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Reciprocally interacting domains of protein phosphatase 1 and focal adhesion kinase

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Abstract

Protein phosphatase 1δ (PP1δ) localizes to focal adhesions and associates with the focal adhesion kinase (FAK). In the present work we used deletion mutants of PP1δ and FAK to detect their reciprocally interacting domains. Dissection of PP1δ indicated 194–260 as the shortest FAK-interacting domain among those tested. Domain 194–260 encompasses several sites involved in catalysis, indirectly confirming that FAK is a PP1 substrate. Mutation of one of these sites, R220 (R220S or R220Q), did not abolish but on the contrary increased the ability of 194–260 to pull-down FAK. Such property might be exploited to detect new potential PP1 substrates. Among the FAK deletion mutants, only the C-terminal domain (684–1053, also known as FRNK) pulled-down a significant amount of PP1. The PP1 eluted from a GST-FRNK affinity column displayed Mr of 35,000 when analyzed by gel-filtration on FPLC Superose 12, indicating the presence of an isolated PP1 catalytic subunit. (Mol Cell Biochem **272**: 85–90, 2005)

Key words: focal adhesion kinase, focal adhesions, phosphatase 1, protein kinase, protein phosphatase

Abbreviations: FAK, focal adhesion kinase; FRNK, FAK-related nonkinase, C-terminal domain of FAK; PP1, type-1 protein serine/threonine phosphatase; LB-broth, Luria–Bertani broth; IPTG, isopropyl b-D-thiogalactopyranoside; GST, glutathione-S-tranferase; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; TRIS, Tris-(hydroxymethyl)aminomethane; EDTA, Ethylenediaminetetracetic acid; TPCK, L-1-p-tosylamino-2-phenylethyl chloromethyl ketone; PMSF, phenylmethyl sulfonyl fluoride; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid

Introduction

Signaling at focal adhesions (FA) involves several Tyr kinases, including the focal adhesion kinase FAK [1, 2]. Ser/Thr phosphoproteins are also found in FA, and FAK itself has consensus sequences for protein kinase A and proline-directed kinases [2–4]. Other FA proteins are phosphorylated by protein kinase C [2, 5] or by the integrin-linked Ser/Thr kinase [6]. On the other hand little information is available on the Ser/Thr phosphatases that may balance the activity of Ser/Thr kinases at FA. We reported that protein Ser/Thr phosphatase-1 δ (PP1δ) localizes to FA and associates with FAK [7]. PP1δ also dephosphorylates Ser-phosphorylated FAK at the exit from mitosis, a process that is completed by the time the cells are fully spread [8]. Further studies indicated that the FAK-directed PP1 is also regulated during cell spreading on fibronectin and following cell treatment with the phorbol ester TPA (M. Bianchi, S. De Lucchini,

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PP1 is a major protein Ser/Thr phosphatase of mammalian cells [9]. PP1 is indeed a family of enzymes, consisting of a catalytic subunit interacting with a variety of regulatory subunits, which target PP1 to specific structures (e.g. muscle glycogen particles, sarcoplasmic reticulum, myofibrils, ribosomes, neurofilaments and the nucleus [10, 11]). The R/KxV/IxF/W consensus sequence is often found in PP1 regulatory subunits or binding proteins, including some substrates [9]. Three isoforms of the catalytic subunit exist (α, $\gamma 1$ and δ), which diverge only at the C-termini [12] and display differential tissue localizations and functions (e.g. [13, 14]).

In the present work we investigated the PP1 and FAK domains involved in the interaction between these two proteins. We identified a PP1 domain whose affinity for FAK is increased upon point-mutation of residue R220 (R220S or R220Q), a property that might be exploited to identify new PP1 substrates. We also found that the PP1 that associates with the C-terminal domain of FAK (684–1053, FRNK) elutes as isolated catalytic subunit from a Superose 12 FPLC gel filtration column.

