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Multi-directional function of the protein phosphatase 1 regulatory subunit TIMAP

Micheal J. Shopik [b], Laiji Li [a], Hue-Anh Luu [b], Marya Obeidat [a], Charles F.B. Holmes [b], Barbara J. Ballermann [a,*]

[a] Department of Medicine, University of Alberta, Edmonton, AB, Canada
[b] Department of Biochemistry, University of Alberta, Edmonton, AB, Canada

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ABSTRACT

TIMAP is an endothelial-cell predominant member of the MYPT family of PP1c regulatory subunits. This study explored the TIMAP–PP1c interaction and substrate specificity *in vitro*. TIMAP associated with all three PP1c isoforms, but endogenous endothelial cell TIMAP preferentially co-immunoprecipitated with PP1cβ. Structural modeling of the TIMAP/PP1c complex predicts that the PP1c C-terminus is buried in the TIMAP ankyrin cluster, and that the PP1c active site remains accessible. Consistent with this model, C-terminal PP1c phosphorylation by cdk2-cyclinA was masked by TIMAP, and PP1c bound TIMAP when the active site was occupied by the inhibitor microcystin. TIMAP inhibited PP1c activity toward phosphorylase *a* in a concentration-dependent manner, with half-maximal inhibition in the 0.4–1.2 nM range, an effect modulated by the length, and by Ser333/Ser337 phosphomimic mutations of the TIMAP C-terminus. TIMAP-bound PP1cβ effectively dephosphorylated MLC2 and TIMAP itself. By contrast, TIMAP inhibited the PP1cβ activity toward the putative substrate LAMR1, and instead masked LAMR1 PKA- and PKC-phosphorylation sites. This is direct evidence that MLC2 is a TIMAP/PP1c substrate. The data also indicate that TIMAP can modify protein phosphorylation independent of its function as a PP1c regulatory subunit, namely by masking phosphorylation sites of binding partners like PP1c and LAMR1.

1. Introduction

TIMAP (TGF-β1 Inhibited Membrane Associated Protein), first identified as a target of TGF-β1 mediated transcriptional repression in renal glomerular endothelial cells (EC) [1], is highly expressed in EC and in neuronal cell bodies [2]. A consensus protein phosphatase 1 catalytic subunit (PP1c)-binding "RVXF" motif at $Lys^{63}Val^{64}Ser^{65}Phe^{66}$, immediately followed by a cluster of ankyrin repeats, places TIMAP into the MYPT family of PP1c regulatory subunits [3]. The TIMAP sequence encodes a C-terminal CAAX box, predicting prenylation, similar to its closest family member, MYPT3 [4], and membrane localization is prenylation-dependent [1,5].

The PP1c family of Ser/Thr phosphatases consists of 3 highly conserved isoforms, PP1cα, PP1cβ (also referred to as PP1cδ) and PP1cγ (γ1 and γ2), and has hundreds of cellular substrates. To restrict and control PP1c catalytic activity toward appropriate substrates and sites, PP1c isoforms associate with many distinct regulatory subunits [6]. Among these, MYPT family members target PP1cβ to regulatory myosin light chains (MLC). In the case of MYPT1, the holoenzyme MYPT1/PP1cβ/M20 is a complex, which regulates MLC2 phosphorylation, opposing myosin light chain kinase (MLCK) [3].

MYPT1, MYPT2 and M85 are phosphorylated at a conserved Thr residue in the C-terminus resulting in inhibition of MLC phosphatase activity [3,7]. MYPT3 and TIMAP lack the extended C-terminus containing the conserved Thr residue, but a protein kinase A (PKA)-sensitive phosphorylation cluster in MYPT3 (Ser340/341/353) [8] and a PKA/GSK-3β sensitive (Ser333/Ser337) phosphorylation site in TIMAP [9] increase the activity of the associated PP1c. While MYPT3 inhibits PP1cβ catalytic activity toward MLC2 *in vitro*, MYPT3 phosphomimic mutants lacking the prenylation site reduce MLC2 phosphorylation in cells [8], and MYPT-75D, an ortholog of MYPT3 in *Drosophila*, has MLC2 phosphatase activity *in vitro* and *in vivo* [10]. So far, proof that TIMAP can regulate MLC phosphorylation is lacking.

TIMAP interacts directly with the 37LRP/67LR non-integrin laminin receptor-1 (LAMR1) [5], a type II transmembrane protein associated with metastatic tumors and active angiogenesis [11]. Introduction of TIMAP into TIMAP-null MDCK cells results in recruitment of the PP1c into a LAMR1/TIMAP complex and is

Abbreviations: TIMAP, TGF-β1-inhibited-membrane-associated-protein; LAMR1, laminin receptor-1; MYPT, myosin phosphatase targeting subunit; MLC2, myosin light chain 2; GST, glutathione S-transferase; MLC, myosin light chain; PP1c, protein phosphatase 1 catalytic subunit; WT, wild-type.

* Corresponding author. Address: Department of Medicine, University of Alberta, 13-103 Clinical Sciences Building, 11350-83rd Ave., Edmonton, AB, Canada. Fax: +1 780 248 1637.
E-mail address: barbara.ballermann@ualberta.ca (B.J. Ballermann).

