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Identification of a Cytoskeleton-bound Form of Phospholemman with Unique C-Terminal Immunoreactivity

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Abstract. Phospholemman (PLM) is a 72-amino acid transmembrane protein thought to function in Na,K-ATPase regulation or assembly, similar to other members of the FXYD family of proteins. Unique to PLM among these regulatory proteins are sites for C-terminal phosphorylation by PKA and PKC, although a role for phosphorylation in PLM function remains unclear. To study PLM phosphorylation, we used PLM phosphopeptides to generate antibodies to specifically detect phosphorylated PLM. Peptide affinity chromatography isolated two populations of antibodies: one reacting with standard PLM, a collection of closely-spaced 15-kDa protein bands by SDS-PAGE. About 20% of PLM antibodies reacted specifically with a single distinct form of PLM. Levels of this second immunological form (PLM-b) were increased with overexpression of PLM cDNA, and also reacted with a monoclonal antibody against the PLM N-terminus. In complete contrast to standard PLM, however, PLM-b was quantitatively insoluble in nonionic detergents and was released from tight binding by colchicine. Antibodies to PLM-b were present in two different antisera raised to the phosphorylated C-terminal peptide (residues 57–70), but not in antiserum raised to the non-phosphorylated C-terminal peptide. Despite an apparent relationship between PLM-b and phosphorylated PLM, PLM-b levels were not affected by treatment of heart cells with isoproterenol. PLM-b appears to represent a cytoskeleton-attached detergent-insoluble form of PLM with distinctive C-terminal immunoreactivity that might have implications for PLM structure and function.

Key words: PKA — Antibodies — Plasma membrane — Detergent insoluble — Signal transduction

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Introduction

Phospholemman (PLM)[1] is a small (72-amino acid) transmembrane protein and prominent phosphoprotein first purified and characterized in plasma membranes of heart (Jones et al., 1979; Jones, Maddock & Hathaway, 1981; Palmer, Scott & Jones, 1991), skeletal muscle (Walaas et al., 1988a, 1988b), smooth muscle (Boulanger-Saunier, Kattenburg & Stoclet, 1985; Boulanger-Saunier & Stoclet, 1987), and liver hepatocytes (Cooper, Kobayashi & Williamson, 1984). Sweadner and Rael (2000) identified PLM as part of a family of seven proteins present in the mammalian genome, which they have termed FXYD proteins, which exhibit common sequences and similar topologies. Thus, distinct tissue distributions of individual homologs may represent tissue-specific regulation of Na,K-ATPase (Geering et al., 2003; Sweadner et al., 2003). Among FXYD family members, PLM alone possesses a cytosolic domain rich in phosphorylation sites for several protein kinases (Palmer et al., 1991; Sweadner et al., 2003).

PLM cDNA reveals a molecular mass of 8409, but the prominent polypeptide migrates by SDS-PAGE with M_r = 15,000 (Chen et al., 1998; Palmer et al., 1991). Hydropathy and structural analyses indicate an amino terminus, which constitutes the protease-insensitive extracellular region and membrane-spanning alpha helix (residues 1–17 and 18–

[1]Abbreviations: Ad.PLM, recombinant adenovirus encoding canine PLM; DMEM, Dulbeccos' modified Eagle's medium; ECL, enhanced chemilumenescence; HEK, human embryonic kidney; HRP, horseradish peroxidase; KLH, keyhole limpet hemocyanin; PAC, pulmonary arterial cells; PLM, phospholemman; PLM-b, tightly bound form of PLM; P-S, PLM 57–70 containing phospho-Ser[68]; P-T, PLM 57–70 containing phospho-Thr[69]; PVDF, polyvinyl difluoride.

