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Phosphorylation of the regulatory subunit of smooth muscle protein phosphatase 1M at Thr850 induces its dissociation from myosin

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Abstract **Rho kinase is known to control smooth muscle contractility by phosphorylating the 110 kDa myosin-targetting subunit (MYPT1) of the myosin-associated form of protein phosphatase 1 (PP1M). Phosphorylation of MYPT1 at Thr695 has previously been reported to inhibit the catalytic activity of PP1. Here, we show that the phosphorylation of Thr850 by Rho kinase dissociates PP1M from myosin, providing a second mechanism by which myosin phosphatase activity is inhibited.**

Key words: Protein phosphatase 1; Rho kinase; Smooth muscle; Myosin

1. Introduction

Protein phosphatase 1 (PP1), one of the major serine/threonine-specific protein phosphatases of eukaryotic cells, is regulated by a variety of targetting subunits that direct the catalytic subunit (PP1C) to particular subcellular locations, modify its substrate specificity and allow its activity to be regulated in a substrate-specific manner in response to extracellular signals (reviewed in [1–3]). For example a glycogen-associated form present in muscle, termed PP1G, comprises PP1C complexed to a glycogen-binding G_M subunit. [4]. Phosphorylation of the G_M subunit by cyclic AMP-dependent protein kinase triggers the dissociation of PP1C from G_M. This releases PP1 from glycogen, which is thought to inhibit the dephosphorylation of glycogen-bound substrates (glycogen phosphorylase and glycogen synthase) [5,6].

The form of PP1 associated with myosin (PP1M) consists of PP1C complexed to a myosin-targetting subunit, termed MYPT1 (myosin phosphatase targetting 1) or M_{110}, which is itself complexed to another protein, termed M_{21} [7–10]. The binding site for PP1C is close to the N-terminus of MYPT1 [9], a myosin-binding site is situated between residues 714 and 933, while the C-terminal 72 residues of MYPT1 bind the M_{21} subunit [10].

The Rho-activated kinase (termed ROK or ROCK) is thought to control the level of phosphorylation of the myosin P-light chain [11–13]. One way in which this is thought to occur is via the ROCK-catalysed phosphorylation of MYPT1, which is reported to inhibit PP1M activity and so increase the level of phosphorylation of the myosin P-light chain. This is believed to induce the contraction of smooth muscle and to induce stress fibre formation in non-muscle cells [14]. ROCK has been reported to phosphorylate MYPT1 at Thr695/Thr697 (chicken/rat), Ser 849/854 and Thr850/855 [13]. Based on experiments in which Thr695 and Thr850 were mutated to Ala, the phosphorylation of Thr695 appears to be required and sufficient for the inhibition of PP1M catalytic activity [15]. However, the role that phosphorylation of Thr850 and Ser849 may play in the regulation of PP1M function has not yet been determined. Here, we show that the phosphorylation of PP1M by the ROCK-II/ROKα isoform [16] dissociates PP1M from myosin and implicates Thr850 in this process.

2. Materials and methods

2.1. Materials

Oligonucleotides were from Oswell (Cambridge, UK), enzymes from Roche (Basel, Switzerland), baculovirus expression kit from Life Technologies (Paisley, UK), Vydac 218TP54 C_{18} column from the Separations Group (Hesperia, CA, USA), and okadaic acid and microcystin-LR from Calbiochem-Novabiochem Corp. (La Jolla, CA, USA). All other chemicals were from BDH Chemicals or Sigma (Poole, UK). Smooth muscle PP1M and myosin were purified from chicken gizzard [7,9] and residues 714–1004 of chicken gizzard MYPT1 were expressed as a maltose-binding protein (MBP) fusion protein [7].

2.2. Antibody production

The PP1M holoenzyme was injected into sheep and the antisera affinity-purified on Sepharose to which the C-terminal fragment comprising residues 714–1004 of MYPT1 had been attached covalently, in order to obtain antibodies that recognise the MYPT1 subunit specifically [7]. Antibodies that recognise chicken gizzard MYPT1 only when it is phosphorylated at Thr850 were raised against the peptide Glu-Lys-Arg-Arg-Ser-pThr-Gly-Val-Ser-Phe-Trp (where pThr denotes phosphothreonine). The peptides were conjugated separately to bo-

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Abbreviations: PP1, protein phosphatase 1; PP1M, smooth muscle protein phosphatase 1; PP1C, protein phosphatase 1 catalytic subunit; MYPT1, myosin phosphatase targetting 1; ROKα/ROCK-II, Rho-dependent protein kinase

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vine serum albumin (BSA) and keyhole limpet haemocyanin, mixed and injected into sheep at Diagnostics Scotland (Penicuik, UK). The antisera were affinity-purified on peptide CH-Sepharose columns.

