Phosphorylation of the myosin phosphatase target subunit by integrin-linked kinase

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A mechanism proposed for regulation of myosin phosphatase (MP) activity is phosphorylation of the myosin phosphatase target subunit (MYPT1). Integrin-linked kinase (ILK) is associated with the contractile machinery and can phosphorylate myosin at the myosin light-chain kinase sites. The possibility that ILK may also phosphorylate and regulate MP was investigated. ILK was associated with the MP holoenzyme, shown by Western blots and in-gel kinase assays. MYPT1 was phosphorylated by ILK and phosphorylation sites in the N- and C-terminal fragments of MYPT1 were detected. From sequence analyses, three sites were identified: a primary site at Thr^{709}, and two other sites at Thr^{695} and Thr^{495}. One of the sites for cAMP-dependent protein kinase (PKA) was Ser^{694}. Assays with the catalytic subunit of type 1 phosphatase indicated that only the C-terminal fragment of MYPT1 phosphorylated by zipper-interacting protein kinase, and ILK inhibited activity. The phosphorylated N-terminal fragment activated phosphatase activity and phosphorylation by PKA was without effect. Using full-length MYPT1 constructs phosphorylated by various kinases it was shown that Rho kinase gave marked inhibition; ILK produced an intermediate level of inhibition, which was considerably reduced for the $Thr^{695} \rightarrow$ Ala mutant; and PKA had no effect. In summary, phosphorylation of the various sites indicated that Thr^{695} was the major inhibitory site, Thr^{709} had only a slight inhibitory effect and Ser^{694} had no effect. The findings that ILK phosphorylated both MYPT1 and myosin and the association of ILK with MP suggest that ILK may influence cytoskeletal structure or function.

Key words: myosin dephosphorylation, regulation of myosin phosphatase, smooth-muscle contraction.

INTRODUCTION

Smooth-muscle contraction and relaxation are regulated by the reversible phosphorylation of myosin [1]. Phosphorylation of the 20 kDa myosin light chains (LC20) is catalysed by Ca^{2+}/calmodulin-dependent myosin light-chain kinase (MLCK) [2] and dephosphorylation by myosin light-chain phosphatase (MP) [3]. Both enzymes are subject to regulation, including that by phosphorylation–dephosphorylation. For example, MLCK is activated by members of the mitogen-activated protein kinase ('MAP kinase') family [4,5] and inhibited by Ca^{2+}/calmodulin-dependent protein kinase II [6], whereas MP is inhibited by Rho-associated kinase (ROK) [7] and myosin phosphatase target subunit-1 (MYPT1) kinase [8]. Inhibition of MP is one of the mechanisms thought to be responsible for Ca^{2+} sensitization of smooth-muscle contraction [9].

Integrin-linked kinase (ILK) has been implicated in integrin-mediated signal transduction and is a component of large protein complexes linking the extracellular matrix to the actin cytoskeleton [10]. Recently we identified a distinct population of ILK that is associated with the myofilaments of smooth muscle and phosphorylates LC20 (at the MLCK sites) in a Ca^{2+}/calmodulin-independent manner, causing activation of contraction [11]. ILK could therefore account for Ca^{2+}-independent contraction of smooth muscle, as well as contributing to Ca^{2+} sensitization. Both myosin and the MP target subunit (MYPT1) are phosphorylated by ROK [7,12] and by ZIP/MYPT1-kinase [8,13,14]. However, it was not known whether ILK could phosphorylate and regulate MYPT1. Here we show that ILK is associated with the MP holoenzyme, which may explain the association of ILK with the contractile apparatus, and phosphorylates MYPT1 at several sites and thus could modify MP activity.

EXPERIMENTAL

Materials

Chemicals and their suppliers: [γ-^{32}P]ATP, PerkinElmer Life Sciences (Boston, MA, U.S.A.); ATP, Sigma (St. Louis, MO, U.S.A.); adenosine 5′-[γ-thio]triphosphate (ATP[S]), Calbiochem (San Diego, CA, U.S.A.); Talon affinity resin, BD Biosciences Clontech (Palo Alto, CA, U.S.A.); GSH–Sepharose 4B, Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.); and nitrocellulose (0.2 μm pore size), Bio-Rad Laboratories (Hercules, CA, U.S.A.). Other chemicals/reagents were of the highest grade available.