37), and a highly charged protease-sensitive intracellular carboxyl terminus (residues 38–72) (Chen et al., 1998). The C-terminus containing the phosphorylation sites, including the sites modified by PKA (Ser^{68}) (Chen et al., 1998; Walaas et al., 1994) and PKC (Thr^{69}) (Walaas et al., 1994), projects into the cytoplasm (Chen et al., 1998). PLM phosphorylation has been reported primarily in vitro (Boulanger-Saunier et al., 1985; Boulanger-Saunier & Stoclet, 1987; Chen et al., 1998; Cooper et al., 1984; Jones et al., 1979; Jones et al., 1981; Walaas et al., 1988b), although its phosphorylation in intact heart (Presti, Jones & Lindemann, 1985) and skeletal muscle (Walaas et al., 1994) were reported in the past. More recently, Fuller et al. (2004) showed indirect evidence for PLM phosphorylation in response to cardiac ischemia.

In order to investigate the pharmacological regulation of PLM, we raised antibodies to Ser^{68}-phosphorylated PLM 57–70 or Thr^{69}-phosphorylated PLM 57–70. Fractionation of serum antibodies by peptide affinity chromatography generated two populations of IgG molecules. A minor IgG fraction reacted specifically with a distinct 15 kDa protein band with higly similar mobility by SDS-PAGE but with unique biochemical properties. While the identity of this novel form of PLM as phospho-PLM remains uncertain, it nevertheless strongly suggests that a fraction of PLM protein contains a modified or otherwise altered C-terminus that results in detergent insolubility and altered immunoreactivity.

Materials and Methods

Antibodies

The PLM peptide 57–70 containing phospho-Ser^{68} and an N-terminal Cys residue (NH2-CEGTFRSSIRRLS(P)TR-COOH) was coupled to thyroglobulin through Cys using M-maleimidobenzoyl sulfosuccinimide ester (Green et al., 1982) (sulfo-MBS) (Pierce Biotechnology, Rockford, IL), and injected into male New Zealand rabbits along with Freunds adjuvant. Besides a Ser^{68} phosphate (S-P) containing immunogen, a second peptide containing Thr^{69} phosphate (T-P) was also used. Immunizations involved 300 μg initial injections and subsequent 100 μg booster injections. Affinity columns were constructed by coupling of PLM peptides to ω-aminooctylagarose (Sigma Aldrich, St. Louis, MO) using sulfo-MBS. Antiserum (5–10 ml) was diluted with Tris-buffered saline, pH 8, centrifuged at $75{,}000 \times g$ for 20 min, then applied to a T-P column to bind and remove anti-PLM antibodies. Anti-PLM antibodies were eluted from the T-P column using 50 mM glycine, pH 2.4 and immediately neutralized by 1/10 volume of 1 M Tris base, pH 8. The column was regenerated and the flow-through fraction was re-applied to the T-P column to assure complete removal of anti-PLM antibodies. This second flow-through fraction, now devoid of standard anti-PLM IgG, was applied to the S-P column to purify any other antibodies. A second fraction of antibodies was eluted from this column; this fraction is referred to as anti-PLM-b antibodies in this study. Both antibody fractions were concentrated and dialyzed by repeated filtration using a Centricon-50 centrifugal filter unit (Millipore Corp., Bedford, MA) and stored in PBS plus 50% glycerol at −20°C. To purify anti-T-P antibodies, antiserum from a rabbit injected with (Thr^{69})PLM 57–70 conjugate was treated as described above but with the two peptide columns used in reverse order. For one experiment (*see* Fig. 4), unfractionated anti-PLM antibodies (anti-PLM plus anti-PLM-b) were purified by applying antiserum directly to the S-P column, which binds both types of antibodies. Antibodies to PLM 58–73 containing no phosphorylated residues (NH2-CGTFRSSIRRLSTRRR-COOH) (Song, Sussman & Seldin, 2000) were the generous gift of Dr. J. Cheung, Geisinger Medical Center, Danville, PA. Monoclonal anti-PLM antibody B8 was a kind gift of Dr. Larry Jones, Indiana University School of Medicine, Indianapolis, IN. Anti-(Ser^{16}-P)-PLB were from Upstate Biotechnology (Lake Placid, NY, USA). Immunoblotting (Towbin, Staehelin & Gordon, 1979) was carried out as previously described (Cala & Miles, 1992) in PBS containing 0.2% Tween-20. Detection of antibodies on immunoblots was routinely carried out by ^{125}I-protein A (PerkinElmer Life Sciences, Boston) (Burnette, 1981) binding and autoradiography using Biomax MS film (Eastman Kodak Co.). For detection of immune complexes by enhanced chemiluminescence's (ECL) horse radish peroxidase (HRP)-coupled goat anti-rabbit antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) was developed with ECL substrate (Amersham Biosciences). Laemmli buffer was (Laemmli, 1970) used for all SDS-PAGE.