2.3. Construction of vectors and expression of ROCK-II in Sf9 cells

The plasmid pGEX4T-ROKα^{1-543} (a generous gift from Dr Louis Lim, IMCB, Singapore) encoding the catalytic domain of rat ROCK-II was the template for PCR amplification using the oligonucleotides: Nt: 5′-CGGGATCCGAATTCGCCACCATGTACCCATACGATG-TGCCAGATTACGCCCCCGGCGCCCCCGAGGCC-3′, Ct: 5′-C-CATCGATTTATATCTGAGAGCTCTGGTTTC-3′.

A baculovirus vector was generated according to the manufacturer's instructions (Life Technologies, Paisley, UK) and used to infect insect cells. The cells were collected, lysed in buffer containing 50 mM Tris–HCl pH 8.5, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulphonyl fluoride and 1% Nonidet P40. The 6-His-tagged protein was then purified from the extracts by affinity chromatography on nickel-nitrilotriacetate agarose. The His-ROCK-II is available from Upstate (www.upstate.com).

2.4. Phosphorylation by ROCK-II

0.5 μM PP1M or 1 μM MBP-MYPT1[714–1004] were incubated for 10 min with 50 nM ROCK-II for the myosin-binding assays, or 0.2 μM PP1M was incubated with 0.5 nM ROCK-II to study the effect of phosphorylation on PP1M activity. The phosphorylation reactions were carried out in 50 mM Tris–HCl, pH 7.5, 0.1 mM EGTA, 0.1% (by vol.) 2-mercaptoethanol, 0.03% (by mass) Brij 35, 10 mM Mg acetate and either 200 μM ATP[γS] or 100 μM ATP. The thiophosphorylated PP1M or phosphorylated MBP-M_{110}(714–1004) were then diluted in buffer A (50 mM Tris–HCl, 0.1 mM EGTA, 0.1% (by vol.) 2-mercaptoethanol, 0.03% (by mass) Brij 35, 1 mg/ml BSA) and used to study the binding to myosin or to measure PP1M activity.

2.5. Protein phosphatase assays

These were carried out by measuring the dephosphorylation of ^{32}P-labelled myosin P-light chain or ^{32}P-labelled glycogen phosphorylase as described [7].

2.6. Myosin-binding experiments

These were performed as described [10]. Briefly, myosin (0.5 mg/ml) in 10 mM HEPES, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.1% (by vol.) 2-mercaptoethanol was mixed with PP1M or MBP-MYPT1[714–1004] that had been phosphorylated with or without ROCK-II. After incubation for 30 min at 0°C, the suspensions were centrifuged for 2 min at 13 000×*g* and the supernatants collected. The myosin pellets were then washed twice in 10 mM HEPES, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.1% (by vol.) 2-mercaptoethanol, before redissolving to the same volume as the supernatant fraction in 50 mM Tris–HCl, pH 7.5, 0.1 mM EGTA, 0.03% (by mass) Brij 35, 0.6 M NaCl, 0.1% (by vol.) 2-mercaptoethanol. Aliquots of the supernatant and the resuspended