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associated with decreased LAMR1 phosphorylation, suggesting that phosphorylated LAMR1 is a TIMAP/PP1c substrate [5]. However, direct evidence that the TIMAP/PP1c holoenzyme functions as a LAMR1 phosphatase is lacking.

In human pulmonary artery EC (HPAEC), TIMAP co-localizes and co-immunoprecipitates with moesin, a member of the ezrin–radixin–moesin (ERM) family. TIMAP silencing in HPAEC blocked inhibition by forskolin of thrombin-stimulated ERM phosphorylation and monolayer permeability, suggesting that moesin is a substrate of PKA-activated TIMAP/PP1c [12,13].

This study explored the interaction of TIMAP with PP1c, and determined whether TIMAP/PP1c can function as a holoenzyme toward LAMR1 and MLC2 *in vitro*.

2. Material and methods

2.1. Materials

Reagents were from Sigma (Oakville, ON) or Fisher Scientific (Ottawa, ON) unless noted. Restriction enzymes and DNA polymerase were from Invitrogen (Burlington, ON) or Stratagene (Santa Clara, CA), DNA primers from Integrated DNA Technologies (San Diego, CA), Glutathione Sepharose 4B and chromatography columns from GE Healthcare (Ottawa, ON), and recombinant myosin regulatory light chain-2 (MLC2) from Calbiochem (Billerica, MA).

2.2. Cell culture

Primary bovine glomerular EC were prepared as described [14], and Madin–Darby canine kidney (MDCK; CCL-34, American Type Culture Collection, Manassas, VA) cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 50 U penicillin and 50 μg streptomycin at 37 °C in humidified air with 5% CO_2.

2.3. Generation of a structural model

A three-dimensional structural model of $TIMAP^{51-322}$ bound to PP1cβc was generated with Swiss-Model[15,16], which produces structural predictions on the basis of sequence homology. The previously solved structure of MYPT1 (residues 1–299) [17] in complex with PP1βc was used as the template for TIMAP, which shares significant domain, and 31% sequence homology with MYPT1.

2.4. Plasmids and recombinant proteins

The cDNAs encoding amino acids 1–568 of wild-type TIMAP ($TIMAP^{WT}$), 46–453 ($TIMAP^{46-453}$) or 46–292 ($TIMAP^{46-292}$) were PCR-amplified from pBlueScript-bovine-TIMAP [9], and sub-cloned into pGEX-4T3 to generate GST-$TIMAP^{WT}$, GST-$TIMAP^{46-453}$, and GST-$TIMAP^{46-292}$ proteins. Site-directed mutagenesis was performed to substitute Ser333 and Ser337 with Aspartic acid (D) or Glutamic acid (E) of GST-$TIMAP^{46-453}$ in order to mimic phosphorylations by Glycogen Synthase Kinase 3β (GSK-3β) and Protein Kinase A (PKA) [9]. The cDNA constructs were expressed in *Escherichia coli* BL-21 Rosetta cells (Novagen Billerica, MA) and resulting GST-TIMAP fusion proteins were purified on glutathione Sepharose beads.

Human PP1cβ and rat PP1cγ were sub-cloned into the pCW vector, expressed in *E. coli* DH5α cell and purified using inhibitor-2-Sepharose as described by Zhang et al. [18].

A cDNA encoding residues 1–200 of human LAMR1 was amplified from full length LAMR1 in the pACT2 vector [5] and sub-cloned into the His-6 tag *E. Coli* expression vector pET-28a (EMD Biosciences, Billerica, MA). The recombinant His-LAMR1 fusion protein was expressed in *E. Coli* BL21 (DE3) cells and purified on Ni-NTA resin (Qiagen, Mississauga, ON).

Sequence fidelity and in-frame cloning were verified by sequencing all cDNAs. Protein concentration was quantified with the Bradford assay (Bio-Rad, Mississauga, ON).

2.5. Affinity co-precipitation

Microcystin Sepharose was generated according to Moorhead et al. [19] and incubated with PP1c (1.4 nmol) and equimolar GST-TIMAP in buffer A (50 mM Tris–HCl, 0.1 mM EDTA, 0.5 mM $MnCl_2$ and 0.2 M β-mercaptoethanol, final volume 500 μl) on an end-over-end rotator for 90 min at 4 °C. The microcystin Sepharose resin was washed four times with 1 ml buffer A supplemented with 100 mM NaCl. Proteins were released with 2× Laemmli buffer, followed by SDS–PAGE analysis.

GST-TIMAP fusion proteins or GST were immobilized on Glutathione Sepharose 4B in the presence of recombinant PP1cβ, LAMR1, phosphorylase *b* or both of PP1cβ and LAMR1 (20 mM HEPES pH 7.5, 100 mM KCl, 10% glycerol, 1 mM EDTA, 0.1% Triton X-100 and 1 mM DTT) by rotation for 90 min at 4 °C. To completely remove free GST-TIMAP or GST, the beads were washed once with PBS containing 0.1% Nonidet P-40 and 1 mg/ml bovine serum albumin, once with PBS containing 0.1% Tween 20, and twice with PBS. Proteins were eluted with 2× Laemmli buffer for subsequent SDS–PAGE electrophoresis and immunoblotting.