Cell Culture

Human embryonic kidney (HEK) 293 (Graham et al, 1977) and pulmonary arterial (PAC-1) cells (Rothman et al., 1992) were grown at 37°C (5% CO_2) in Dulbeccos' modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) containing 25 mM HEPES, 25 mM glucose, 10% fetal bovine serum, 100 U/ml penicillin G, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B solution. Adult rat hearts were removed from heparinized animals anesthetized by i.p. injection of pentobarbital (40–60 mg/kg). All procedures conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Ventricular heart cells were enzymatically dissociated using collagenase (Type I, Sigma) using a Langendorff perfusion apparatus, then cultured on laminin-coated 60 mm diameter dishes in modified Medium 199 (Ellingsen et al., 1993) containing 2% bovine serum albumin. After any experimental treatments, cells were rinsed free of serum, then scraped into a solution of 1% SDS for protein concentration analysis, followed by SDS-PAGE.

Construction of Recombinant PLM Viruses

Replication-deficient adenovirus containing wild-type canine PLM cDNA (Ad.PLM) was kindly provided by Dr. Jeff O'Brian, Jefferson Medical College, Philadelphia, PA. The PLM insert was amplified from the λgt10 clone (Palmer et al., 1991). Recombinant virus was expanded in HEK 293 cells (Graham et al., 1977), and purified by CsCl density centrifugation (Becker et al., 1994). Titers of viral stocks were determined by plaque assay using HEK 293 cells. Recombinant baculovirus encoding canine PLM was used as previously described (Chen et al., 1998).

Overexpression of Recombinant PLM

For standard expression of PLM, cultured cells were grown to 50% confluence and infected with Ad.PLM. HEK cells were infected with recombinant adenoviruses at MOI $\sim$ 1.0 in DMEM for 2 h without serum, then incubated under our normal conditions for

48–72 h. Primary rat heart cells and PAC-1 cells were infected at a multiplicity of infection (MOI) ~ 50 (or as indicated) in their standard medium, and virus was left on throughout the infection period. Sf21 insect cells were infected with recombinant baculoviras and PLM purified as previously described (Chen et al, 1998).

Nonionic Detergent Extractions

Cultured cells were rinsed free of serum proteins, then scraped into a solution of 1% Zwittergent 3–14 (Calbiochem, San Diego, CA), 20 mM MOPS, pH 7.5, 100 mM NaCl, 0.5 mM EGTA, and 0.1% of a protease inhibitor cocktail (Sigma). Included in the buffer were 10 mM NaF and 10 mM β-glycerophosphate to inhibit protein phosphatases. A homogenate fraction was generally saved in detergent for SDS-PAGE, and detergent mixtures were centrifuged at 80,000 $\times$ *g* for 20 min and mixed with SDS for gel analyses. Insoluble protein was resuspended in a similar mixture of Zwittergent and SDS for SDS-PAGE.

Colchicine Treatments

Colchicine (Sigma Aldrich) was added to cultured HEK cells in 6-well dishes in DMEM with 10% FBS for 4 h, then cells were scraped, and cells spun down at 2000 $\times$ *g* for 10 min and the medium was removed. Pelleted cells were resuspended in 1% Zwittergent 3-14, 20 mM MOPS, pH 7.5, 100 mM NaCl, 0.5 mM EGTA, 0.1% protease inhibitor cocktail, 10 mM NaF, and 10 mM β-glycerophosphate. Detergent mixtures were centrifuged at 80,000 $\times$ *g* for 20 min and the pellets containing insoluble protein were resuspended in SDS and analyzed by immunoblotting with anti-PLM-b antibodies. ^{125}I-protein A binding was quantified by gamma counting of excised PVDF pieces.