Proteins

ILK was partially purified from chicken gizzard [11]. A synthetic peptide, residues 689–701 of MYPT1 (numbering for the chicken M133 isoform [15]) with phosphothreonine at position 695 and an additional C-terminal cysteine residue, was synthesized at the Peptide Synthesis Core Facility (University of Calgary, AB, Canada) and used to generate monoclonal antibodies. An IgG fraction recognizing only MYPT1 phosphorylated at Thr^{695} was

Abbreviations used: ATP[S], adenosine 5′-[γ-thio]triphosphate; GST, glutathione S-transferase; ILK, integrin-linked kinase; LC20, 20 kDa light chain of smooth-muscle myosin; MLCK, myosin light-chain kinase; MP, myosin phosphatase; MYPT, myosin phosphatase target subunit; PKA, cAMP-dependent protein kinase; PP1c, catalytic subunit of protein phosphatase type 1; ROK, Rho-associated kinase; TFA, trifluoroacetic acid; TOF, time-of-flight; ZIPK, zipper-interacting protein kinase.

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purified by G-Sepharose chromatography [16]. To determine the specificity of the antibody, excess phosphopeptide (antigen) or dephosphopeptide, both at 0.1 μg/ml, were incubated for 1 h with the diluted (1:100) antibody. Blots were then carried out with gizzard MYPT1 phosphorylated by ILK or, dephosphorylated MYPT1, as control (see Figure 4B below). Other controls to characterize this antibody are also shown in Figure 4(B) below. Rabbit polyclonal anti-ILK, the catalytic subunit of cAMP-dependent protein kinase (PKA) and Rho-associated kinase (ROKα/ROCKII) were from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.). A polyclonal antibody to gizzard MYPT1 (F38) was from Covance Inc. (Richmond, CA, U.S.A.). The catalytic domain of ZIP kinase was expressed as a glutathione S-transferase (GST) fusion protein (GST–ZIPK) and isolated as described previously [8]. Purification procedures for the following are given in [17]: type 1 protein phosphatase catalytic subunit (PP1c), 20 kDa myosin light chain (LC20), ^{32}P-labelled LC20, full-length MYPT1 (M133 chicken isoform) as a GST-fusion protein (termed GST–MYPT1) and a hexahistidine-tagged C-terminal fragment (residues 514–963 for the M130 chicken isoform [15]) of MYPT1 (termed C130). Note that the C-terminal sequence in C130 is identical with that of M133 [15], but the numbering differs because of the presence of a central insert in M133. For convenience the given sequences are adjusted to those in M133. Hexahistidine-tagged N-terminal MYPT1 fragments, residues 1–296 (termed E296) and residues 1–511 (termed N511) were described in [18]. Preparation of the full-length Thr^{695} → Ala mutant (termed GST–MYPT1 T695A) was given in [7]. The MP holoenzyme from turkey gizzard was isolated as described previously [15]. A partially purified MYPT1 (gizzard MYPT1) was isolated by DEAE-Sephacel and phosphocellulose chromatography of a 30 mM $MgCl_2$ extract of chicken gizzard myofilaments [19]. MYPT1 was detected using the F38 antibody.

Methods

Phosphorylation of GST–MYPT1, C130 and N511 by native ILK were carried out in a kinase assay buffer containing 30 mM Tris/HCl, pH 7.5, 85 mM KCl, 5 mM $MgCl_2$, 10 mM EGTA, 10 mM dithiothreitol, 1 % Tween-80, 1 μM microcystin-LR and 0.1 mM [γ-^{32}P]ATP (200–500 c.p.m./pmol). For some experiments ATP[S] was used. ^{32}P-labelled samples were digested overnight at 37 °C with trypsin (0.5 μg). Digests were acidified with trifluoroacetic acid (TFA) and applied to a reverse-phase column (Aquapore OD-300 C_{18}, 1.0 mm inner diameter × 100 mm long) equilibrated in 0.1 % TFA. The column was washed with 0.1 % TFA and peptides eluted with a linear gradient of acetonitrile (0–60 % in 100 min). Fractions (200 μl each) were collected and phosphopeptides identified by Cerenkov radiation. Part of the ^{32}P-containing fractions was used for phosphoamino acid analysis [20]. Fractions containing the major peaks of radioactivity were subjected to nanospray mass spectrometry on a QSTAR-Pulsar mass spectrometer (Applied Biosystems, Foster City, CA, U.S.A.). Peptide whole masses from precursor ion scans were determined by time-of-flight (TOF) MS in the negative mode (TOFNEG), which identified those parent ions that liberated the characteristic m/z 78.997 corresponding to the phosphate ion. The positive mode (TOFPOS) was then used to measure by tandem MS/MS the masses of peptide fragments produced by collision-induced dissociation and, ultimately, to predict the sequences of the unknown phosphopeptides.

