP1 δ was found to localize to FAs in adherent cells it is possible that PP1 as well as Ser/Thr kinases are also involved in the regulation of FAK or of other phosphoproteins that also localize to FAs during cell attachment or spreading.

The financial support of Telethon-Italy and MURST, Rome, to the project is gratefully acknowledged. We thank Dr N. Berndt, Dr J. W. Ludlow, Dr M. Nagao and Dr M. Ito for providing the full-length PP1 clones, Dr E. Y. C. Lee for providing purified recombinant PP1 isoforms used as positive controls in immunoblotting, and Dr Stefano Biffo, (Department of Medical Sciences, University of Alessandria and Novara, Italy) for helping in preparing the deletion mutants.

REFERENCES

- Cohen, P. and Cohen, P. T. W. (1989) Protein phosphatases come of age. *J. Biol. Chem.* **264**, 21435–21438
- Bollen, M. and Stalmans, W. (1992) The structure, role and regulation of Type 1 Protein Phosphatases. *Crit. Rev. Biochem. Mol. Biol.* **27**, 227–281
- Hubbard, M. and Cohen, P. (1993) On target with a new mechanism for the regulation of protein phosphorylation. *Trends Biochem. Sci.* **18**, 172–177
- Villa-Moruzzi, E. and Heilmeyer, Jr., L. M. G. (1987) Phosphorylase phosphatase from skeletal muscle membrane. *Eur. J. Biochem.* **169**, 659–667
- Beullens, M., Van Eyde, A., Stalmans, W. and Bollen, M. (1992) The isolation of novel inhibitory polypeptides of protein phosphatase 1 from bovine thymus nuclei. *J. Biol. Chem.* **267**, 16538–16544
- Tognarini, M. and Villa-Moruzzi, E. (1998) Analysis of the isoforms of protein phosphatase 1 (PP1) with polyclonal peptide antibodies. In *Methods in Molecular Biology*, vol. 93, Protein phosphatase protocols (Ludlow, J. W., ed.), pp. 169–183. The Humana Press, Totowa NJ, U.S.A.
- Sasaki, K., Shima, H., Kitagawa, Y., Irino, S., Sugimura, T. and Nagao, M. (1990) Identification of members of the protein phosphatase 1 gene family in the rat and enhanced expression of protein phosphatase 1 α gene in rat hepatocellular carcinomas. *Jpn. J. Cancer Res.* **81**, 1272–1280