Materials and methods

Deletion mutants

The constructs to express the GST-fusion proteins of domains 2 (159–295), 3 (295–327), 6 (159–212) and 7 (194–260) of PP1δ were described previously [8, 15]. For constructs 4 (159–260) and 5 (194–295) amplimers were produced by pcr, using oligonucleotides derived from rat PP1δ cDNA sequence: 5′-CTA GGA TCC CCT ATA GCT GCT AT-3′ and 5′-ATC GAA TTC TCG TTT AGC AAA AA-3′ (antisense) for construct 4 and 5′-CTA GGA TCC GTA CCT GAT ACA GG-3′ and 5′-ATC GAA TTC CAA TAT CTG GAA TG-3′ (antisense) for construct 5. The template DNA for amplification was full-length rat PP1δ cDNA in pBluescript SK, supplied by Dr. M. Nagao (Tokyo, Japan). The fragments were cloned into the Bam HI and EcoRI sites of the pGEX4T-1 vector. For constructs F1 (1–350) and F2 (340–690) of FAK amplimers were produced by pcr, using oligonucleotides derived from chicken FAK cDNA sequence: 5′-CTA GGA TCC ATG GCA GCA GCT TA-3′ and 5′-ATC CTC GAG CAG TCG GCA GTA TC-3′ (antisense) for construct F1 and 5′-CTA GGA TCC ATG GCT GAC TTG AT-3′ and 5′-ATC GAA TTC TCG TTC CTC TTG CT-3′ (antisense) for construct F2. The template DNA for amplification was full-length chicken FAK cDNA in pBluescript SK, supplied by Dr. J.T. Parsons (Charlottesville, VA). The fragments were cloned into the Bam HI and XhoI sites for F1 and Bam HI and EcoRI for F2 of the pGEX4T-1 vector. GST-FRNK was described elsewhere [8]. All the GST-fusion proteins were produced in BL-21 *E. coli*.

Point mutations

Point mutations were introduced in the pGEX vectors (PP1δ or construct 7) using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, USA). The following PAGE-purified synthetic oligonucletides (Sigma Genosys, UK) were used: 5′-TTG GGG TGA AAA TGA TAG TGG GGT CTC TTT TAC T -3′ and 5′-AGT AAA AGA GAC CCC ACT ATC ATT TTC ACC CCA A-3′ (antisense) for R220S of PP1δ; 5′-GGC TGG GGA GAA AAT GAT CAG GGT GTT TCT TTT ACT TTT-3′ and 5′-AAA AGT AAA AGA AAC ACC CTG ATC ATT TTC TCC CCA GCC-3′ (antisense) for R220Q of PP1δ; 5′-TTG CTT TGT GAT TTA CTG TTT TCC GAC CCA GAT AAG GAT-3′ and 5′-ATC CTT ATC TGG GTC GGA AAA CAG TAA ATC ACA AAG CAA-3′ (antisense) for W205F of PP1δ; 5′-TTA CTG TGG TCC GCC CCA GAT AAG GAT-3′ and 5′-ATC CTT ATC TGG GGC GGA CCA CAG TAA-3′ (antisense) for D207A of PP1δ; 5′-CAT CAG GTG GTA GCA GAT GGA TAT GAA-3′ and 5′-TTC ATA TCC ATC TGC TAC CAC CTG ATG-3′ (antisense) for E251A of PP1δ; 5′-GAG CAA AAG ATT TGC ACA TAG AGA TAT TGC-3′ and 5′-GCA ATA TCT CTA TGT GCA AAT CTT TTG CTC-3′ (antisense) for V543A of construct F2. The mutations were confirmed by DNA sequencing (Primm, Italy).