2.6. Co-immunoprecipitation (IP) and Western blotting

Cell lysates were prepared by homogenization in ice-cold Buffer B (50 mM Tris–Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate) supplemented with complete protease inhibitors (Roche, Laval, QC). Particulates were removed by centrifugation. Chicken anti-TIMAP or non-specific chicken IgY was added to the cell lysates, incubated for 1 h at 4 °C followed by addition of goat-anti-chicken Ig-Y coated agarose beads (AVES, Tigard, Oregon) and incubation overnight. The beads were extensively washed, suspended in 2× Laemmli buffer and boiled for 10 min. Proteins were separated by electrophoresis on 8% or 10% SDS–PAGE gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). Blots were probed with appropriate antibodies, and bands were visualized with ECL chemiluminescent substrate (GE Healthcare, Ottawa, ON).

2.7. PP1c catalytic activity

The concentration-dependent effect of various GST-TIMAP proteins on PP1c catalytic activity was determined using ^{32}P-phosphorylase *a* or *p*-Nitrophenol Phosphate (PNPP) as substrates. Phosphorylase *a* assays [20,21] were performed by pre-incubating PP1cβ (0.96 nM) or PP1cγ (0.25 nM) with or without increasing concentrations of GST-TIMAP in 30 μl phosphatase buffer (50 mM Tris–HCl, pH 7.0; 0.1 mM EDTA; 1 mg/ml BSA; 1 mM $MnCl_2$; 0.2% β-mercaptoethanol) for 10 min at 30 °C, followed by addition of ^{32}P-phosphorylase *a* and caffeine (final concentration: 10 μM and 3.75 mM, respectively). After 10 min at 30 °C, the reaction was stopped with ice-cold 20% trichloroacetic acid (TCA). The TCA precipitated proteins were sedimented 18,000 g and ^{32}P released into the supernatant was quantified in a scintillation counter.

For the *p*-nitrophenol phosphate (PNPP) assay, PP1cβ (60 nM) or PP1cγ (30 nM) with or without increasing concentrations of GST-TIMAP were suspended in PNPP assay buffer (50 mM Tris–HCl (pH 8.3), 0.1 mM EDTA, 30 mM $MgCl_2$, 1 mg/ml BSA, 0.2% β-mercaptoethanol, and 0.5 mM $MnCl_2$) in a total volume of 60 μl. After pre-incubation for 15 min at 30 °C the reaction was initiated by adding PNPP substrate (5 mM) and allowed to proceed for 45 min at 30 °C before the measuring absorbance at 405 nm

(A_{405}) in a Perkin Elmer plate reader. All reactions were performed in duplicate, and assays were repeated 3–4 times.

2.8. In vitro phosphorylation

Cdk2-cyclinA phosphorylation reactions were carried out as described by Dohadwala [22] with some modification. Recombinant GST-TIMAP (6 μM) was pre-incubated with or without PP1cγ (4 μM) in reaction buffer (40 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 2 mM DTT, 0.5 mM $MnCl_2$ 0.38 μM γ-^{32}P-ATP, 7.5 μl final volume) for 15 min at 30 °C. The reaction was initiated by adding cdk2-cyclinA (50 U, New England Biolabs, Pickering, ON). After 30 min at 30 °C proteins were released with 2× Laemmli buffer and boiling for 5 min. Parallel reactions were performed in the presence of the PP1c inhibitor microcystin (4.2 μM).

For LAMR1 phosphorylation, 2.0 μg His-LAMR1$^{1-200}$ were incubated with 2500 U PKA catalytic subunit (New England Biolabs, Pickering, ON) in 30 μl of reaction buffer (50 mM Tris–Cl pH 7.5, 10 mM $MgCl_2$, 100 μM ATP and 5 μCi γ-^{32}P-ATP), or with 12.5 ng of PKC catalytic fragment (Enzo Life Sciences, Inc, Farmingdale, NY) in PKC reaction buffer (40 mM MES, pH 6.0, 1 mM EGTA, 10 mM $MgCl_2$, 100 μM ATP and 5 μCi γ-^{32}P-ATP) for 60 min at 30 °C.

For MLC2 phosphorylation *in vitro*, 1.0 μg MLC2 was incubated with myosin light chain kinase (active fragment, aa 1425–1776, Sigma, St. Louis, MO) in 25 μl of reaction buffer (7.0 mM MOPS pH 7.0, 7 mM $MgCl_2$, 1.4 mM EGTA, 0.56 mM EDTA, 10 μM DTT, 0.5 mM $CaCl_2$, 750 ng Calmodulin, 100 μM ATP and 5 μCi γ-^{32}P-ATP) for 60 min at 30 °C.

The phosphorylation reactions were stopped by addition of 2 volumes of 2× Laemmli buffer, followed by SDS–PAGE (10%), blotting onto a PVDF membrane and autoradiography.