Results

Fractionation of Anti-PLM Peptide Antisera

Rabbit antiserum raised against PLM phosphopeptide was comprised of two populations of IgG, which were readily separated on peptide columns as described in Materials and Methods. The major fraction of PLM antibodies (anti-PLM) was extracted from antisera using bound PLM peptide 57–70, and from the remaining material we isolated a second fraction of antibodies that bound to and eluted from the peptide (Ser^{68}-P)PLM 57–70. Based upon peptide reactivities of the two IgG fractions, we expected that this second population of antibodies (termed anti-PLM-b) would be sensitive to phospho-PLM.

Immunoblot Analysis of Anti-PLM and Anti-PLM-b Antibodies against Heart Cell Homogenates

To test whether anti-PLM-b antibodies could be used to detect phosphorylated PLM after pharmacological treatments, we treated cultured heart cells with isoproterenol, a beta-adrenergic receptor agonist expected to increase phosphorylation of PLM, as previously reported for perfused Guinea pig hearts (Presti et al, 1985). As a positive control, we showed that isoproterenol led to an increase in phosphorylation of the SR protein phospholamban, as determined using phospho-specific antibodies obtained commercially (Fig. 1*A*, left panel). In spite of the fact that the phospholamban phosphorylation sequence is nearly identical to that found in PLM (Palmer et al., 1991), no band was seen at 15 kDa using the phospho-specific phospholamban antibody. When cell homogenates were analyzed with our anti-PLM-b antibodies, a 15 kDa band was observed in both untreated controls and isoproterenol-treated cells (Fig. 1*A*, right panel), but no increase in immunoreactivity was observed that might denote an increase in Ser^{68}-phosphate. In fact, in each of three experiments, we observed no change or a slight decrease, suggesting either that anti-PLM-b immunoreactivity did not detect (Ser^{68}-P)-PLM, or that isoproterenol did not lead to PLM phosphorylation.

It was also surprising that our anti-PLM-b antibodies detected what appeared to be standard (unphosphorylated) PLM on this immunoblot (panel *A*, 3rd lane). Antibodies reacting to unphosphorylated PLM were removed from this preparation of IgG molecules by repeated passage through a PLM peptide column (*see* Materials and Methods). To further characterize anti-PLM-b immunoreactivity in heart cells, we therefore analyzed homogenates of cultured heart cells (Fig. 1*B*). Overexpression of PLM by treatment with Ad.PLM significantly increased the levels of a diffuse band of PLM immunoreactivity (lane *2*) compared to untreated heart cells. Basal levels of PLM in homogenates in this experiment were below detection threshold (*left panel*, lane *1*) even though heart cells are known to contain PLM (*compare* Fig. 6). Immunoblot analysis with anti-PLM-b again yielded a similar unexpected result, specifically labeling a relatively sharp immunoreactive band with a mobility very similar to but distinct from that of PLM (lanes *3* and *4*). PLM cDNA overexpression increased expression of both immunoreactive forms of PLM, but PLM-b underwent smaller increases (~50%) compared to the massive increase observed for anti-PLM immunoreactivity.

To examine whether PLM cDNA overexpression would increase PLM-b in cells outside of heart, we treated two other cell types, HEK 293 cells and PAC-1 cells (Fig. 2). HEK (kidney) cells were not expected to contain significant endogenous PLM, and while anti-PLM antibodies substantiated this notion (Fig. 2*A*, lane *1*), anti-PLM-b antibodies detected significant levels of PLM-b (lane *3*). Ad.PLM treatment again led to large increases in PLM levels (lane *2*) and only minor increases in levels of PLM-b (*compare* lanes *3* and *4*). In contrast to HEK cells, PAC-1 cells were found to contain endogenous PLM (Fig. 2*B*, lane *1*); yet, just as for heart cells, these cells also contained PLM-b immunoreactivity (lane *4*).

