Phosphorylation of a Novel Myosin Binding Subunit of Protein Phosphatase 1 Reveals a Conserved Mechanism in the Regulation of Actin Cytoskeleton*

Ivan Tan‡, Chong Han Ng‡, Louis Lim‡§, and Thomas Leung‡§
From the ‡Glaxo-IMCB Group, Institute of Molecular and Cell Biology, 30 Medical Dr., Singapore 117609, Singapore and §Institute of Neurology, University College London, London WC1N 1PJ, United Kingdom

The myotonic dystrophy kinase-related kinases RhoA binding kinase and myotonic dystrophy kinase-related Cdc42 binding kinase (MRCK) are effectors of RhoA and Cdc42, respectively, for actin reorganization. Using substrate screening in various tissues, we uncovered two major substrates, p130 and p85, for MRCK kinase. p130 is identified as myosin binding subunit p130, whereas p85 is a novel related protein. p85 contains N-terminal ankyrin repeats, an α-helical C terminus with leucine repeats, and a centrally located conserved motif with the MRCKα-kinase phosphorylation site. Like MBS130, p85 is specifically associated with protein phosphatase 1Δ (PP1Δ), and this requires the N terminus, including the ankyrin repeats. This association is required for the regulation of both the catalytic activities and the assembly of actin cytoskeleton. The N terminus, in association with PP1Δ, is essential for actin depolymerization, whereas the C terminus antagonizes this action. The C-terminal effects consist of two independent events that involved both the conserved phosphorylation inhibitory motif and the α-helical leucine repeats. The former was able to interact with PP1Δ only in the phosphorylated state and result in inactivation of PP1Δ activity. This provides further evidence that phosphorylation of a myosin binding subunit protein by specific kinases confers conformational changes in a highly conserved region that plays an essential role in the regulation of its catalytic subunit activities.

The Rho subfamily of GTPases are biological regulators of actin cytoskeleton. In adherent cells, RhoA induces stress fiber formation, Rac-1 generates lamellipodia, and Cdc42 produces filopodia and actin microspikes (1). A variety of effectors of these cytoskeletal switches has been isolated and characterized (see Refs. 2 and 3 for reviews), some of which are directly involved in regulation of actin dynamics. We and others have reported serine/threonine kinases related to the myotonic dystrophy kinase, which play effector roles for the perspective Rho GTPase in cytoskeletal reorganization (4–7). ROKs or Rho kinase family proteins involve in the regulation of actinomyosin dynamics in cultured cells (7). Precisely how these occur is not clear, although a number of proteins are known to be effective substrates for these kinases. These include the non-muscle myosin light chain 2 (MLC2), whose phosphorylation state is crucial for actinomyosin contractility and polymerization (7, 8), the myosin binding subunit p130 (9–11), Ezrin, Radixin, and Moesin (ERM) family proteins (12), adducin (13), and intermediate filament proteins (14–17), which are directly or indirectly linked to the actin cytoskeleton. In particular, the myosin binding subunit MBS130 appears to play a unique role in the regulation of the activity of the associated PP1 catalytic subunit. The specific binding of MBS130 to RhoA may link this regulatory subunit to Rho-dependent events (9). Indeed chicken gizzard MBS130 is found to be effectively and specifically phosphorylated by ROK at threonine 695 and serine 854 (10, 11). Phosphorylation at threonine 695 resulted in inhibition of the intrinsic phosphatase activity. Other proteins that can interact with and phosphorylate MBS130 include the cGMP-dependent protein kinase Iα and an unidentified mitotic kinase, but in contrast, such phosphorylation resulted in activation of protein phosphatase activity (18, 19). MBS130 therefore appears to serve as a scaffold for multiple protein interactions as well as phosphorylation regulation. Indeed, a recent report has indicated that a number of proteins involve in the Rho signaling pathways including Ezrin, Radixin and Moesin family proteins, adducin, and MBS130 can be found to colocalize at cell periphery upon stimulation. It is possible that MBS130 may provide a bridge for various Rho-dependent components to function in a coordinated manner (20). Since the myotonic dystrophy kinase-related Cdc42-binding kinases (MRCKs) also consist of similar catalytic domains with differential cellular localization and cellular functions (7, 21), it is of interest to investigate if these kinases could share similar and different sets of substrates for their cellular activities.