The C130 fragment was phosphorylated by PKA (0.5 μg/ml) under the above conditions to ≈ 2 mol of P_i/mol and digested with lysyl endopeptidase. ^{32}P-labelled peptides were separated as described above. Sequential Edman degradation of ^{32}P-labelled peptides was completed on a vapour-phase amino acid sequencer (Procise 494; Applied Biosystems,). The phosphopeptide samples were immobilized on Immobilon (Millipore) membrane by following the manufacturer's instructions. Phosphorylated residues within phosphopeptides were located by determining the cycles in which ^{32}P was released when samples were subjected to sequential Edman chemistry under conditions that optimize recovery of ^{32}P [21].

Samples for Western blotting with the antibody specific for phospho-Thr^{695} were prepared by phosphorylation/thiophosphorylation of MYPT1 and fragments as described previously [18] using ILK, PKA (0.5 μg/ml) and ROK (50 munits/ml) as control. Phosphatase assays with [^{32}P]LC20, SDS/PAGE, Western blots and determination of protein concentration are documented in [17]. The in-gel kinase assay, using various MYPT1-derived substrates, was described previously [22].

RESULTS AND DISCUSSION

It is known that the MP holoenzyme contains endogenous kinase activity [8,23]. As shown in Figure 1, at least part of this activity is due to the presence of ILK. The anti-ILK antibody recognized a band of ≈ 59 kDa in the holoenzyme and a band of similar mobility was found in the ILK preparation (Figure 1A). (The molecular mass of mammalian ILK is about 59 kDa [24]). An in-gel kinase assay, using the C130 fragment as substrate, detected three bands of kinase activity (Figure 1B). Of these three kinases the band at ≈ 59 kDa corresponded to ILK. The band at ≈ 35 kDa was identified previously as MYPT1-kinase (previously termed ZIP-like kinase [8]) and the higher-mobility band at ≈ 70 kDa was not identified. In addition, a single band of activity at ≈ 59 kDa was detected in the ILK preparation by an in-gel kinase assay using GST–MYPT1 as substrate (results not shown). In-gel kinase assays using LC20 as substrate detected the 35 and 59 kDa bands (results not shown) and this is consistent with earlier observations that both ILK [11] and zipper-interacting protein kinase (ZIPK) [25] phosphorylate myosin. These data suggest that part of the endogenous kinase activity in MP preparations is due to ILK, and thus it is likely that ILK associates with one or more of the MP subunits.

The next point was to determine whether ILK phosphorylated the MP holoenzyme. Previously it was shown [23] that the endogenous kinases in the MP holoenzyme phosphorylated predominantly the large subunit, i.e. MYPT1 and not PP1c. Several subsequent studies (reviewed in [3]) have focused on regulation of MP activity via phosphorylation of MYPT1. Therefore, time courses of phosphorylation were carried out using the ILK preparation and MYPT1 and its N- or C-terminal fragments (Figure 1C). The full-length MYPT1 construct, GST–MYPT1, was phosphorylated to about 1.3 mol of P_i/mol of MYPT1. The C-terminal fragment, C130, and the N-terminal fragment, N511, were also phosphorylated, both to about 0.8 mol of P_i/mol (Figure 1C). The N-terminal fragment, E296, which contains the PP1c-binding motif and the ankyrin repeats [3], was not phosphorylated. Thus the N-terminal phosphorylation site(s) was within the sequence 297–511. The major phosphorylation site on MYPT1 responsible for inhibition of PP1c with Rho-kinase [7], MYPT1-kinase [8] or myotonic dystrophy protein kinase [17] is Thr^{695}. It is noteworthy that phosphorylation by ILK of a full-length mutant of MYPT1 in which Thr^{695} is changed to Ala, GST–MYPT1 T695A, shows only slightly reduced phosphorylation levels compared with wild-type MYPT1. This indicates that although Thr^{695} may contribute to overall phosphorylation levels, it is not the major site of phosphorylation in MYPT1 for ILK.