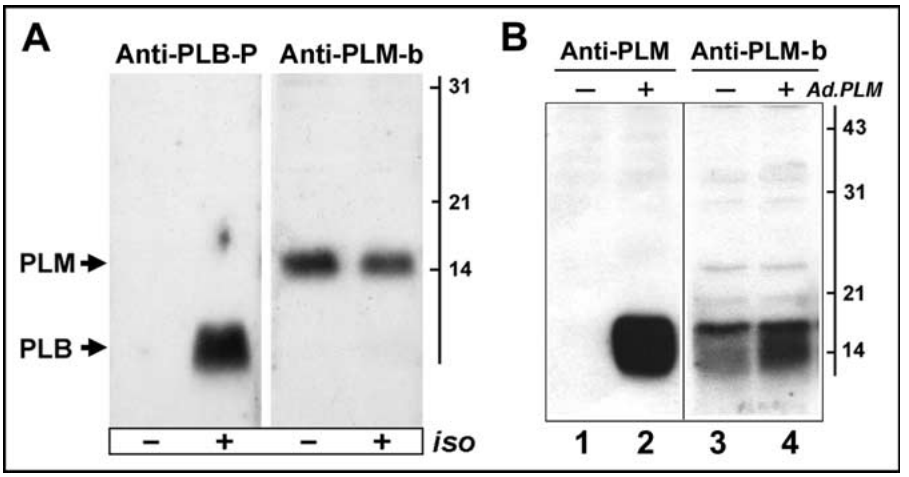

Fig. 1. Immunoblot analysis of heart cells. (***A***) Cultured adult rat heart cells were incubated in the presence (+) or absence (−) of 10^{-7} M isoproterenol for 30 min before harvesting in SDS-containing buffer for immunoblot analysis with either a phospho (Ser^{16}-P)-specific anti-phospholamban (anti-P-PLB) or anti-PLM-b antibodies. (***B***) Cultured adult rat heart cells were incubated in the absence (lanes *1* and *3*) or presence (lanes *2* and *4*) of Ad.PLM at an MOI∼50 for 48 h. Cells were harvested in SDS, and homogenates (80 μg) were analyzed by immunoblotting with anti-PLM (lanes *1* and *2*) or anti-PLM-b antibodies (lanes *3* and *4*). Detection of antibodies was by ^{125}I-protein A binding and autoradiography. Mass standards (*right edge*) are in kDa.

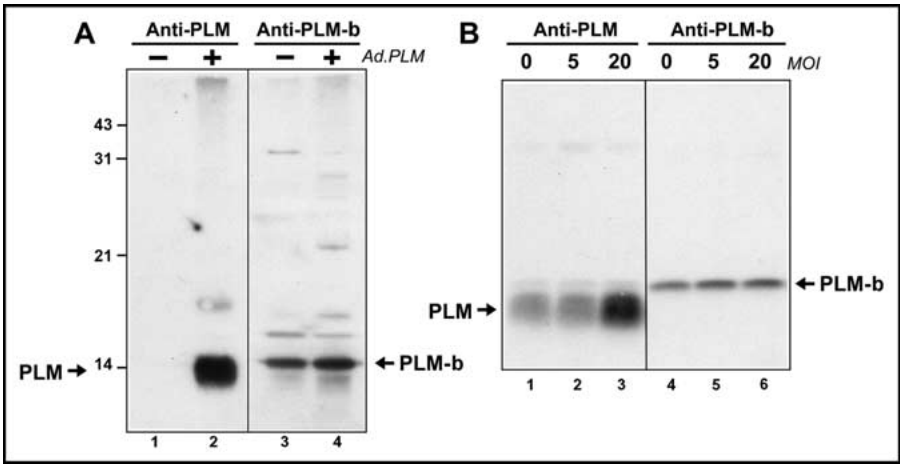

Fig. 2. Immunoblot analysis of HER and PAC-1 cells. (***A***) Cultured HEK cells were incubated in the absence (lanes *1* and *3*) or presence (lanes *2* and *4*) of Ad.PLM at an MOI ∼1 for 48 h. Cells were harvested in SDS, and homogenates (80 μg) were analyzed by immunoblotting with anti-PLM (lanes *1* and *2*) or anti-PLM-b antibodies (lanes *3* and *4*). Molecular weight standards (in kDa) are indicated along the left edge. (***B***) Cultured PAC-1 cells were incubated with increasing amounts of Ad.PLM (MOI = 0,5, or 20) for 48 h. Cells were harvested in SDS, and homogenates (60 μg) were analyzed by immunoblotting with anti-PLM (*left panel*) or anti-PLM-b antibodies (*right panel*).