- 8 Zhang, Z., Bai, G., Shima, M., Zhao, S., Nagao, M. and Lee, E. Y. C. (1993) Expression and characterization of rat protein phosphatase-1 α , -1 γ 1, -1 γ 2 and -1 δ . *Arch. Biochem. Biophys.* **303**, 402–406
- 9 Shima, H., Hatano, Y., Chun, Y.-S., Sugimura, T., Zhang, Z., Lee, E. Y. C. and Nagao, M. (1993) Identification of PP1 catalytic subunit isotypes PP1 γ 1, PP1 δ and PP1 α in various rat tissues. *Biochem. Biophys. Res. Commun.* **192**, 1289–1296
- 10 Villa-Moruzzi, E., Puntoni, F. and Marin, O. (1996) Activation of protein phosphatase-1 isoforms and glycogen synthase kinase- β in muscle from mdx mice. *Int. J. Biochem. Cell Biol.* **28**, 13–22
- 11 Ouimet, C. C., Da Cruz e Silva, E. F. and Greengard, P. (1995) The α and γ 1 isoforms of protein phosphatase 1 are highly and specifically concentrated in dendritic spines. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 3396–3400
- 12 Da Cruz e Silva, E. F., Fox, C. A., Ouimet, C. C., Gustafson, E., Watson, S. J. and Greengard, P. (1995) Differential expression of protein phosphatase 1 isoforms in mammalian brain. *J. Neurosci.* **15**, 3375–3389
- 13 Hashikawa, T., Nakazawa, K., Mikawa, S., Shima, H. and Nagao, M. (1995) Immunohistochemical localization of protein phosphatase isoforms in rat cerebellum. *Neurosci. Res.* **22**, 133–136
- 14 Chu, Y., Wilson, S. E. and Schlender, K. K. (1994) A latent form of protein phosphatase 1 α associated with bovine heart myofibrils. *Biochim. Biophys. Acta.* **1208**, 45–54
- 15 Shima, H., Haneji, T., Hatano, Y., Kasugai, I., Sugimura, T. and Nagao, M. (1993) Protein phosphatase 1 γ 1 is associated with nuclei of mitotic cells in rat testis. *Biochem. Biophys. Res. Commun.* **194**, 930–937
- 16 Murata, K., Hirano, K., Villa-Moruzzi, E., Hartshorne, D. J. and Brautigan, D. L. (1997) Differential localization of myosin and myosin phosphatase subunits in smooth muscle cells and migrating fibroblasts. *Mol. Biol. Cell.* **8**, 663–673
- 17 Villa-Moruzzi, E. (1992) Activation of type-1 protein phosphatase by cdc2 kinase. *FEBS Lett.* **304**, 211–215
- 18 Dohadwala, M., Da Cruz e Silva, E. F., Hall, F. L., Williams, R. T., Carbonaro-Hall, D. A., Nairn, A. C., Greengard, P. and Berndt, N. (1994) Phosphorylation and inactivation of protein phosphatase 1 by cyclin-dependent kinases. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 6408–6412
- 19 Villa-Moruzzi, E., Dalla Zonca, P. and Crabb, J. W. (1991) Phosphorylation of the catalytic subunit of type-1 protein phosphatase by the v-abl tyrosine kinase. *FEBS Lett.* **293**, 67–71
- 20 Villa-Moruzzi, E., Tognarini, M., Cecchini, G. and Marchisio, P. C. (1998) Protein phosphatase 1 δ is associated with focal adhesions. *Cell Adhes. Commun.* **5**, 297–305
- 21 Burridge, K. and Chrzanowska-Wodnicka, M. (1996) Focal adhesions, contractility and signalling. *Annu. Rev. Cell Dev. Biol.* **12**, 463–519
- 22 Clark, E. A. and Brugge, J. S. (1995) Integrins and signal transduction pathways: the road taken. *Science (Washington, D.C.)* **268**, 233–239
- 23 Schaller, M. D. and Parsons, J. T. (1993) Focal adhesion kinase: an integrin-linked protein tyrosine kinase. *Trends Cell Biol.* **3**, 258–262
- 24 Schaller, M. D. and Parsons, J. T. (1994) Focal adhesion kinase and associated proteins. *Curr. Opin. Cell Biol.* **6**, 705–710
- 25 Malik, R. K. and Parsons, J. T. (1996) Integrin-mediated signalling in normal and malignant cells: a role of protein tyrosine kinases. *Biochim. Biophys. Acta* **1287**, 73–76
- 26 Ma, A., Richardson, A. and Parsons, J. T. (1997) Serine phosphorylation of FAK and FRNK. In *Signal Transduction and Cell Adhesion Receptors. Keystone Symposium*, Keystone CO, U.S.A., March 31–April 6, abstract 122
- 27 Machesky, L. M. and Hall, A. (1996) Rho: a connection between membrane receptor signalling and the cytoskeleton. *Trends Cell Biol.* **6**, 304–310
- 28 Dedhar, S., Williams, B. and Hannigan, G. (1999) Integrin-linked kinase (ILK): a regulator of integrin and growth-factor signalling. *Trends Cell Biol.* **9**, 319–323
- 29 Villalobos Campos, S. and Schonthal, A. H. (2000) Induction of protein phosphatase type 2A in response to disruption of cell-matrix interactions. *J. Cell. Physiology.* **182**, 88–96
- 30 Puntoni, F. and Villa-Moruzzi, E. (1997) Protein phosphatase-1 α , γ 1 and δ : changes in phosphorylation and activity in mitotic HeLa cells and in cells released from the mitotic block. *Arch. Biochem. Biophys.* **340**, 177–184
- 31 Fernandez, A., Brautigan, D. L. and Lamb, N. (1992) Protein phosphatase type 1 in mammalian cells mitosis: chromosomal localization and involvement in mitotic exit. *J. Cell Biol.* **116**, 1421–1430
- 32 Ludlow, J. W., Glendening, C. L., Livingston, D. M. and DeCaprio, J. A. (1993) Specific enzymatic dephosphorylation of retinoblastoma protein. *Mol. Cell. Biol.* **13**, 367–372
- 33 Puntoni, F. and Villa-Moruzzi, E. (1997) Association of protein phosphatase-1 delta with the retinoblastoma protein and reversible phosphatase activation in mitotic. HeLa cells and in cells released from mitosis. *Biochem. Biophys. Res. Commun.* **235**, 704–708
- 34 Resink, T. J., Hemmings, B. A., Tung, H. Y. L. and Cohen, P. (1983) Characterization of a reconstituted Mg-ATP-dependent protein phosphatase. *Eur. J. Biochem.* **133**, 455–461
- 35 Schaller, M. D., Borgman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B. and Parsons, J. T. (1992) pp125^{FAK}, a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5192–5196
- 36 Kamps, P. M. (1991) Determination of phosphoamino acids composition by acid hydrolysis of protein blotted to immobilon. *Methods Enzymol.* **201**, 21–27
- 37 Richardson, A., Malik, R. K., Hildebrand, J. D. and Parsons, J. T. (1997) Inhibition of cell spreading by expression of the C-terminal domain of Focal Adhesion Kinase (FAK) is rescued by coexpression of src or catalytically inactive FAK: a role for paxillin tyrosine phosphorylation. *Mol. Cell. Biol.* **17**, 6906–6914
- 38 Tognarini, M. and Villa-Moruzzi, E. (1998) Protein phosphatase 1 isoforms in differentiating C2C12 myocytes. *Eur. J. Cell Biol.* **76**, 212–219
- 39 Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K., Iwamatsu, A. and Kaibuchi, K. (1996) Regulation of protein phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science (Washington, D.C.)* **273**, 245–248
- 40 Eglhoff, M.-P., Johnson, D. F., Moorhead, G., Cohen, P. T. W., Cohen, P. and Barford, D. (1997) Structural basis for the recognition of regulatory subunits by the catalytic subunit of protein phosphatase 1. *EMBO J.* **16**, 1876–1887

Received 23 January 2001/5 June 2001; accepted 21 June 2001