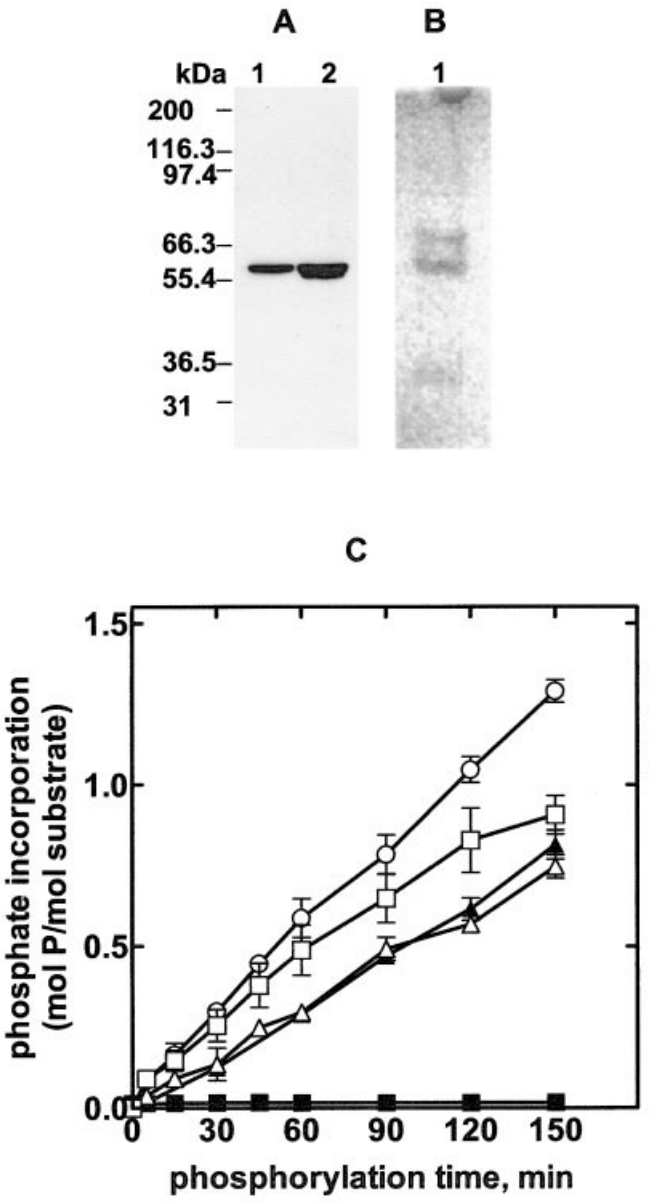

Figure 1 Phosphorylation of MYPT1 and its fragments by ILK

(**A**) Western blots with anti-ILK. Lane 1, MP holoenzyme; lane 2, ILK preparation. About 2 μg of protein was loaded per lane. (**B**) In-gel kinase assay. C130 (100 μg/ml) was incorporated throughout the gel by addition to the gel buffers prior to polymerization. MP holoenzyme (2 μg) was then subjected to SDS/PAGE and proteins were renatured in the gel. The kinase reaction was started by incubating the gel in kinase assay buffer containing 100 μCi of [γ-^{32}P]ATP for 1.5 h at 30 °C. Incorporation of ^{32}P into C130 was identified by autoradiography. (**C**) Phosphorylation of various substrates by ILK. The substrate concentration was 100 μg/ml. ○, GST–MYPT1; □, GST–MYPT1 T695A; △, C130; ▲, N511 (MYPT1 fragment 1–511); ■, E296 (MYPT1 fragment 1–296). Values are means ± S.E.M. ($n = 3$ or 4).

To identify the sites of phosphorylation on MYPT1, different MYPT1 preparations (see the Experimental section) were phosphorylated by ILK and digested with trypsin. ^{32}P-labelled peptides were separated by HPLC (Figure 2A). A major peptide (C3) was present in the full-length MYPT1 and the C130 digests. The C3 peptide from C130 was subjected to analysis by nanospray MS (Figure 2B). The peptide sequence was determined from the b- and y-series ions and the phosphorylated residue identified as Thr^{709}. Identical results were obtained with the C3 peptide from GST–MYPT1. Another site contained in the C1 peptide (from GST–MYPT1) was identified as the inhibitory site, Thr^{695} (Figure 2C). However, on the basis of the results shown in Figure 1(C) and the fact that the levels of phosphorylation for the Thr^{695} → Ala mutant were only slightly lower than wild-type, it is proposed that Thr^{695} is a minor/secondary site for ILK on MYPT1. A third sequence was determined from the N1 peak of the N511 digest and corresponded to residues 490–498, i.e. Ala-Tyr-Val-Ala-Pro-Thr-Ile-Pro-Arg, suggesting Thr^{495} as a third phosphorylation site.