Bacterial growth and extracts

The bacteria were grown at 37 °C in LB-Ampicyllin, induced with 0.1 mM IPTG for 2 h, collected by centrifugation and resuspended in 25 mM TRIS-HCl, pH 7.5, 150 mM NaCl, 15 mM 2-mercaptoethanol (bacterial lysis buffer) added with protease inhibitors (0.02% w/v benzamidine, 0.02% w/v PMSF, 0.02% w/v TPCK and 4 μg/ml leupeptine). Following quick freezing and thawing, 0.25% v/v Triton X-100, 50 U/ml DNase, 10 mM $MnCl_2$ and 10 mM $MgCl_2$ were added, followed by 30 min rotation at 4 °C and 10,000 $\times$ g centrifugation for 20 min. The extract thus obtained was either used immediately in pull-down assays or stored at -20 °C.

Cell growth and extracts

Fisher rat fibroblasts were grown at 37 °C in water-saturated CO_2, in DMEM (Sigma, USA) added with10% v/v fetal calf serum (Invitrogen, USA). Following two washes in cold PBS, 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), 7.5 mM 2-mercaptoethanol, 1 mM orthovanadate and protease inhibitors as described for bacterial extracts.

Pull-down assays, immunoprecipitations and Western blots

GST-fusion proteins from bacterial extracts were bound to 50 μl of glutathione-Sepharose beads, pre-treated with lipid-free BSA. After 90 min rotation at 4 °C and three washes with bacterial lysis buffer (added with 0.1%Triton X-100, 0.02% w/v benzamidine and 0.02% w/v PMSF) the beads were mixed either with cell extract (1.5 mg/pull-down) or with PP1 catalytic subunit (purified from skeletal muscle and diluted in cell extraction buffer; [8]). This was followed by incubation at 4 °C for 90 min with shaking, three washes in cell lysis buffer added with 0.02% w/v benzamidine and 0.02% w/v PMSF and boiling in Laemmli buffer. For immunoprecipitation anti-FAK purified antibodies (Santa Cruz, USA; 10 μl/ml of extract) or anti-PP1δ hyperimmune rabbit serum (10 μl/ml of extract) were mixed with Protein A-Sepharose (Sigma) [13]. The subsequent procedure was as performed in the pull-down assays. Electrophoresis was on 10% polyacrylamide–SDS gel and Immobilon-P membranes (Millipore, USA) were used for transblotting. The membranes were probed with the indicated antibodies, followed by protein A-peroxidase (Sigma, USA) and the enhanced chemiluminescence ECL system (Amersham Biosciences, UK). For re-probing with another antibody, the membranes were previously incubated in 5 mM phosphate buffer, 2% SDS and 2 mM 2-mercaptoethanol at 60 °C for 30 min [8].

Affinity and gel filtration columns

GST-FRNK (25 ml of bacterial extract) was coupled to 0.8 ml of glutathione-Sepharose (Amersham Biosciences, UK) according to the manufacturer's instructions. The resin was washed three times in bacterial lysis buffer, mixed with 7 mg of rat fibroblast extract, rotated for 90 min at 4 °C, washed extensively with cell lysis buffer containing 150 mM NaCl, 15 mM 2-mercaptoethanol, 0.02% w/v benzamidine and 0.02% w/v PMSF and transferred to a 2 ml column. The column was run at 5.5 ml/h and the PP1 was eluted in cell lysis buffer as above, but containing 250 mM NaCl. 0.275 ml fractions were collected and assayed for PP1 activity with the substrate [^{32}P]Phosphorylase *a* [8]. Fractions were pooled as indicated in Fig. 4A, concentrated in Amicon Centricon (Millipore, USA) and loaded on an FPLC Superose 12 HR 10/30 gel filtration column (Amersham, UK), equilibrated in 10 mM imidazole, pH 7.5, 5% glycerol, 0.01% Brij-35, 100 mM NaCl, 15 mM 2-mercaptoethanol, 0.02% w/v benzamidine and 0.02% w/v PMSF. The column was run at 24 ml/h. 0.20 ml fractions were collected after discarding 6 ml and assayed for PP1 activity.