Similar to HEK and heart cells, Ad.PLM produced a large increase in PLM levels in PAC-1 cells (Fig. 2B, *left panel*) but only minor increases in PLM-b levels (*right panel*).

Variability of PLM and PLM-b mobility by SDS-PAGE analysis

The mobility of PLM-b by SDS-PAGE relative to standard PLM varied depending upon the percent bis-acrylamide used, with PLM-b migrating at the slow edge (0.8% bis-acrylamide, Fig. 2*A*) or closer to the middle of the diffuse PLM band (1.5% bis-acrylamide, Fig. 3). Furthermore, the diffuse band that constitutes PLM immunoreactivity could sometimes be resolved into individual constituent protein bands by SDS-PAGE (Fig. 3), a feature further illustrated below (*see* Fig. 5). When visualized in adjacent lanes on high bis-acrylamide gels, PLM-b immunoreactivity was seen to exhibit the appearance of one of the several discrete components that comprise the diffuse PLM protein band.

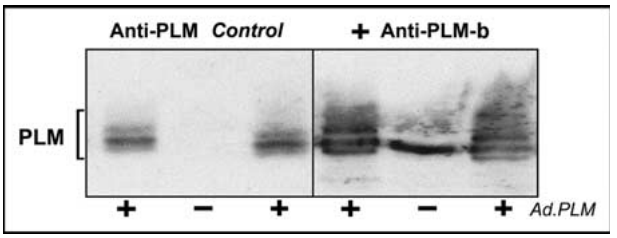

Fig. 3. Resolution of PLM polypeptide components. HEK cells were treated with or without Ad.PLM and analyzed by SDS-PAGE and immunoblotting as in Fig. 2*A*. Immunoblotting was first carried out with anti-PLM antibodies, visualized by ^{125}I-protein A binding and autoradiography (*left panel*). Next, the nitrocellulose was immunoblotted with anti-PLM-b, then visualization of immune complexes was achieved with ECL and autoradiography (*right panel*).

Nevertheless, PLM-b represented a distinct form of PLM as it exhibited variable mobility relative to standard PLM upon changing bis-acrylamide concentrations.

Separation of PLM and PLM-b Immunoreactivities Based upon Detergent Solubility

HEK cells were infected with Ad.PLM and the cell pellets were extracted with an amount of detergent previously found to solubilize PLM (Chen et al., 1998). Analyzed with purified but unfractionated PLM antibodies, the broad diffuse PLM band found in homogenates was found to contain, in addition to detergent-soluble PLM, a detergent-insoluble component that exhibited a mobility on gels typical of that seen for PLM-b (Fig. 4*A*, lane *3*). Subsequent immunoblot analysis of detergent-soluble and -insoluble fractions using purified anti-PLM or anti-PLM-b antibodies showed that PLM was quantitatively extracted by non-ionic detergent, whereas PLM-b was quantitatively retained in the detergent-insoluble fraction (*panel B*). Treatment with 2% Zwittergent 3–14 produced the greatest degree of solubilization among detergents tested, and the quantitative separations of immunoreactivities were generally carried out using this detergent, although 2% CHAPS or 2% Triton X-100 produced similar results. This same separation of PLM and PLM-b forms by detergent was observed for PAC-1 cells (*data not shown*).

With a biochemical method of separating the two forms of PLM, we were able to test other PLM antibodies for reactivity with the PLM-b form (Fig. 5). Antiserum raised to PLM 57–70 from a second rabbit (P-T serum, *see* Materials and Methods) also contained antibodies to a detergent insoluble form similar in mobility on gels to those seen using the P-S antiserum described above. In fact, the P-T immunoreactive band differed slightly in mobility from that visualized by P-S antiserum. Whether this represents yet another distinct component of PLM-b was not further investigated. Antibodies raised to PLM 57–70 without either phosphoserine or phosphothreonine, on the other hand, did not react with the PLM-b. Finally, a monoclonal PLM antibody (B8) that is known to react with the PLM N-terminus[2] also reacted with both PLM and PLM-b.