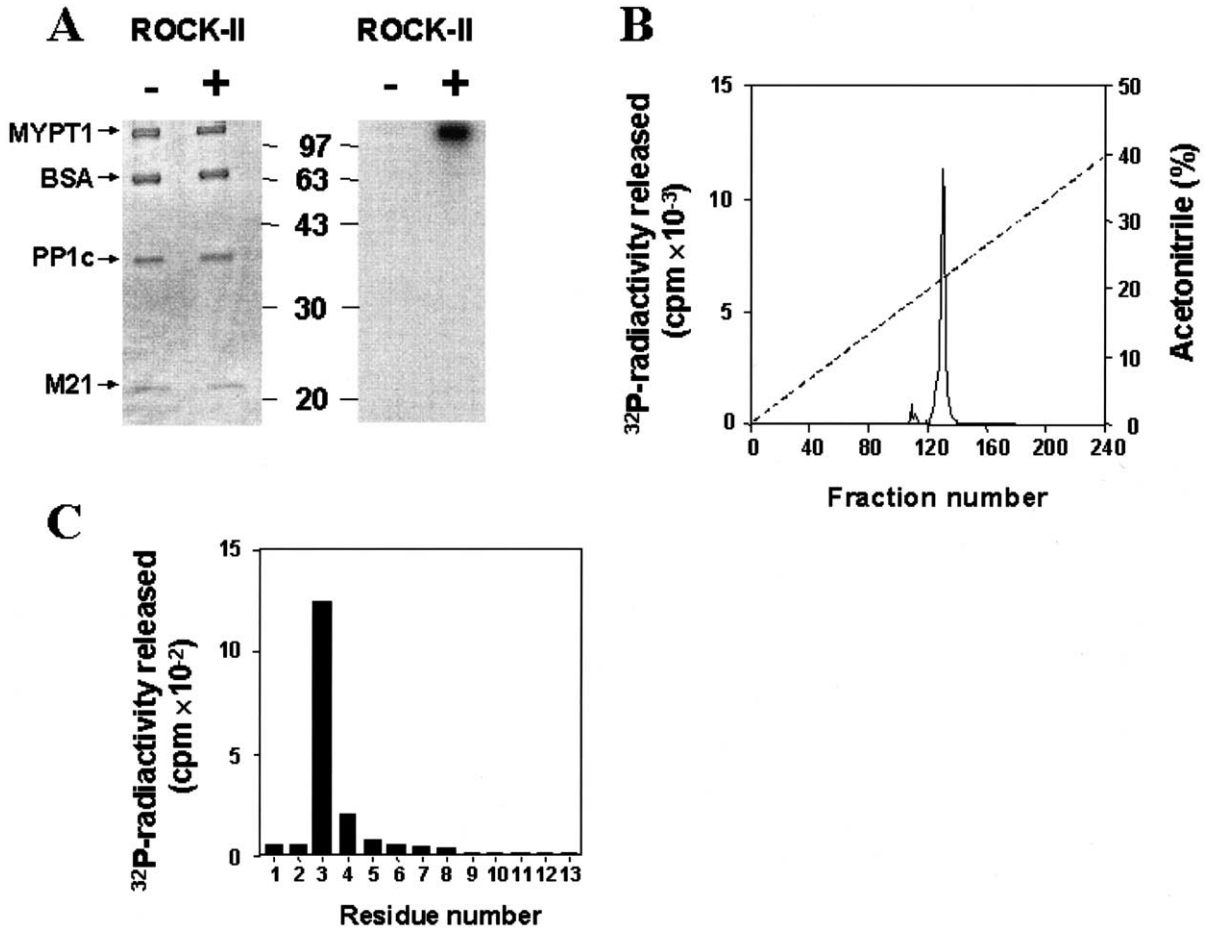

Fig. 1. ROCK-II phosphorylates the MYPT1 subunit of chicken gizzard PP1M at Thr695 and Thr850. A: The chicken gizzard PP1M complex (1 μM) was incubated for 15 min with 50 nM ROCK-II in 50 mM Tris–HCl, pH 7.5, 0.1 mM EGTA, 0.1% (by vol.) 2-mercaptoethanol, 0.03% (by mass) Brij 35, 1 μM okadaic acid, 10 mM Mg acetate and 100 μM [^{32}P]ATP, then denatured in SDS, separated by SDS–PAGE and either stained with Coomassie blue (left hand gel) or autoradiographed (right hand gel). B: The ^{32}P-labelled band from A was excised, eluted from the gel piece and digested with trypsin as described [22]. The supernatant containing >90% of the ^{32}P radioactivity was chromatographed on a Vydac 218TP54 C_{18} column equilibrated with 0.1% (by vol.) trifluoroacetic acid and developed with a linear acetonitrile gradient (broken line). The ^{32}P radioactivity is shown by the full line. The flow rate was 0.8 ml/min, and fractions of 0.4 ml were collected. C: The ^{32}P-labelled phosphopeptide from B was subjected to solid phase sequencing and ^{32}P radioactivity released after each cycle of Edman degradation was measured by Cerenkov counting to identify the sites of phosphorylation [22].

pellets were denatured in SDS and the presence of PP1M or MBP-MYPT1[714–1004] analysed by SDS–PAGE and immunoblotting.