As a first step toward identification of new substrates for kinases such as MRCKs, we derived a filter assay to screen for potential candidates. This assay allows repressed proteins on the filter to be phosphorylated by specific recombinant kinases. Using this assay for MRCKα kinase, we identified two potential substrate proteins, one of which was MBS130; the other was a novel related protein.

**EXPERIMENTAL PROCEDURES**

Construction of Expression Vectors—Full-length p85 cDNA was obtained by first performing a PCR reaction of the presumed initiation

Cdc42 binding kinase; ROK, RhoA binding kinase; MLC2, myosin light chain 2; MBS, myosin binding subunit; PP1, protein phosphatase 1; PIM, phosphorylation inhibitory motif; αLZ, α-helical leucine zipper; CAT, catalytic domain; PCR, polymerase chain reaction; HA, hemagglutinin; PVDF, polyvinylidene difluoride; GST, glutathione S-transferase; ATPγS, adenosine 5’-O-(thiotriphosphate); EST, expressed sequence tag.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AF312028.

‡ To whom correspondence should be addressed. Tel.: 65-874 6167; Fax: 65-774 0742; E-mail: mcblehm@imcb.nus.edu.sg.

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codon from the first exon of the human genomic DNA using two adaptor primers, 5'-CAGGATCATGTCGGGAGAGGCTG3'- and 5'-GGACTGCGTGGTACGTCG3'- was then joined to a StuI/NotI fragment from EST clone AI825921 that contains the rest of the 3' end of p85. p85AA was derived from a BamHI/PalI DNA fragment of p85s (encoding residues 1-554), which was obtained by PCR from a cDNA from chicken gizzard M130 (which is identical to the flanking sequence around threonine 560 of p85). Enzymatic Measurements—Kinase assays were carried out in kinase buffer with 0.15 mM ATP/s in the presence or absence of 0.5 mM of GST-MRCKA-CAT for 30 min at 30 °C. Phosphatase assays were initiated by the addition of 5 μM 32P-GST-MLC2 in 30 mM Tris-HCl, pH 7.5, 0.1 mM KCl, 2 mM MgCl2, and 0.1 mM bovine serum albumin. The reactions were stopped by adding an equal volume of SDS sample buffer at each time point indicated and boiling for 5 min before gel loading. To show PP1 inhibition by phosphorylated GST-PIM50, 10 μM of the fusion protein was first incubated in kinase buffer (as above using ATP) with and without GST-MRCKA-CAT for 30 min at 30 °C. These phosphorylated and nonphosphorylated GST-PIM50 proteins were then separately mixed with the immunoprecipitated FLAG-p85 and HA-PP1 and proteinase kinase A (Sigma). Phosphatase activities were quantified using the Molecular Dynamics PhosphorImager system. Immunoprecipitation and in Vitro Binding Assays—COS-7 cells co-expressing various FLAG-p85 and HA-PP1 constructs were lysed in buffer containing 25 mM HEPES, pH 7.3, 0.15 mM NaCl, 0.5 mM MgCl2, 0.2 mM EDTA, 20 mM β-glycerol phosphate, 1 mM sodium vanadate, 5% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.1% Triton, 1 μg/ml each aprotinin, leupeptin, and pepstatin A, and 1× protease inhibitor mixture and incubated with anti-FLAG-conjugated agarose beads (Sigma) for 1 h at 4 °C. After an extensive wash, the immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis and subjected to immunoprecipitation analysis using anti-FLAG or anti-HA immunoblotting reactions. Protein MBS130—Both peptide sequencing and subsequent mass spectrometry experiments were performed with a QSTAR Mass-spectrometer (PerkinElmer). RESULTS Identification of p130 and p85 as Major in Vitro Substrates for MRCKa-CAT—To identify and characterize potential substrates for ROKα and MRCKα, we made GST fusion proteins of the catalytic domains of both ROKα (1–432) and MRCKα (1–473). The yield and catalytic activities of MRCKα were consistently higher than ROKα, and subsequent experiments were thus carried out with GST-MRCKα-CAT. Here we observed that renatured proteins separated on SDS-polyacrylamide electrophoresis gels and transferred onto PVDF membrane filters were readily phosphorylated by MRCKα-CAT. Two proteins of 135 and 85 kDa were prominently and specifically phosphorylated by MRCKα-CAT (Fig. 1) but not by α-p21-activated kinase or protein kinase A (data not shown). These two proteins are not abundant in tissues such as brain and testis but could easily be enriched by passing through an affinity-dye Reactive Brown 10-Sepharose column (Sigma), and this constitutes a simple one-step enrichment of these proteins for further purification (Fig. 1A). Further separation of these two proteins was achieved with two-dimensional gel electrophoresis (Fig. 1B), and the Coomassie Blue-stained spots corresponding to the phosphorylated proteins were excised for peptide sequencing. P85 Is a Novel Protein Related to Myosin Binding Subunit Protein MBS130—Both peptide sequencing and subsequent immunoblotting analyses showed that the p130 corresponds to the previously reported MBS130 (data not shown). Peptide sequence analysis also indicated that the p85 is a novel protein (Fig. 2A). A 2.8-kilobase human EST clone AI825921 contains most of the coding sequence except for the extreme N terminus. The complete match of this cDNA with two overlapping