3. Results and discussion

3.1. The TIMAP-PP1c interaction

Swiss-Model [15,16] was used to produce a structural model of TIMAP (residues 51–322) bound to PP1cβ (Fig. 1A), based on the

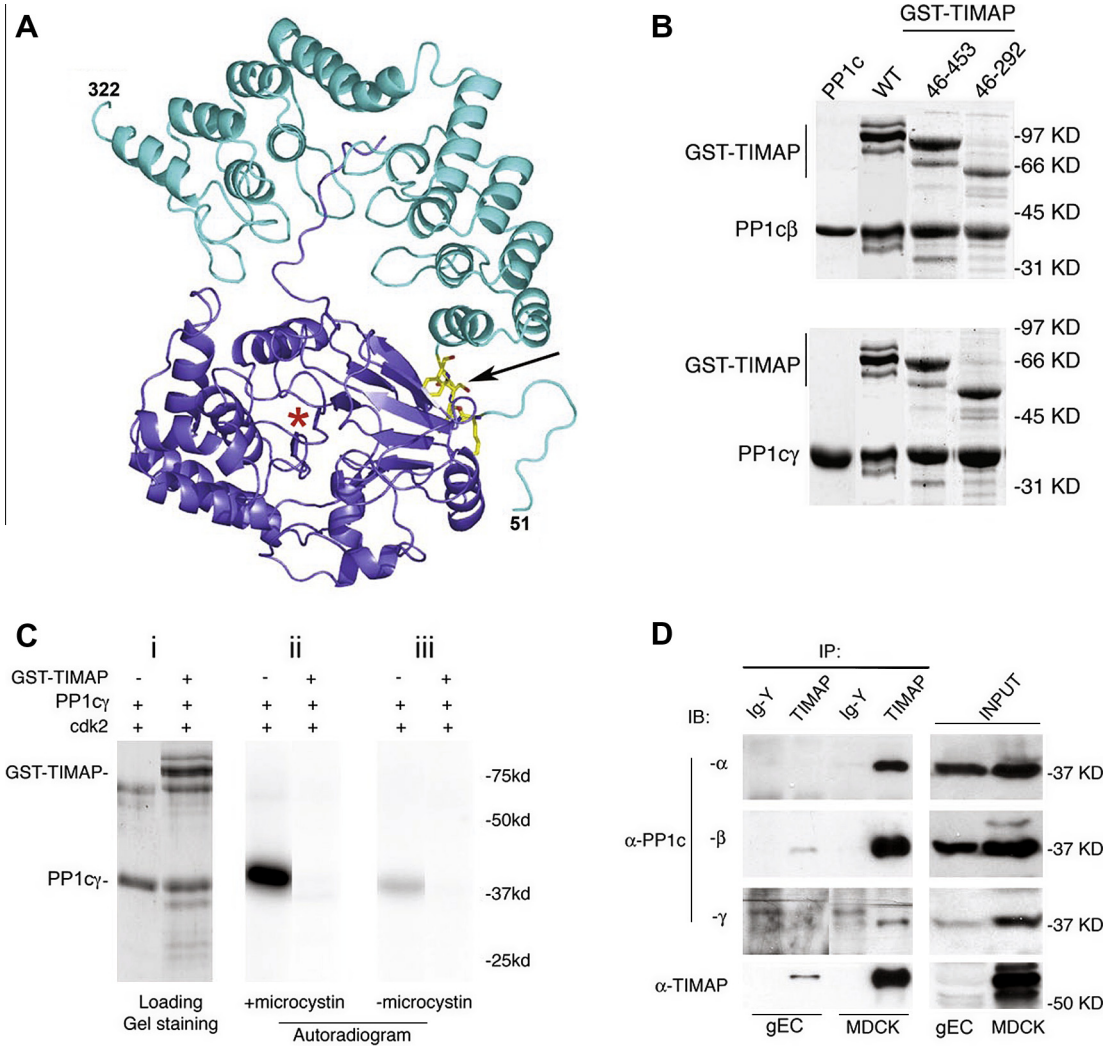

Fig. 1. TIMAP Interaction with PP1c. (A). Predicted structural model of the TIMAP and PP1cβ interaction. TIMAP (top, cyan, residues 51–322); PP1cβ (bottom, purple). Red asterisk: active site of PP1cβ. Arrow: ^{63}KVSF^{66} (yellow sticks) of TIMAP, predicted to interact with the RVXF binding groove of PP1cβ. Eight α-helices of TIMAP form 4 ankyrin repeats that wrap around the PP1cβ C-terminus. (B). Interaction of purified, recombinant GST-TIMAP^{WT}, GST-TIMAP$^{46-453}$, and GST-TIMAP$^{46-293}$ with PP1cβ or PP1cγ immobilized on microcystin-Sepharose. (C). GST-TIMAP^{WT} blocks cdk2-cyclinA mediated phosphorylation of PP1cγ. i: PP1c and GST-TIMAP input on Coomassie-blue stained SDS–PAGE gels. ii, iii: PP1cγ phosphorylation, visualized by ^{32}P autoradiography, in the presence (ii) or absence (iii) of the phosphatase inhibitor microcystin. PP1cγis strongly phosphorylated by cdk2-cyclinA in the absence, but not in the presence of TIMAP. In the absence of microcystin, PP1cγ, auto-dephosphorylation is evident. (D). Co-immunoprecipitation of PP1c with TIMAP from living cells. Immunoprecipitation of endogenous TIMAP from bovine glomerular EC (gEC) co-precipitated PP1cβ. Immonoprecipitation of TIMAP stably overexpressed in MDCK cells co-precipitated PP1cα, PP1cβ and PP1cγ. Immunoblot of cell lysates (input) shows expression of PP1c isoforms in gEC and MDCK cells.