Previously it was shown that MYPT1 possesses at least three sites of phosphorylation by PKA [26]. To determine if any of these sites are in the same region as the ILK sites (i.e. Thr^{709} and Thr^{695}) the C130 fragment was phosphorylated by PKA and a ^{32}P-labelled peptide subjected to sequencing (see the Experimental section). One phosphorylation site was identified as Ser^{694}. Two other putative sites for PKA in the C-terminal fragment of MYPT1 are Ser^{849} and Ser^{986} (based on the consensus sequence for PKA).

The above results indicate that MYPT1 has at least three sites of phosphorylation by ILK: one in the N-terminal half, namely Thr^{495}; and two in the C-terminal half, a major/primary site at Thr^{709} and a minor/secondary site at Thr^{695}. Also it is shown that, within the sequence 694–709, there are three phosphorylation sites, Ser^{694} for PKA and the two ILK sites. It was suggested [3,7] that Thr^{695} is the inhibitory site, but the effect(s) of the other sites on PP1c activity have not been established. Previously it was reported that phosphorylation of MYPT1 by cGMP-dependent protein kinase and PKA did not inhibit MP activity [27], but the sites of phosphorylation were not identified.

Truncation mutants of MYPT1 were used to investigate which phosphorylation sites had an inhibitory effect on PP1c. Initially the N-terminal fragment N511 was tested following phosphorylation by ILK. As shown in Figure 3, this caused activation of PP1c activity (the activity of PP1c alone was taken as 100 %) and this is consistent with previous results using dephosphorylated N-terminal fragments [18]. Thus the focus of further experiments was on the C-terminal mutant, C130. The dephosphorylated C130 and C130 phosphorylated by PKA were not inhibitory (Figure 3). However, C130 phosphorylated by GST–ZIPK and by ILK both inhibited PP1c activity, with the former being slightly more effective. These results demonstrate that the C-terminal truncation mutants, e.g. C130, can be used to study inhibition of PP1c. The structural requirements for inhibition (i.e. minimum size) have not been established. However, it was shown that a peptide containing Thr^{695} (sequence 685–702) phosphorylated by either GST–ZIPK or ILK was not inhibitory at the concentrations used in Figure 3 (results not shown).

Although the use of C-terminal truncation mutants is a useful investigative tool, there are distinct differences between the modes of inhibition induced by phosphorylated full-length MYPT1 and by the phosphorylated truncation mutants. The extent of inhibition is different. With the phosphorylated mutants essentially complete inhibition is attained, compared with about 20 % residual activity for the PP1c–MYPT1 complex (see Figure 4A). Full inhibition of PP1c would also be observed with the traditional PP1c inhibitors, e.g. inhibitor 1 (reviewed in [3,28]). In addition, the two modes of inhibition are achieved by markedly different concentrations of MYPT1 or the C-terminal mutant. For the full-length phosphorylated MYPT1 maximum inhibition is obtained at close-to-molar stoichiometry with PP1c, in the 5 nM range [17]. The phosphorylated C130, by contrast, requires