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brane filters for phosphorylation reactions with GST-MRCK deletion mutants of p85 in COS-7 cells (Fig. 3).

After extensive washes, the bound fraction was eluted with sample buffer and separated either on a one- or two-dimensional gel (B) for Coomassie Blue staining (top panel) or transferred onto PVDF membrane filters for phosphorylation reactions with GST-MRCKα-CAT and [*-32P]ATP. In A, each lane was loaded with 100 μg each of soluble extract (1), flow-through (2), wash (3), and eluents (4, 5). Arrows indicate equivalent positions of p85 and p130 on filters. Mr, molecular mass marker.

genomic clones, S51329 and AC005782, from human chromosome 19 (Fig. 2C) suggested that the N terminus is confined within the first exon. A full-length cDNA for p85 was thus constructed from the PCR product of the first exon of human p85 genomic DNA and subsequently joined to the truncated cDNA. The amino acid sequence derived from this cDNA indicated that p85 is structurally related to MBS130 (Fig. 2B). The N terminus of p85 consists of a closely related structure with 6 ankyrin repeats and 48% identity to MBS130, which has been reported to have 7–8 repeats (23, 24). A putative PP1 binding consensus sequence, RTVRF (25), was also present immediately before the ankyrin repeats. The C terminus contains a conserved α-helical structure with leucine zippers at the C-terminal end (αLZ). This structure can form homodimers or heterodimers (e.g. with M20, which also contains this structural motif (26)). Of most striking similarity (87% identity) is a central motif, which contains the sole phosphorylation site for MRCKα-CAT (refer to Fig. 3).

The genomic organization of p85 showed that the mRNA is derived from 22 exons (Fig. 2C). Intriguingly, a number of integration hotspots (AAVS1) of adenovirus-associated virus (AAV) was found in the first exon/intron regions of p85 genomic sequence (Fig. 2D, Refs. 27 and 28). The consequence of these integrations is not known but is expected to disrupt the expression of this gene. Rearrangements and disruption of a nearby troponin gene were also observed upon adenovirus-associated virus integration (29). Northern blot analysis indicated that the 3-kilobase p85 mRNA was ubiquitously expressed and is especially high in the heart (Fig. 2F).

P85 protein expressed in serum-starved HeLa cells mainly showed cytoplasmic punctate distribution (Fig. 2F, a) but was readily redistributed to cell peripheral upon treatment with lysophosphatidic acid and phorbol myristic acetate (Fig. 2F, b and c).