crystal structure of MYPT1-bound PP1cβ [17]. For MYPT1/PP1cβ, the architecture of the PP1cβ active site remains relatively unchanged compared to the free phosphatase [17,23]. Our model is similar and predicts a 1:1 stoichiometry of TIMAP:PP1cβ with a series of 4 TIMAP ankyrin repeats that wrap around the PP1cβ C-terminus, an interaction of the TIMAP ^{63}KVSF^{66} motif with the RVXF binding groove of PP1cβ, and an accessible PP1cβ active site.

The marine toxin microcystin binds the active site of PP1c, but leaves the RVXF binding groove of PP1c unoccupied [24]. Here, PP1cβ and PP1cγ immobilized on microcystin Sepharose [25] bound GST-TIMAP^{WT}, GST-TIMAP$^{46–292}$ and GST-TIMAP$^{46–453}$ (Fig. 1B), in keeping with prediction that TIMAP does not bind the PP1c active site. In the absence of PP1c, TIMAP did not bind microcystin (not shown). Also consistent with the structural model, substitution mutations of Phe^{66} and/or Val^{64} to Ala abolish the TIMAP interaction with PP1c [9].

Interactions at sites other than the RVXF motif contribute to binding and modification of PP1c function by regulatory subunits [4,26,27]. The C-terminus of PP1c can be phosphorylated by cyclin-dependent kinases, with consequent inactivation of the