Effect of Cytoskeleton Disruption on PLM-b Solubilization

Because protein insolubility in nonionic detergents is commonly attributed to association with cytoskeleton, we tested whether PLM-b solubility could be affected by the microtubule-disrupting agent colchicine. Ad.PLM-transfected HEK cells were treated with 2 μM colchicine for 4 h, then cell homogenates were extracted with Zwittergent detergent as shown above. Colchicine led to the release of PLM-b from the detergent-insoluble pellet and an approximate doubling of the amount of PLM-b solubilized by detergent (Fig. 6).

Tissue Distribution of PLM-b

As PLM-b was apparent in HEK cells in the absence of detectable levels of PLM, we analyzed several tissues from rat to determine whether the b-form of PLM would exhibit a similar distribution as that obtained for standard PLM. Indeed, analysis of five different tissues showed that PLM and PLM-b exhibit distinct distributions (Fig. 7). Both forms were found in heart, but in other tissues (skeletal muscle, aorta, lung, and kidney) more PLM-b than PLM immunoreactivity was visualized. Aorta, lung, and kidney contained more PLM-b than did heart, in contrast to previously reported enrichment of PLM in heart compared to other tissues.

Discussion

PLM is composed of multiple mobility forms by SDS-PAGE, although the identities of the various forms are unknown. In this report, we have identified one of these forms in cell homogenates as being uniquely and quantitatively insoluble and sensitive to cytoskeleton-disrupting agents. Moreover, we have isolated antibodies that react specifically and selectively to this form of PLM by employing procedures intended to isolate phospho-specific antibodies. Repeated attempts to prove that this form of PLM, PLM-b, is phosphorylated PLM were unsuccessful.

[2]S. Cala and L. Jones, unpublished observations.

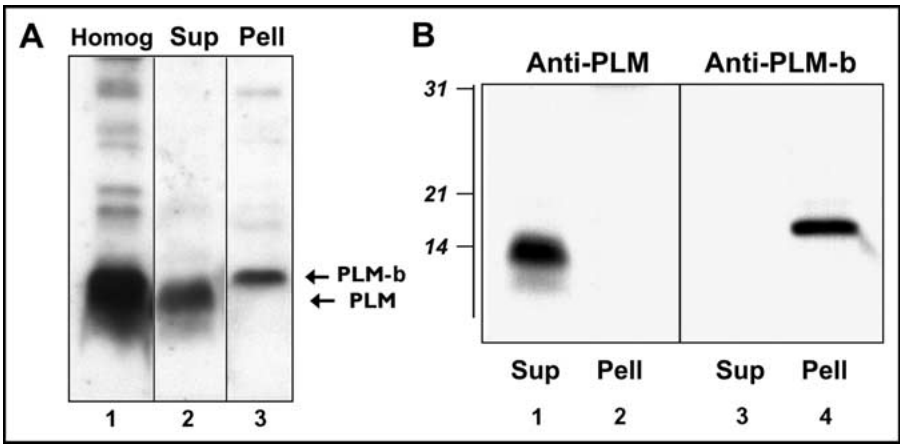

Fig. 4. Detergent insolubility of PLM-b. (***A***) HEK cells were incubated with Ad.PLM (MOI = 1) for 48 h, then harvested in buffer containing 2% Zwittergent detergent, and the cell homogenate (*Homog*) was centrifuged at 75,000 × *g* for 20 min. A supernatant (*Sup*) fraction was removed and the detergent-insoluble pellet fraction (*Pell*) was resuspended back to the original volume. Equal volumes of homogenate, supernatant, and pellet were analyzed by SDS-PAGE and transferred to PVDF. Immunoblotting was carried out using unfractionated (anti-PLM plus anti-PLM-b) antibodies, and visualized by ^{125}I-protein A-binding and autoradiography. (***B***) Identity of supernatant and pellet fractions as containing PLM and PLM-b, respectively, was verified by testing each pair of fractions with purified anti-PLM and anti-PLM-b antibodies.