**Identification of the Phosphorylation Site of p85 on Threonine 560 by MRCKα-CAT**—To confirm the nature of phosphorylation of p85, we expressed the FLAG-tagged wild-type and deletion mutants of p85 in COS-7 cells (Fig. 3A). The immuno-

precipitated proteins were phosphorylated with MRCKα-CAT to map the phosphorylation site(s). It is clear that the major site(s) is within the central conserved region, as mutants de-
phosphorylated this region were not phosphorylated (Fig. 3B). To confirm this, we expressed GST fusion protein containing the wild-type conserved PIM motif (PIM50; also refer to Fig. 4) and a mutant S559A/T560A (PIM50AA) and showed that the mutant was not phosphorylated (Fig. 3B, lane 8), unlike the wild-type control (lane 7). This in vitro phosphorylation site of p85 on threonine 560 by MRCKα-CAT was further confirmed by mass spectrometry on the tryptic phosphopeptides derived from p85 phosphorylation.

Further evidence was obtained by probing p85 protein expressed from COS-7 cells that were co-transfected with either MRCKα-CAT or ROKα-CAT with an antibody that specifically recognizes phosphorylated threonine 560 of p85. Clearly, threonine 560 of p85 can be phosphorylated by both MRCKα-CAT and ROKα-CAT in vitro (Fig. 3C).

p85 Is Specifically Associated with PP1δ and Its Substrate MLC2, and the Phosphorylation by MRCKα Regulates the Phosphatase Activity—To see if p85 can associate with PP1δ, we co-transfected FLAG-tagged p85 and HA-tagged PP1δ in COS-7 cells. Proteins immunoprecipitated (IP) with mouse anti-FLAG beads were separated on 10% SDS-polyacrylamide electrophoresis gels, transferred to PVDF membranes, and detected with a rabbit-anti-FLAG antibody for p85 and a rabbit-anti-HA antibody for associated PP1δ. Overexpressed PP1δ present in the cell lysate was also shown for comparison. WB, Western blot. B, the N terminus of p85 binds MLC-2. FLAG-tagged p85N (lane 1) or p85NΔ2 (lane 2) was co-expressed with HA-tagged MLC-2. Immunoprecipitated proteins with anti-FLAG antibody transferred onto PVDF membrane were detected with anti-FLAG or anti-HA as described in A. C, phosphorylation of p85 by GST-MRCKα-CAT inhibits associated PP1β activities. Immunoprecipitated wild-type p85 (p85WT) or a phosphorylation-deficient mutant, p85S559A/T560A (p85AA), coexpressed with PP1β were phosphorylated with GST-MRCKα-CAT in the presence of 0.1 mM ATPγS. The nonphosphorylated and phosphorylated proteins were used to initiate the dephosphorylation activities of the associate PP1β toward 32P-MLC2 at different time intervals.
Actin polymerization (p85CT in Fig. 5), whereas a deletion mutant of a single ankyrin repeat (lane 5) can dramatically reduce such interaction. Deletion mutants devoid of N terminus are totally ineffective (Fig. 4A, lanes 6 and 7).

The N terminus of p85 could also interact with MLC2 (Fig. 4B), and the C terminus is totally ineffective. Hence PP1\(d\) can form a tight complex with p85 and substrate MLC2 through its N terminus.

Next, to examine if the phosphorylation of threonine 560 by MRCK\(d\)-CAT can regulate PP1 activity, we measured the time course of dephosphorylation toward 33\(^3\)P-MLC2. As shown in Fig. 4C, nonphosphorylated wild-type p85 (p85\(^{WT}\)) or the phosphorylation-defective mutant (p85\(^{AA}\)) were equally active in MLC2 dephosphorylation. Wild-type p85 but not the mutant p85\(^{AA}\), when phosphorylated in vitro with MRCK\(d\)-CAT, showed significant reduction in the rate of MLC2 dephosphorylation. These results confirm a similar observation with MBS130 where phosphorylation of a conserved threonine 695 within a highly conserved motif was essential for the inhibition of phosphatase catalytic activity (11). Based on the biochemical and functional similarities between p85 and MBS130, we therefore designate p85 as MBS85.