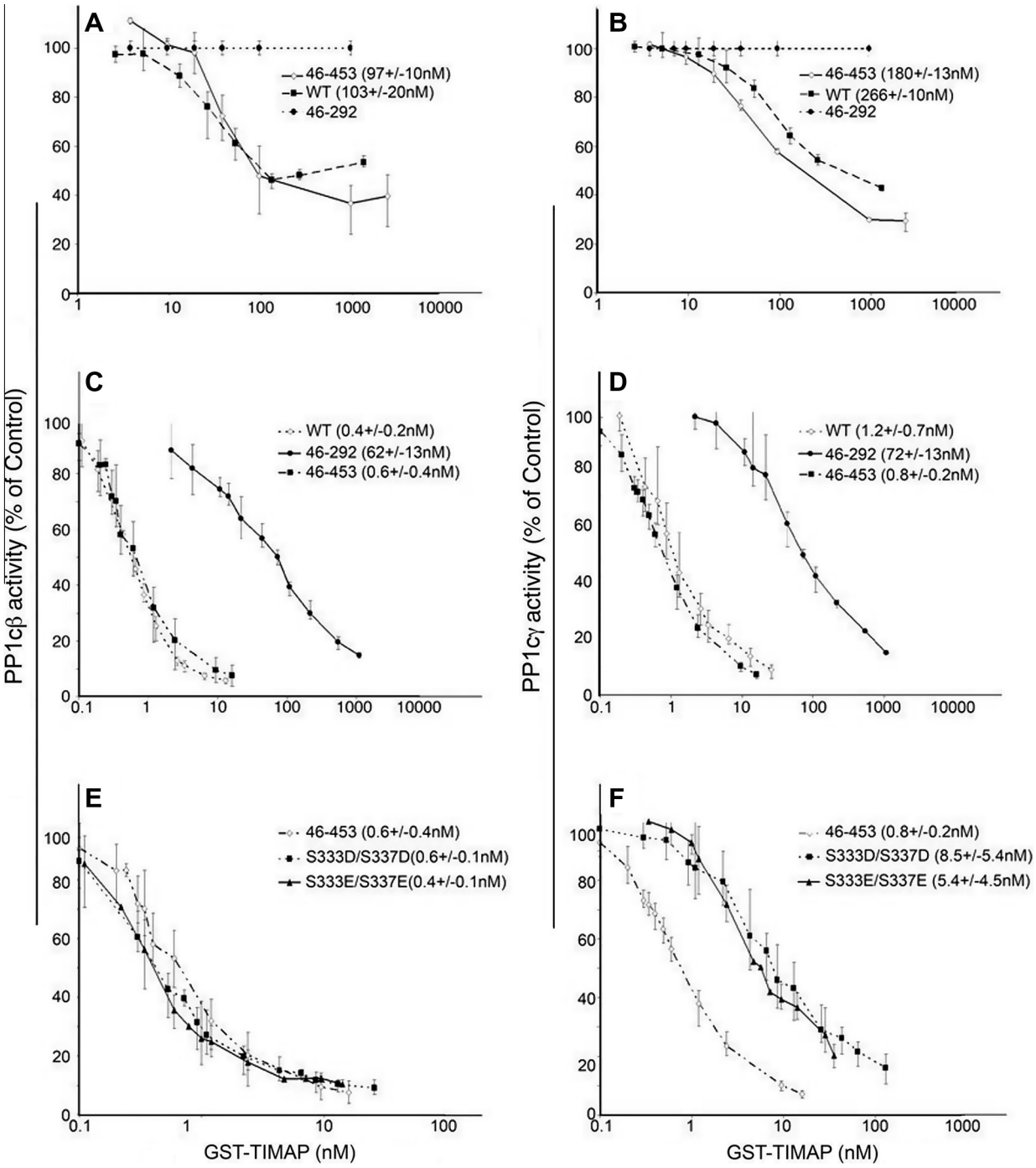

Fig. 2. GST-TIMAP inhibits PP1c activity toward phosphorylase a. (A and B). Concentration-dependent effect of GST-TIMAP^{WT} (■), GST-TIMAP$^{46–453}$ (◇) and GST-TIMAP$^{46–292}$(●) on PNPP-directed PP1cβ (A) and PP1cγ (B) activity. (C and D). Concentration-dependent inhibition of PP1cβ(C) and PP1cγ (D) activity toward phosphorylase *a* by GST-TIMAP^{WT}(◇), GST-TIMAP$^{46–453}$(■) and GST-TIMAP$^{46–292}$(●). (E and F). Concentration-dependent inhibition of PP1cβ (E) and PP1cγ (F) activity toward phosphorylase *a* by GST-TIMAP$^{46–453}$(◇), GST-TIMAP^{S333DS337D}(■) and GST-TIMAP^{S333ES337E}(▲). The IC_{50}, for each construct was calculated from 3 separate experiments (mean ± SE).

phosphatase [22,28]. However, the structural model suggests that the PP1c C-terminus may not be accessible to kinases when bound to TIMAP. In the presence of microcystin, free PP1cγ was strongly phosphorylated by cdk2-cyclinA (Fig. 1C). Phosphorylation of PP1cγ in the absence of microcystin was less pronounced, in keeping with auto-dephosphorylation. However, when PP1cγ was bound to TIMAP^{WT}, its phosphorylation by cdk2-cyclinA was blocked with and without microcystin (Fig. 1C). Not shown, GST-TIMAP$^{46–453}$ and GST-TIMAP$^{46–292}$ similarly prevented cdk2-cyclinA mediated phosphorylation of PP1cγ. These observations indicate that the interactions between the TIMAP ankyrin cluster and the PP1cγ C-terminus mask the cdk2-cyclinA phosphorylation site.

The PP1c isoforms differ at their N- and C-termini, raising the possibility that TIMAP might discriminate between them as previously reported for MYPT1 [29], MYPT2 [19], MYPT3 [8] and TIMAP [12]. We observed that both, PP1cα and PP1cβ were present in glomerular EC lysates, but only PP1cβ co-immunoprecipitated with endogenous EC TIMAP (Fig. 1D). By contrast, when TIMAP was stably over-expressed in MDCK cells [1,5] all three PP1c isoforms co-precipitated with TIMAP (Fig. 1D). Also, GST-TIMAP bound both PP1cβ and PP1cγ immobilized on microcystin Sepharose, and the C-terminal half of TIMAP did not substantially alter PP1c binding (Fig. 1B). Hence, TIMAP can bind all three isoforms of PP1c, though endogenous EC TIMAP shows a preference for PP1cβ. These observations leave open the possibility that TIMAP regulates not only PP1cβ but potentially also PP1cα and PP1cγ, depending on cell type and potentially also intracellular location.

3.2. TIMAP inhibits PP1cβ and PP1cγ activity toward phosphorylase a

We next probed the effect of GST-TIMAP on PP1c activity. The phosphatase activity of PP1cβ and PP1cγ toward PNPP was not altered by GST-TIMAP$^{46–292}$, but GST-TIMAP$^{46–453}$ and GST-TIMAP^{WT} inhibited PNPP dephosphorylation (Fig. 2A and B). The phosphatase activity of PP1cβ and PP1cγ toward glycogen phosphorylase *a* was strongly inhibited by GST-TIMAP^{WT} and GST-TIMAP$^{46–453}$ and GST-TIMAP$^{46–292}$, though the latter was nearly 100 fold less potent (Fig. 2C and D). Hence, similar to the findings reported for MYPT1 [30] and MYPT3 [4], TIMAP markedly restricts PP1cβ and PP1cγ activity against phosphorylase *a*, an effect modulated by the C-terminal half of TIMAP. In cells, TIMAP is phosphorylated at Ser333 and Ser337 by GSK-3β and PKA, respectively, resulting in apparent activation of the phosphatase [9]. When Ser333 and Ser337 of GST-TIMAP$^{46–453}$ were mutated to mimic phosphorylation, the GST-TIMAP$^{S333D/S337D}$ and GST-TIMAP$^{S333E/S337E}$ mutants inhibited PP1cβ activity toward phosphorylase *a*, with an IC_{50} similar to the that of GST-TIMAP$^{46–453}$ (Fig. 2E), while their inhibitory activity toward PP1cγ was reduced by approximately one order of magnitude (Fig. 2F). These findings suggest that phosphorylation of TIMAP at Ser333/Ser337 does not alter the inhibitory activity toward associated PP1cβ and that GSK3β/PKA dependent modulation is directed toward PP1cγ, at least when phosphorylase *a* is the substrate.