3. Results and discussion

3.1. ROCK-II phosphorylates the MYPT1 subunit at Thr695 and Thr850

We used a constitutively active fragment of ROCK-II comprising residues 1–543 [16] to phosphorylate the PP1M complex. Only the MYPT1 subunit, but not the M_{21} subunit or the catalytic subunit, became phosphorylated (Fig. 1A). The maximally phosphorylated PP1M was digested with trypsin and chromatographed on a C_{18} column, which revealed a single peak of ^{32}P radioactivity (Fig. 1B) that was subjected to mass spectrometry and Edman sequencing. These experiments demonstrated the presence of two species corresponding to monophosphorylated derivatives of the peptides RSTQGVTLTDLQE and RSTGVSFWTQDSD, comprising residues 693–705 and 848–860, respectively. Solid phase sequencing showed that the ^{32}P radioactivity was only released after the third cycle of Edman degradation (Fig. 1C). This directly identified residues 697 and 850 as the sites of phosphorylation, which had previously been inferred from site-directed mutagenesis experiments [15]. No phosphorylation of Ser849 was detected in our experiments.

3.2. Phosphorylation by Rho kinase inhibits PP1M activity

The PP1M complex was phosphorylated by ROCK-II using ATP[γS] to produce a thiophosphorylated derivative that would be relatively resistant to autodephosphorylation by the PP1 catalytic subunit. Under these conditions, there was a time-dependent loss of PP1M activity when either the myosin P-light chain (Fig. 2A) or glycogen phosphorylase (Fig. 2B) were used as substrates, which was abrogated in the presence of Y27632, a relatively specific inhibitor of Rho kinase [17,18]. There was no inhibition of PP1M in control incubations where ATP[γS] was omitted.

The interaction of MYPT1 with PP1C is known to enhance phosphatase activity towards myosin and suppress activity towards glycogen phosphorylase [7]. As a consequence, the dissociation of these subunits stimulates phosphorylase phosphatase but decreases myosin phosphatase activity. Therefore, the finding that the ROCK-II-catalysed phosphorylation of PP1M inhibits phosphorylase phosphatase as well as myosin phosphatase activity implies that inhibition of myosin phosphatase activity does not result from the dissociation of PP1C and MYPT1. This is in contrast to the PKA-catalysed phosphorylation of PP1G, which triggers the dissociation of PP1C from the G_M subunit (see Section 1).

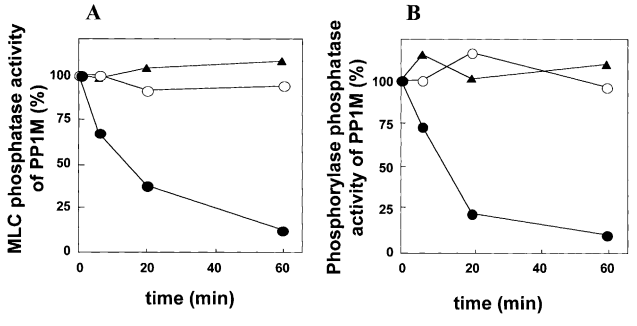

Fig. 2. Phosphorylation by ROCK-II inhibits PP1M activity. PP1M was phosphorylated as described under Section 2 in the presence of 0.5 nM ROCK-II (open circles), 0.5 nM ROCK-II and 0.2 mM ATP[γS] (closed circles), or 0.5 nM ROCK-II, 0.2 mM ATP[γS] and 50 μM Y27632 (closed triangles). Aliquots of the reactions were taken at the times indicated, diluted in buffer A and assayed for myosin light chain (MLC) phosphatase activity (A) or phosphorylase phosphatase activity (B). Results are presented as a percentage of the activity measured in incubations in which ROCK-II and ATP[γS] were omitted. A representative example of five separate experiments is shown.