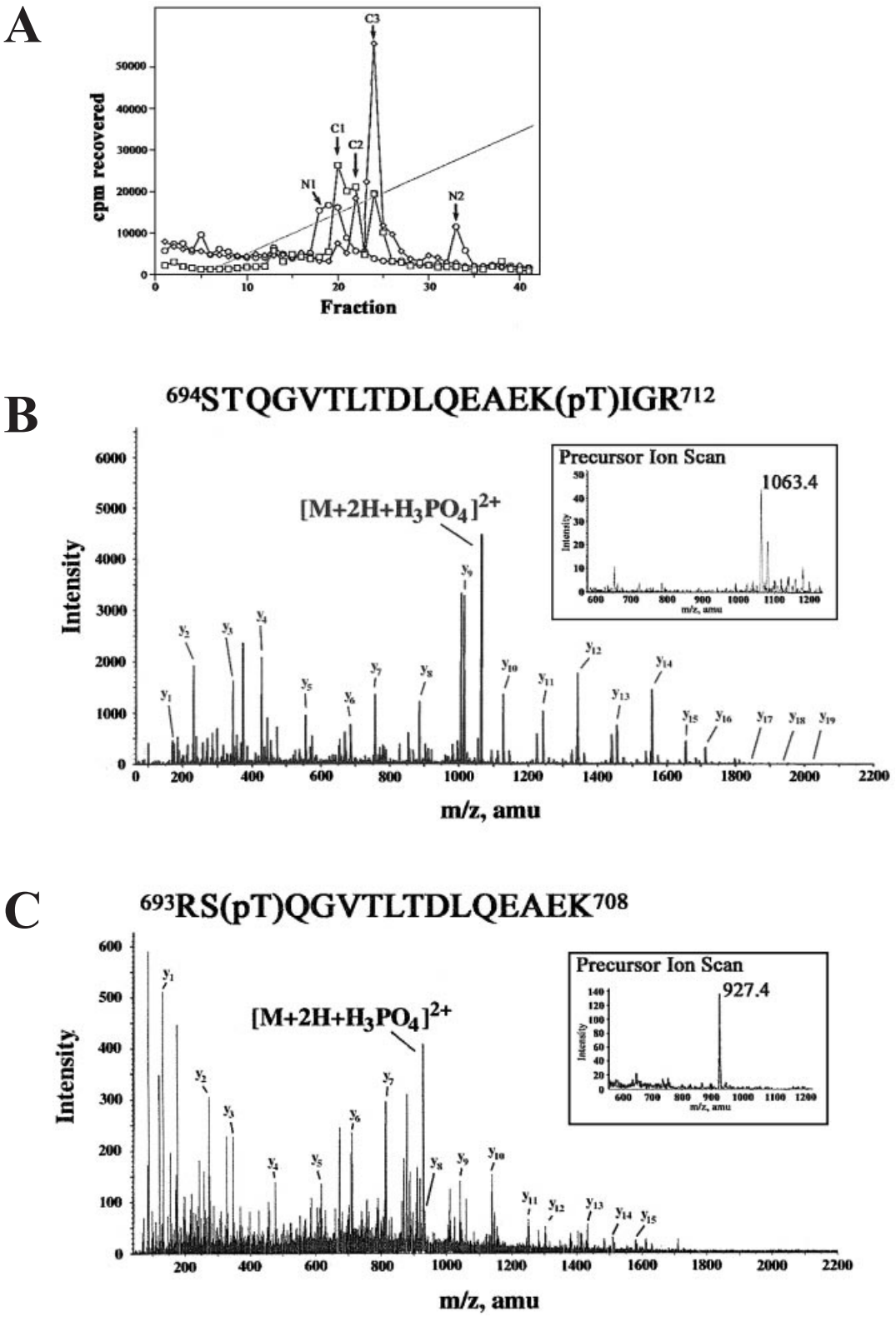

Figure 2 Identification of phosphorylation sites for ILK in GST–MYPT1 and its fragments

(**A**) Separation of ^{32}P-labelled tryptic peptides by HPLC. Results for digests of phosphorylated GST–MYPT1 (□), phosphorylated N511 (MYPT1 fragment 1–511, ○) and phosphorylated C130 (◇) are shown. Selected fractions were analysed on a QSTAR/Pulsar mass spectrometer. (**B**) TOFPOS MS/MS spectrum of peptide C3 from the C130 digest showing doubly charged phosphorylated parent ion with *m/z* 1064.4. The sequence is based on recovered b and y fragment ions. (**C**) TOFPOS MS/MS spectrum for peptide C1 from GST–MYPT1. The sequence of the ion of *m/z* 928.4 is shown. Insets in (**B**) and (**C**): TOFNEG precursor ion scans displaying *m/z* of peptides liberating the 78.997 *m/z* phosphate product: Scanned mass ranges were 400.0 to 1500.0 by 1.0 amu (atomic mass units) with a dwell time of 100.0 ms and a pause time of 5.0 ms.

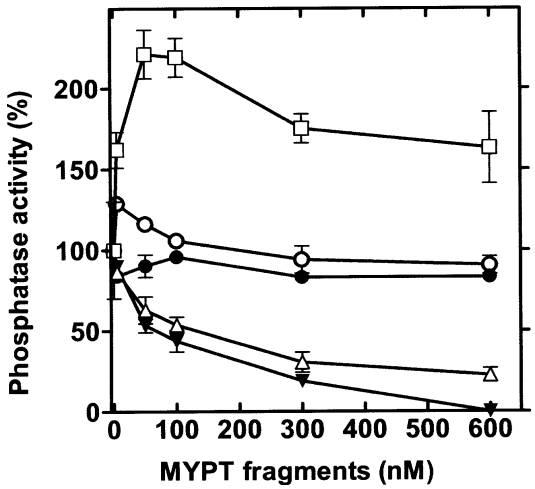

Figure 3 Effect of MYPT1 fragments on PP1c activity

Assays were carried out with PP1c (5 nM) and [^{32}P]LC20 (5 μM). C130 and N511 (MYPT fragment 1–511) were thiophosphorylated with ATP[S] (0.1 mM) prior to phosphatase assays. ○, Dephosphorylated C130; □, N511 thiophosphorylated (0.8 mol of P/mol) by ILK; ●, C130 thiophosphorylated (1.9 mol of P/mol) by PKA; △, C130 thiophosphorylated (0.75 mol of P/mol) by ILK; ▼, C130 thiophosphorylated (1.4 mol of P/mol) by GST–ZIPK. Values are means ± S.E.M. ($n = 3$ or 4).