3.3. MLC2, but not LAMR1, is a substrate for TIMAP/PP1cβ in vitro

The non-integrin laminin receptor (LAMR1) directly binds TIMAP, resulting in de-phosphorylation of LAMR1 in cells [5]. We therefore determined whether LAMR1 is a TIMAP/PP1cβ substrate. The cytoplasmic N-terminus of LAMR1 contains predicted phosphorylation sites for PKA (Ser43) and PKC (Ser78). Recombinant His-LAMR1$^{1–200}$ directly bound GST-TIMAP, consistent with published results [5], whereas glycogen phosphorylase did not (Supplemental data). His-LAMR1$^{1–200}$ was phosphorylated *in vitro* by PKA and PKC, and effectively de-phosphorylated by free PP1cβ, while the phosphatase activity toward LAMR1$^{1–200}$ was inhibited when PP1cβ was pre-associated with GST-TIMAP^{WT} (Supplemental data). As expected, the PP1c inhibitor calyculin A blocked His-LAMR1$^{1–200}$ dephosphorylation by free PP1cβ. Similar to the findings with phosporylase *a*, GST-TIMAP$^{46–292}$ was much less effective in inhibiting PP1cβ activity toward LAMR1$^{1–200}$ than TIMAP^{WT} and TIMAP$^{46–453}$ (Supplemental data).

Since TIMAP is a member of the MYPT family we also determined whether TIMAP/PP1cβ can act as a phosphatase toward MLC2 *in vitro*. We observed that MLC2 pre-phosphorylated with MLCK was effectively dephosphorylated by PP1cβ in the presence of TIMAP under conditions that inhibited phosphatase activity toward pre-phosphorylated LAMR1 and phosphorylase *a* (Fig. 3A). Also, when MLC2 or LAMR1 were phosphorylated in the presence of the pre-assembled TIMAP/PP1cβ complex, TIMAP and MLC2 were dephosphorylated and LAMR1 was not (Fig 3B). Thus, at least *in vitro*, TIMAP/PP1cβ is a robust MLC2–directed phosphatase.

3.4. TIMAP inhibits PKA- and PKC mediated phosphorylation of LAMR1

The findings that TIMAP inhibits PP1cβ activity toward LAMR1 did not seem consistent with the observation that LAMR1 is

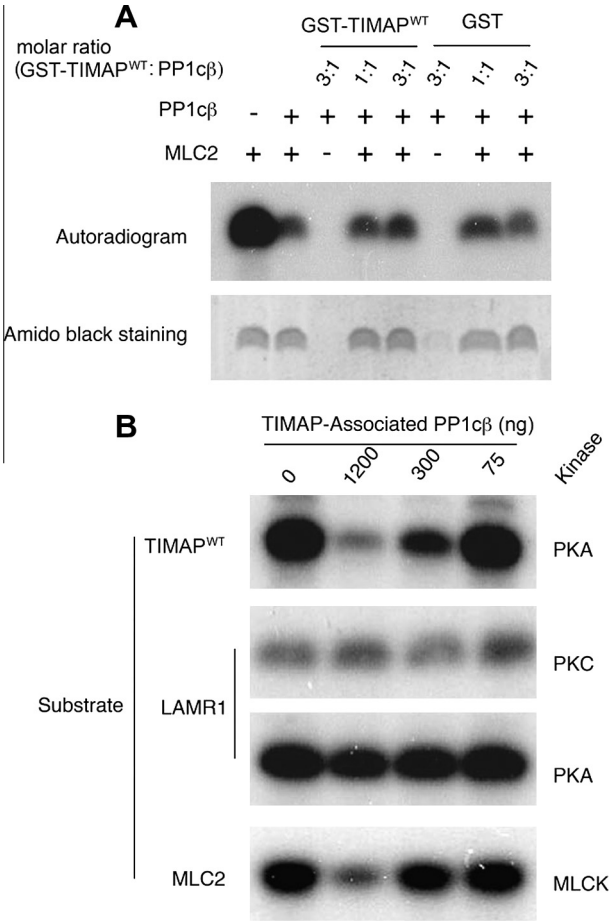

Fig. 3. GST-TIMAP does not inhibit PP1cβ activity toward MLC2. (A). Effect of TIMAP on MLC2-directed PP1cβ phosphatase activity. MLCK pre-phosphorylated MLC2 was incubated with PP1cβ without TIMAP, or in the presence of GST-TIMAP^{WT} or GST, each at molar ratios of 1:1 and 3:1. The ^{32}P-autoradiogram is shown at the top and the amido black stained gel showing MLC2 loading, at the bottom. (B). Concentration-dependent activity of PP1cβ complexed with TIMAP toward TIMAP, LAMR1 and MLC2. PP1cβ and immobilized TIMAP^{WT} were pre-incubated followed by extensive washing. MLC2 or LAMR1 in their kinase reaction buffers were then added to the TIMAP/ PP1cβ complex. TIMAP phosphorylation shown here occurred after addition of LAMR1/PKA.