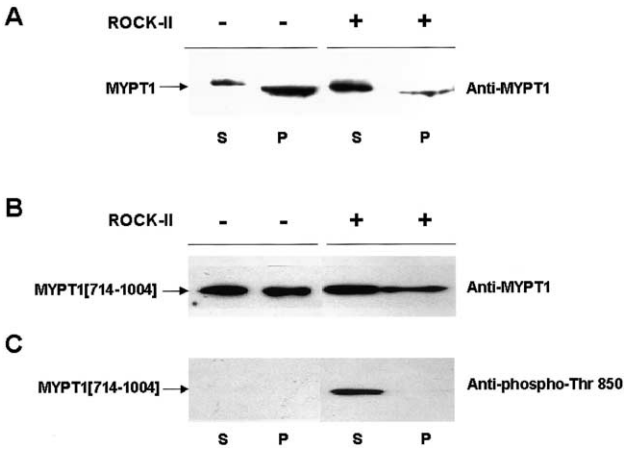

Fig. 3. Phosphorylation of PP1M and MYPT[714–1004] by ROCK-II prevents binding to myosin. 1 nM PP1M (A) or 0.15 nM MYPT1[714–1004] (B and C) were phosphorylated for 30 min at 30°C with or without 1 μM myosin as described under Section 2 and centrifuged. The supernatant (S) and resuspended pellets (P) were analysed by SDS–PAGE on 10% or 7.5% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with antibodies raised against the PP1M holoenzyme (A and B) or a phosphopeptide corresponding to the sequence surrounding Thr850 (C). A representative experiment is shown for each panel. Similar results were obtained in three independent experiments. No PP1M or MYPT1[714–1004] were pelleted if myosin was omitted from the incubations (data not shown).

3.3. Phosphorylation on Thr850 triggers the dissociation of PP1M from myosin

As reported previously [10], the PP1M complex bound to myosin, but binding was greatly decreased after phosphorylation by ROCK-II (Fig. 3A). Interestingly, Thr850 lies within a region that has been identified as a myosin-binding domain [10]. In order to examine whether the failure of phosphorylated PP1M to bind to myosin resulted from the phosphorylation of MYPT1 at Thr850, the experiment was therefore repeated using a C-terminal myosin-binding fragment of MYPT1 (expressed as a MBP fusion MBP-MYPT1[714–1004]), which binds to myosin but lacks Thr695. Under the conditions studied, densitometric scanning of the gels revealed that 49% of the MBP-MYPT1[714–1004] was pelleted with myosin, but this was decreased 22% after phosphorylation by ROCK-II (Fig. 3B). Strikingly, the fraction of the MBP-MYPT1[714–1004] that was phosphorylated at Thr850 did not bind to myosin at all (Fig. 3C), demonstrating that phosphorylation of this residue is sufficient to abolish the binding of the C-terminal fragment of MYPT1 to myosin. Similar results were obtained in several independent experiments.

3.4. Concluding remarks

Earlier studies had indicated that the ROCK-II-catalysed

phosphorylation of MYPT1 at Thr695 inhibits PP1M. In this paper, we show that phosphorylation also prevents the binding of PP1M to myosin and that this is mediated, at least in part, by the phosphorylation of Thr850, which is located in a myosin-binding domain. The dissociation of PP1M from myosin may be a 'failsafe' device to prevent PP1M from dephosphorylating the myosin P-light chain in situations where the phosphorylation of Thr695 is incomplete. It would therefore be interesting to know the relative rates at which Thr695 or Thr850 are phosphorylated and dephosphorylated in smooth muscle during contraction and relaxation.

The presence of two phosphorylation sites on MYPT1 that inhibit PP1M activity in different ways also raises the possibility that these residues may be phosphorylated differentially by different protein kinases that respond to distinct signals. In this connection, it should be noted that Thr695 can also be phosphorylated in vitro by a Zip-like kinase associated with PP1M [12,19,20].

Although we were unable to detect any phosphorylation of chicken gizzard MYPT1 at Ser849 by ROCK-II (Fig. 1C), others have reported that this residue is one of the major sites phosphorylated by ROCK [21]. The reason for this discrepancy is unclear, although one possibility is that it could be related to their use of the isolated rat MYPT1 subunit as a substrate, whereas we used the native PP1M complex isolated from chicken gizzard. These investigators also reported that stimulation of mammalian MDCK cells with tetradecanoylphorbol-13-acetate triggered the phosphorylation of mammalian MYPT1 at the residue equivalent to Ser849 of chicken gizzard MYPT1 [21]. However, phosphorylation was only inhibited partially by pharmacological inhibitors of ROCK and it remains possible that Ser849 is targeted by a different protein kinase(s) in cells. In these experiments, phosphorylation of Ser849 correlated with an increase in the cytosolic distribution of myosin phosphatase, suggesting that Ser849, like Thr850, may also trigger the dissociation of PP1M from myosin and change its subcellular distribution.

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