Results and discussion

The shortest PP1δ domain that binds FAK

As previously described [8], the 159–295 deletion mutant of PP1δ (construct 2) binds FAK from a cell extract, likewise full-length PP1δ, whereas the C-terminus 295–327 (construct 3) or GST alone do not bind FAK (see also Fig. 1). To find-out which domain is responsible for binding FAK, we prepared several deletion mutants that represented the dissection of construct 2 (Fig. 1). The mutants were designed in a way that did not interrupt any β-strand or α-helix of the molecule [9] and were expressed in bacteria as GST-fusion proteins [8]. The partially overlapping constructs 4 (159–260) and 5 (194–295) were able to pull-down cellular

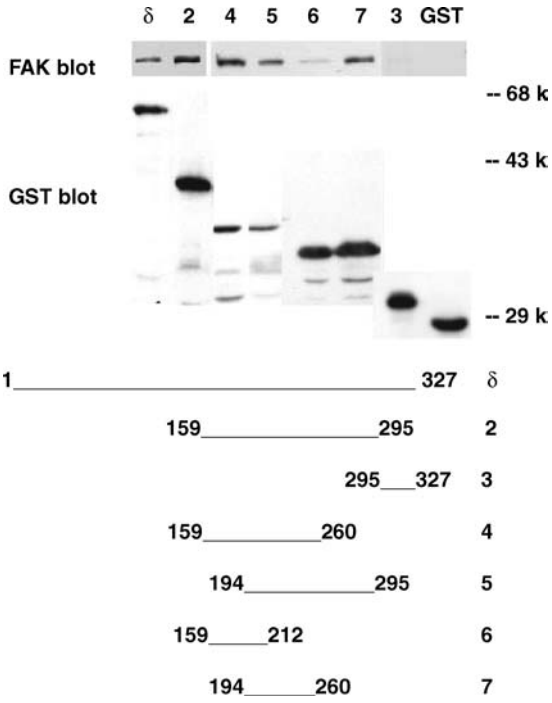

Fig. 1. Pull-down of cellular FAK with PP1δ deletion mutants. GST-PP1δ fusion proteins, either full-length δ or its deletion mutants 2 (residues 159–295), 4 (159–260), 5 (194–295), 6 (159–212), 7 (194–260) and 3 (295–327), described in the bottom part of the figure, or GST were bound to glutathione-Sepharose and used to pull-down FAK from rat fibroblasts extract. This was followed by electrophoresis on SDS-10% polyacrylamide gel and transblotting onto Immobilon-P. Immunodetection used anti-FAK antibodies (FAK blot), followed by removal of the immune complexes and re-probing with anti-GST antibodies (GST blot) to detect the GST-fusion proteins and GST. Molecular weight markers: BSA (68 k), ovalbumin (43 k), carbonic anhydrase (29 k).

FAK. Domain 6 (159–212) did not pull-down FAK, whereas domain 7 (194–260) did (Fig. 1). We conclude that 7 represents the shortest domain able to interact with FAK among those tested. The deletion mutants pulled-down FAK from mitotic cells (which is highly Ser-phosphorylated, [8]) and from asynchronous adherent cells equally well. The amount of interacting FAK represented up to 10–15% of the total FAK immunodetected in the cell extract (not shown).

FAK binding by point-mutants of PP1δ or 7

Domain 7 (194–260) encompasses several residues involved in enzyme catalysis [16, 17]. We analyzed the effect of mutating some of these residues on FAK binding (Fig. 2 and [16] for the location of the residues in a space-filling model of PP1). First we targeted R220, which binds one oxygen of the phosphate group to be removed from the substrate and whose mutation was reported to induce the deepest effect