**N and C Termini of MBS85 Show Independent Morphological Effects Reflecting Their Biochemical Activities**—Because PP1 activities are essential for regulating the phosphorylation states of myosin, it is likely that the biochemical interaction of MBS85 with PP1\(d\) may be correlated with morphological effects in cultured cells. Furthermore, adherent cells mainly exhibit active Rho phenotype in serum medium, and the interference of endogenous PP1\(d\) would be expected to affect this actin structure. As shown in Fig. 5A, expression of wild-type p85 alone did not give any obvious morphological consequence. However, truncation of the C terminus led to various degrees of disassembly of actin stress fibers, with the most striking effects when both the PIM and aLZ motifs were totally removed (p85\(^{NT}\); Fig. 5A and B). Deletion of the PIM motif (p85\(^{AA}\)) or mutation of the phosphorylation site (p85\(^{AA}\)) was significantly effective in inducing similar morphological effects, although this appears to depend on the levels of expression (Fig. 5A, bottom panel). Both the PIM and aLZ motifs at the C terminus appear to exert opposing effects on the N-terminal function. Truncation of the N terminus resulted in general increases in actin polymerization (p85\(^{CT}\) in Fig. 5A) that was resistant to C3 treatment (data not shown). As aLZ alone was also effective, although to a lesser extent (data not shown), it is likely that PIM and aLZ domains have independent activities toward actin assembly. Similar trends were also observed when the various constructs were co-expressed with PP1\(d\) (Fig. 5B). In this case, the morphological effects were more pronounced in the presence of exogenous PP1\(d\). As expected, both ankyrin repeat mutants p85\(^{AA1}\) and p85\(^{AA2}\) that were defective in binding to PP1\(d\) were totally ineffective in inducing morphological changes (Fig. 5B and data not shown). Hence the phenotypic effects of the various MBS85 variants on actin cytoskeleton correlate well with the biochemical data described earlier.

**Phosphorylation Inhibitory Motif (PIM50) of MBS85 Binds PP1\(d\) When Phosphorylated by MRCK\(d\)-CAT and Exerted an Inhibitory Effect on PP1\(d\) Activity**—It is known that phosphorylation of threonine 695 of MBS (which is equivalent to the threonine 560 of p85) is critical for the inhibitory effects on PP1 catalytic function (11). It is likely that such a mechanism may operate for MBS85. To test this possibility, we derived an assay to examine the effect of phosphorylation on the interaction of the highly conserved GST-PIM with p85/PP1\(d\) complex. The phosphorylation-deficient mutant p85\(^{AA}\) was used to eliminate possible competition for binding. As shown in Fig. 6A, phosphorylated GST-PIM, but not the nonphosphorylated form, was detected in the p85/PP1\(d\) immuno-complex. Similarly only glutathione beads with the phosphorylated GST-PIM, but not the nonphosphorylated or phosphorylation-deficient mutant GST-PIM\(^{AA}\), were able to pull down the expressed PP1\(d\) or p85/PP1\(d\) complex (Fig. 6B), clearly indicating that phosphorylation of threonine 560 of MBS85 is essential for its interaction with PP1\(d\).

To examine if such interaction is functional, we measured...
the catalytic activities of the immuno-complex in the presence of in vitro phosphorylated or nonphosphorylated GST-PIM50. Phosphorylated GST-PIM50, but not the nonphosphorylated form, was likewise effective in inhibiting the catalytic activity of p85\(^\text{z}\)PP1\(^d\) complex (Fig. 6C). This provides further evidence that the interaction is functional.

Next we tested the effects of co-expression of this PIM50 motif on the p85\(^\text{NT}\)-induced morphological changes. As shown in Fig. 7, A and B, PIM50 expression is sufficient in reversing the effect of p85\(^\text{NT}\)-induced actin stress fiber losses. The phosphorylation-deficient mutant PIM50\(^\text{AA}\) was totally inefficient (Fig. 7B). These inhibitory effects became less pronounced when p85\(^\text{NT}\) was co-expressed with PP1\(^d\), suggesting that PIM50 may well be competing with the catalytic subunit in regulating actin dynamics. We therefore conclude that from both biochemical and morphological data that the central conserved motif of MBS85 can be regulated by phosphorylation, resulting in conformational changes that affect the associated PP1\(^d\) catalytic property and subsequently effects on actin morphology, probably through the eventual effects on myosin phosphorylation.