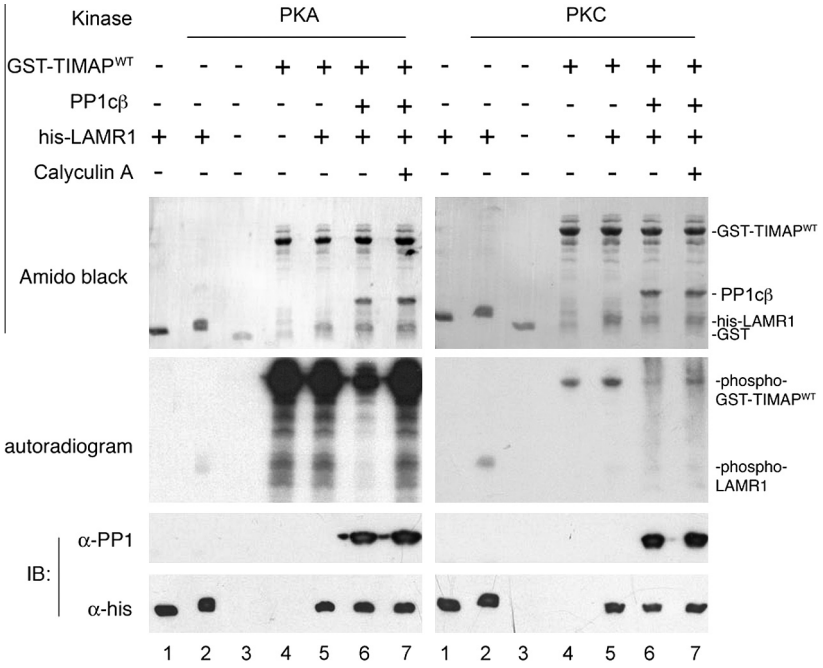

Fig. 4. Phosphorylation of LAMR1 and TIMAP by PKA and PKC in TIMAP/LAMR1 and TIMAP/LAMR1/PP1cβ complexes. Phosphorylation by PKA (left) or PKC (right) was performed *in vitro* using free His-LAMR1 (lane 2), immobilized GST-TIMAP^{WT} (lane 4), immobilized GST-TIMAP^{WT}/LAMR1 (lane 5), immobilized GST-TIMAP^{WT}/LAMR1/PP1cβ (lane 6), and immobilized GST-TIMAP^{WT}/LAMR1/PP1cβ in the presence of calyculin A (lane 7). Amido black stained gels are shown at the top, ^{32}P-autoradiograms in the middle and immunoblots with anti-PP1c and anti-His antibodies at the bottom.

hypo-phosphorylated in cells expressing TIMAP [5]. We therefore determined whether reduced LAMR1$^{1-200}$ phosphorylation could be detected in a GST-TIMAP^{WT}/LAMR1$^{1-200}$ complex, containing PP1cβ or not. The pre-formed complexes were subjected to phosphorylation *in vitro* by PKA or PKC. In response to PKA, there was substantial TIMAP^{WT} phosphorylation, consistent with previous observations [9]. PKC-mediated phosphorylation of TIMAP was much less pronounced (Fig. 4). Free His-LAMR1 was also phosphorylated by PKA or PKC, shown by autoradiography and by retardation of His-LAMR1 migration on SDS–PAGE gels and on anti-His immunoblots (Lane 2, Fig. 4). Whether PP1cβ was present or not, PKC-mediated LAMR1$^{1-200}$ phosphorylation was not observed by autoradiography or by mobility shift in the GST-TIMAP^{WT}/His-LAMR1$^{1-200}$ complex. Autoradiography of PKA-mediated His-LAMR1$^{1-200}$ phosphorylation was not informative because massive TIMAP phosphorylation obscured the appropriate bands on longer exposure. Nonetheless, the lack of His-LAMR1$^{1-200}$ migration retardation in SDS–PAGE gels (Lanes 5–7, Fig. 4) after PKA treatment strongly suggests that phosphorylation of His-LAMR1$^{1-200}$ by PKA is inhibited when it is associated with GST-TIMAP^{WT}. In the same GST-TIMAP^{WT}/His-LAMR1$^{1-200}$ complexes, TIMAP^{WT} itself was phosphorylated by PKA, and dephosphorylated when PP1cβ was present. Calyculin A protected TIMAP^{WT} from dephosphorylation by PP1cβ. By contrast, calyculin A failed to unmask any phosphorylation of His-LAMR1$^{1-200}$ pre-bound to GST-TIMAP^{WT}. These data indicate that LAMR1 is not a substrate for TIMAP-associated PP1cβ, but that LAMR1 phosphorylation sites are masked when LAMR1 is bound to TIMAP.

In conclusion, *in vitro* association of PP1c with TIMAP inhibits phosphatase activity toward phosphorylase *a* and LAMR1, but not toward TIMAP and MLC2. The inhibitory effect is modulated by the TIMAP C-terminus. TIMAP also modifies protein phosphorylation by restricting access of kinases to phosphorylation sites on associated PP1c and LAMR1. Therefore, the function of this PP1c regulatory subunit is broader than the modulation of PP1c activity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.012.

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