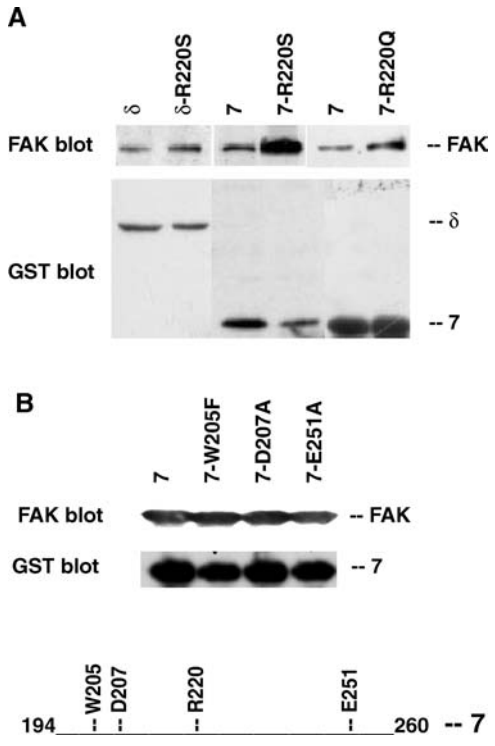

Fig. 2. Pull-down of cellular FAK by point-mutants of PP1δ or 7. (A) GST-PP1δ or the deletion mutant 7 (194–260), or the point-mutants δ-R220S, 7-R220S or 7-R220Q were bound to glutathione-Sepharose and used to pull-down FAK from cell extract. Following electrophoresis and transblotting, immunodetection used first the anti-FAK antibodies (FAK blot), followed by re-probing for GST (GST blot). All the rest was as in Fig. 1. (B) 7 or the point-mutants 7-W205F, 7-D207A or E251A were bound to glutathione-Sepharose and used to pull-down FAK as in A. The sites mutated are summarized in the bottom part of the figure.

on K_m (increase) and V_{max} (reduction) among the mutants tested [16, 17]. To test the role of R220 on FAK association, we prepared the R220S mutants of both PP1δ and 7. Also in our case mutation inactivated PP1 and the GST-R220S-PP1δ displayed 2% of the activity assayed on GST-PP1δ. Pull-down experiments indicated that the R220S mutation did not abolish the ability of PP1δ and 7 to interact with FAK (Fig. 2A). Indeed the mutants pulled-down more FAK than the corresponding wild-type protein. The same results were obtained with the R220Q mutant of 7 (Fig. 2A). Based on these results we conclude that mutation of R220 did not prevent substrate-binding. On the contrary, it even caused some degree of substrate trapping, possibly due to the inability of the enzyme to carry-on catalysis. The results also indirectly confirmed that FAK is a PP1 substrate [8].

We also targeted other PP1 sites reported to affect catalysis, though in a less dramatic way than R220. These were: W205, which interacts with the substrate S or T [16], D207 and E251, both located in the PP1 acidic groove, one of the grooves radiating from the active site [16, 17]. Mutation of these three sites (W205F, D207A, E251A) did not affect the amount of FAK pulled-down by 7 (Fig. 2B), hence yielding results that were clearly different from those obtained by mutating R220. This is possibly explained considering the specific role of R220 in the catalytic mechanism, which is not shared by the other residues (see above and [16]).

The FAK domain that binds PP1

PP1 associates with the C-terminal domain of FAK (684–1053, also known as FRNK [8]). This may be due to the presence of several serine residues potentially targeted by PP1 [4, 8]. However, the presence of additional binding site in FAK cannot be excluded, as reported for other PP1 binding proteins [9, 18]. To test this hypothesis we prepared two GST-fusion proteins reproducing residues 1–350 (F1) and 340–690 (F2) of chicken FAK. The results indicated that FRNK was the only domain that pulled-down a significant amount of PP1 catalytic subunit (purified from skeletal muscle [8]). PP1 was detected either as enzyme activity (Fig. 3) or as protein on immunoblot (Fig. 3, inset). The same results were obtained with PP1 pulled-down from a cell extract (not shown). The results indicated also that PP1 interaction does not require site-phosphorylation, since it occurs also with recombinant FRNK that has not been pre-phosphorylated. We previously reported that also in the interaction between PP1 and the receptor Ron, pre-phosphorylation of Ron was not required for the interaction [19].