**DISCUSSION**

Two candidate proteins from rat brain cytosolic extract were identified in this study on the basis of their in vitro phosphorylation by MRCKα-CAT. The p130 protein was confirmed to be MBS130 by peptide sequencing and immunoreactive toward specific antibodies. The smaller protein p85 is a novel protein that is structurally related to MBS130. Overall p85 shares low
activity on MLC2 substrate (30). p85 (designated here as scaffold for PP1 and myosin and confers specific phosphatase the substrate MLC2 within the N-terminal region. In this previous report for MBS130 (32), we also detected binding of other proteins can interact with the C terminus of MBS130. MBS130 (30). More recently, it has also been reported thatilar helical structure. Indeed, it has been reported that this part of the heterotrimeric complex (28), and it contains a sim-

M20, a small subunit protein of PP1, is found to be an integral involved in dimerization and interaction with other proteins. M20, a small subunit protein of PP1, is found to be an integral part of the heterotrimeric complex (28), and it contains a sim-

A central conserved motif that contains the sole phosphorylation site for MRCKα and ROKα exhibits a more striking similarity. Threonine 560 of p85 is equivalent to threonine 695 of MBS130 and is located within a ~50-amino acid conserved phosphorylation inhibitory motif (PIM50). Phosphoryla-
tion of the threonine 695 by ROKα has been shown to inhibit associated PP1 activity (11, 30). A second phosphorylation site (serine 854) by ROKα has also been described in MBS130, but this is absent in p85 (Fig. 2A and Ref. 10).

Here we have also demonstrated that p85 is functionally similar to MBS130. First it is specifically associated with PP1α, and this depends on the N terminus including the whole of the ankyrin repeats. N-terminal as well as ankyrin-repeat deletion mutants are ineffective in binding PP1α. In agreement with a previous report for MBS130 (32), we also detected binding of the substrate MLC2 within the N-terminal region. In this respect, the N terminus of p85 alone can therefore act as scaffold for PP1 and myosin and confers specific phosphatase activity on MLC2 substrate (30). p85 (designated here as MBS85) is therefore a genuine myosin binding subunit of PP1α, similar to MBS130. Furthermore, the phosphorylation of threo-

FIG. 8. A model for the phosphorylation regulation of threonine 560 of p85MBS on PP1α activity. When threonine 560 (T) MBS85 is not phosphorylated, PP1α assumes an orientation in contact with its substrate MLC2, resulting in an active conformation for the dephosphorylation of MLC2 and subsequent actin-myosin disassembly. Upon phosphorylation (T with an asterisk), it presents a conformation that has a higher affinity to PP1α and disrupts its accessibility or catalytic activity toward MLC2, resulting in a shutdown of MLC2 dephosphorylation that favors myosin phosphorylation and actin-myosin assembly. AR, ankyrin repeats.

similarity to MBS130 (<40%). The N terminus of p85 contains six ankyrin repeats that are known to be involved in protein-protein interactions. This motif shares a 48% identity to MBS130, which has 7–8 repeats (23, 24). Preceding these repeats is a short stretch (RTVRF) that resembles PP1 binding consensus sequence (25). The C terminus of p85 is also conserved and consists of an α-helical structure with four leucine heptad repeats at the C-terminal end. This motif is known to be involved in dimerization and interaction with other proteins. M20, a small subunit protein of PP1, is found to be an integral part of the heterotrimeric complex (28), and it contains a sim-

opposing activities in regulating actin polymerization and that the phosphorylation of threonine 560 in a central motif appears to fine tune these events.

In summary, we have isolated a novel myosin binding subunit that is ubiquitously expressed. Compared with MBS130, the smaller size and simpler arrangement of the regulatory domains of this novel MBS85 allow an easier analysis of structure and function relationships. The identification of an increasing number of these myosin binding subunits, which share a similar regulatory mechanism, should help to understand how each of these are regulated by various diverse signal pathways in the control of the actin cytoskeleton.

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