considerably higher concentrations (Figure 3). The reduced sensitivity of inhibition may reflect the absence of the PP1c-binding motif (residues 35–38) in the C-terminal mutant, an effect that is also observed for mutants of inhibitor 1 [28].

The two phosphorylation sites that remain to be distinguished, with respect to inhibition of PP1c, are Thr^{695} and Thr^{709}, and, for these studies, full-length MYPT1 constructs were used. GST–MYPT1 was thiophosphorylated by ROK and inhibition of PP1c activity was determined as a function of the thiophosphorylation level (at constant GST–MYPT1, concentration 5 nM). As shown in Figure 4(A), a plateau of inhibition was observed at approx. 20 % of control activity (PP1c plus dephosphorylated GST–MYPT1). A similar experiment using GST–MYPT1 thiophosphorylated by ILK also inhibited PP1c activity, but the extent of inhibition was less than that obtained with ROK. However, using the GST–MYPT1 Thr^{695} → Ala mutant thiophosphorylated by ILK, only slight inhibition was observed (Figure 4A). In confirmation of the results shown above, thiophosphorylation of GST–MYPT1 by PKA did not cause inhibition of PP1c.

Western blots using the antibody specific for phospho-Thr^{695} are shown in Figure 4(B). Initially it was shown that incubation of the antibody with excess phosphopeptide antigen eliminated the antibody response, whereas the dephosphopeptide had no effect. Several other controls also are shown, i.e. that the dephosphorylated species of the holoenzyme, GST–MYPT1 and C130 did not react and that GST–MYPT1 phosphorylated by PKA also was negative. Positive antibody reactions were obtained for GST–MYPT1 and C130 phosphorylated by Rho kinase and for ILK phosphorylations of gizzard MYPT1, GST–MYPT1 and C130. Phosphorylation of the MP holoenzyme by endogenous kinases also indicated phosphorylation of Thr^{695}. Previously it was shown that Thr^{695} is a major site for Rho kinase [7], and these results confirm that Thr^{695} also can be phosphorylated by ILK.

The above results indicate that the dominant inhibitory phosphorylation site is Thr^{695}, as illustrated by the results with