Additionally, a small, though consistently present, amount of PP1 was also pulled-down by F2 (Fig. 3, inset). Since F2 displays the KRFVHR sequence (540–545 of FAK) which is similar, though not identical, to the R/KxV/IxF/W of

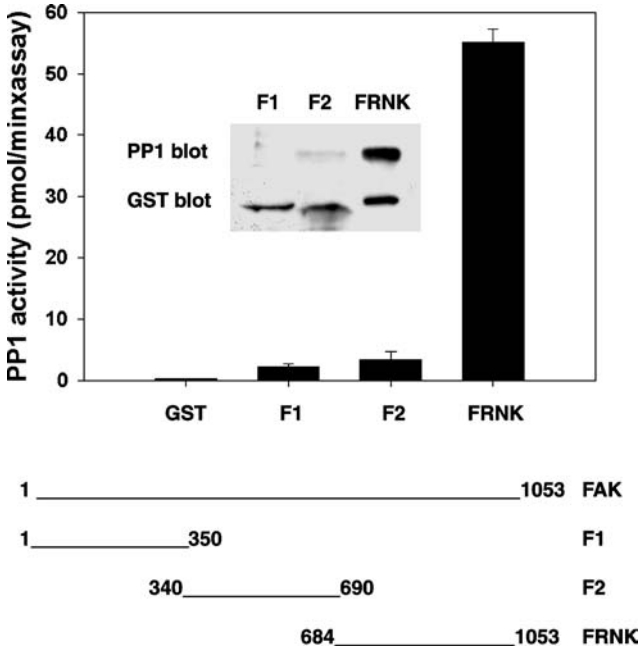

Fig. 3. Pull-down of PP1 catalytic subunit with FAK deletion mutants. Three GST-fusion proteins reproducing residues 1–350 (F1), 340–690 (F2) and 684–1053 (FRNK) of chicken FAK, also described in the bottom part of the figure, were bound to glutathione-Sepharose and used to pull-down muscle-purified PP1 catalytic subunit. This was followed by PP1 assay (mean values ± SEM of four cases are given) or immunoblotting to detect PP1 and the GST-fusion proteins (inset).

several PP1-binding proteins [18], we tested if such sequence might be involved in associating PP1. However, neither the V543A mutation nor additional mutations of the nearby residues abolished the ability of F2 to pull-down PP1 (not shown), suggesting that the KRFVHR sequence was not involved.

Isolation of the PP1 that is pulled-down by FRNK from a cell extract

We have previously shown that, among the PP1 isoforms, GST-FRNK preferentially pulls-down PP1δ [8]. To investigate its aggregation state, the FAK-directed PP1 was isolated from fibroblasts extract using an affinity column prepared with GST-FRNK bound to glutathione-Sepharose. PP1 eluted from the column as a single broad peak (Fig. 4A). The PP1-containing fractions were pooled as indicated, concentrated and applied to an FPLC Superose 12 HR 10/30 gel filtration column (Fig. 4B). Most of the PP1 activity eluted as a single peak of approx. 35,000 Mr, hence representing a free catalytic subunit (Fig. 4B). Limited tryptic digestion of the pool before column loading did not change the elution profile, thus confirming the absence of PP1 binding proteins (not

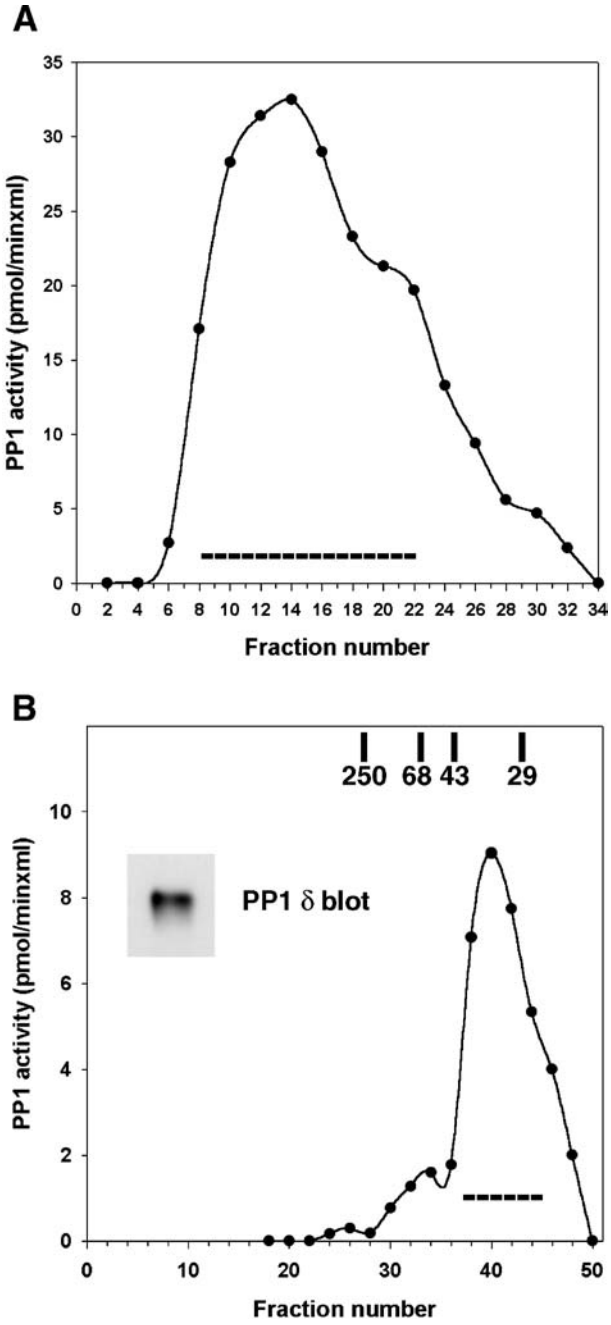

Fig. 4. (A) Affinity chromatography of cellular PP1 on GST-FRNK-Sepharose. Fibroblasts extract was applied to a 0.8 ml GST-FRNK-Sepharose column. This was washed and eluted (starting from fraction 1), as further described in Materials and Methods. The fractions were assayed for PP1 activity and the peak fractions were pooled, as indicated by the bar. (B) Gel-filtration of the PP1 eluted from the affinity column. Following concentration, the pool was applied to an FPLC Superose 12 HR 10/30 gel filtration column (see Materials and Methods). 0.2 ml fractions were collected after discarding 6 ml and PP1 was assayed. Molecular weight markers: catalase (250 k), BSA (68 k), ovalbumin (43 k), carbonic anhydrase (29 k). The peak fractions were pooled as indicated by the bar and concentrated by precipitation in the presence of 7% TCA. This was followed by electrophoresis and immunoblotting to detect PP1δ (inset).

shown; see also [20] for PP1 purification and sizing by gel filtration). Immunoblotting of the peak fractions (Fig. 4B, inset) detected PP1δ, confirming the association of this isoform with FAK.

Conclusions

The results indicated that the 67-aminoacid domain 7 (194–260) of PP1δ is sufficient to associate FAK in a pull-down assay. The several residues of 7 involved in substrate-binding [16] may account for such property. Indeed we have previously shown that 7 binds also pRb (retinoblastoma gene product), another PP1 substrate [15]. R220S or R220Q mutation further improved the ability of 7 to bind FAK. Such property might be exploited to detect new potential PP1 substrates in cell extracts.

The use of FAK deletion mutants confirmed that FAK binds PP1 through the FRNK-domain, where several serine residues are located [4] and indicated that FRNK interacts directly with free PP1δ catalytic subunit.

Acknowledgments

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