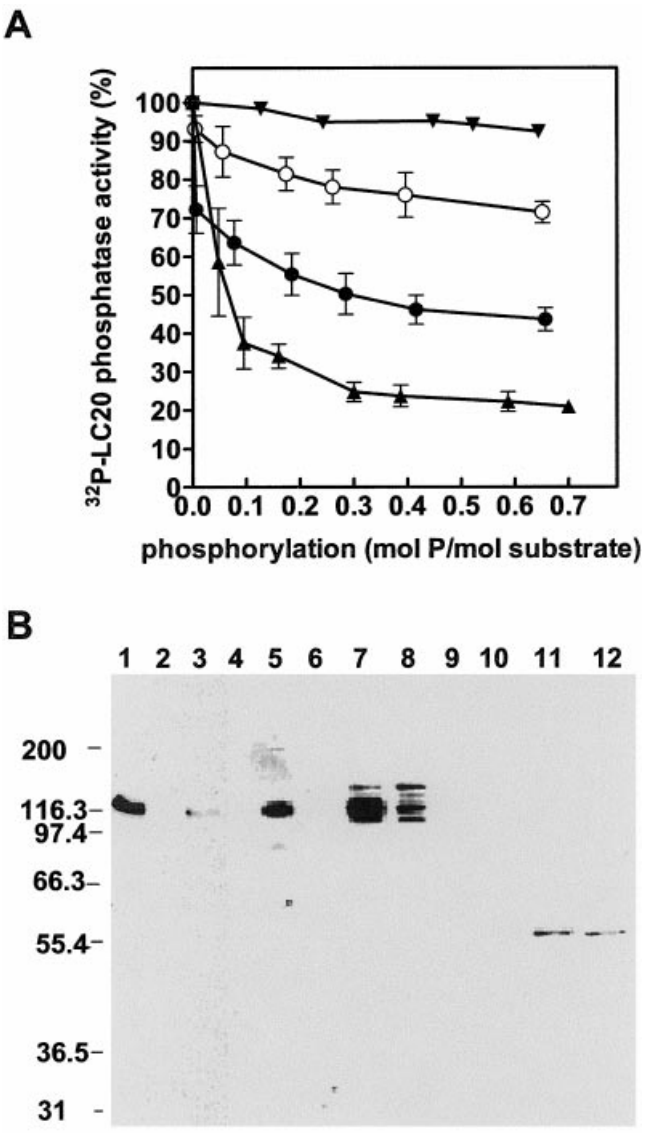

Figure 4 Effect of MYPT1 phosphorylation by various kinases on PP1c activity: phosphorylation dependence

(**A**) GST–MYPT1 (●) or GST–MYPT1 T695A (both at 625 nM) (○) were thiophosphorylated by ILK [20%, v/v; using the active fraction(s) from MonoQ chromatography], and 625 nM GST–MYPT1 was thiophosphorylated by ROK (50 munits/ml) (▲) or PKA (0.5 μg/ml) (▼). The thiophosphorylated samples were diluted to 5 nM in the phosphatase assay with 5 nM PP1c and 5 μM [^{32}P]LC20. Values are means ± S.E.M. ($n = 3$ or 4). (**B**) Western blots using phosphorylation site-specific antibody (phospho-Thr^{695}). The various samples were thiophosphorylated (see Figure 3) or phosphorylated (lanes 1 and 3) by various kinases for 1 h. The phosphospecific antibody was used at a dilution of 1 : 100 in lanes 1 to 3 and 1 : 1000 for others. Lane 1, gizzard MYPT1 phosphorylated by ILK and antibody incubated with dephosphopeptide; lane 2, dephosphorylated gizzard MYPT1; lane 3, gizzard MYPT1 phosphorylated by ILK and antibody incubated with phosphopeptide (antigen); lane 4, dephosphorylated MP holoenzyme; lane 5, MP holoenzyme thiophosphorylated by endogenous kinases (0.6 mol of P/mol); lane 6, non-phosphorylated GST–MYPT1; lane 7, GST–MYPT1 thiophosphorylated by ROK (0.7 mol of P/mol); lane 8, GST–MYPT1 thiophosphorylated by ILK (0.4 mol of P/mol); lane 9, GST–MYPT1 thiophosphorylated by PKA (1.2 mol of P/mol); lane 10, non-phosphorylated C130; lane 11, C130 thiophosphorylated by ROK (0.68 mol of P/mol); lane 12, C130 thiophosphorylated by ILK (0.25 mol of P/mol). lanes 1–3, 0.5 μg of protein/lane, lanes 4–12, 1.5 μg of protein/lane.

ROK and as suggested previously [7]. In addition, they allow a better definition of the inhibitory sequence. It is surprising that phosphorylation by PKA of Ser^{694}, adjacent to the inhibitory

site, has no apparent effect. This may reflect the requirement for phosphothreonine as the inhibitory species, also found with inhibitor 1 [28]. The inhibitory sequence appears restricted, however, as phosphorylation of Thr^{709} (by ILK) has only a slight inhibitory effect. The inhibition of PP1c activity by the GST–MYPT1/ILK combination was interpreted to reflect a reduced fractional phosphorylation of Thr^{695} by ILK, as compared with ROK, and additional phosphorylation at Thr^{709}.

Since MP acts on phosphorylated myosin, it is reasonable to expect co-localization of MP and myosin in the cytoskeleton. Indeed, localization of MYPT1 on stress fibres has been observed [29,30]. However, it was also shown that MYPT1 localized at the plasmalemma where its association with myosin was not apparent [29,30]. In addition, it was demonstrated recently that, in response to specific agonist stimulation, the phosphorylated MYPT1 was translocated to the smooth-muscle cell membrane [31]. These data and the reported binding of MYPT1 to phospholipids [26] suggest that MYPT1 can bind to components of the plasmalemma and that MP may have substrates other than myosin. Thus, with the inclusion of the above results, the possibility is raised that it may be involved with ILK in the multimeric complexes that link the extracellular matrix to the actin cytoskeleton [10]. ILK is known already to interact with several proteins, including β1 and β3 integrins, PINCH, affixin, paxillin and CH-ILK BP. The latter three link ILK directly to actin filaments [10]. The association of ILK with MP and a membrane location for MYPT1 suggest a scenario in which MP regulates cytoskeletal interactions in a manner that is controlled by ILK.

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