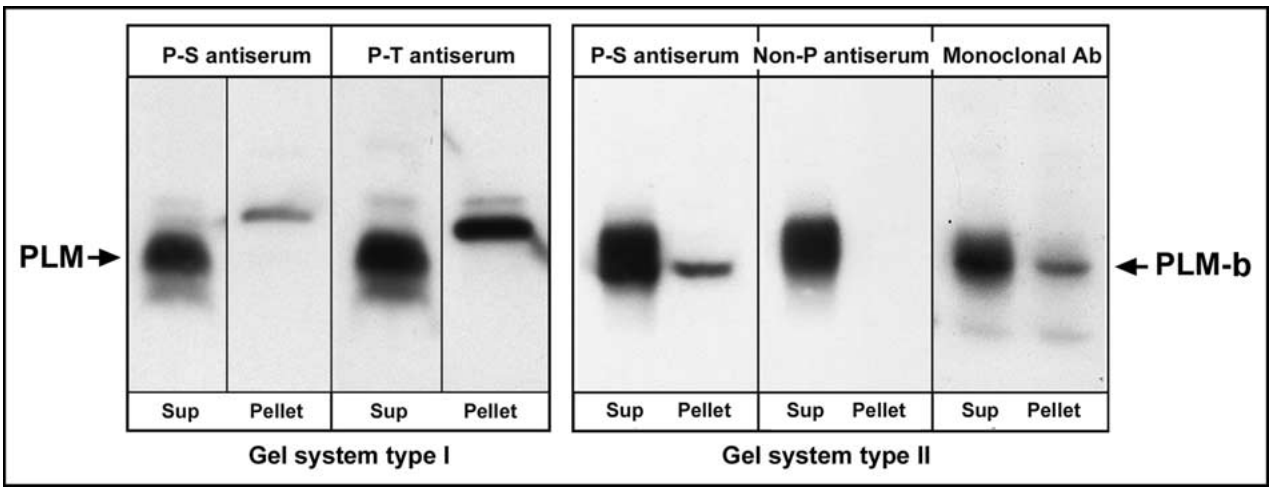

Fig. 5. Analysis of detergent-insoluble PLM-b by other PLM antibodies. Fractionation of PLM and PLM-b based upon detergent solubility allowed subsequent analysis by antibodies raised by us and others. HEK cells were treated with Ad.PLM, and cell pellets extracted by 1% Zwittergent buffer. Supernatants and insoluble pellets were analyzed by several different antibodies. Antisera raised to (Ser^{68}-P)PLM 57–71 and (Thr^{69}-P)PLM 57–71 (P-S and P-T antisera) contained antibodies that react with both standard PLM and PLM-b, whereas antiserum produced to unphosphorylated PLM 57–71 (non-P) contained no anti-PLM-b antibodies. A monoclonal antibody (B8) that reacts with the PLM N-terminus also detected both forms. Gel systems type I and II correspond to gel solutions where the acrylamide:bis-acrylamide ratio equals 37.5 or 20, respectively, leading to different relative mobilities of PLM versus PLM-b.

Antisera raised to PLM 57–70 contain two populations of antibodies that can be isolated by peptide affinity chromatography. Employing a strategy designed to isolate phospho-specific antibodies, we purified antibodies that react specifically and strongly with a protein highly similar but distinct from standard PLM. In our antiserum, roughly one fifth of IgG purified from the P-S antiserum was specific to PLM-b, based upon the yields from the two peptide column chromatography steps, as quantified by ^{125}I-protein A binding (*data not shown*), although the anti-PLM-b antibody appeared to have greater avidity. The standard form of PLM appeared on gels and immunoblots as a diffuse protein band that was sometimes resolved as multiple bands having slight differences in mobility, while PLM-b antibodies reacted strongly with a single discrete form of PLM, here termed PLM-b. PLM-b exhibited biochemical

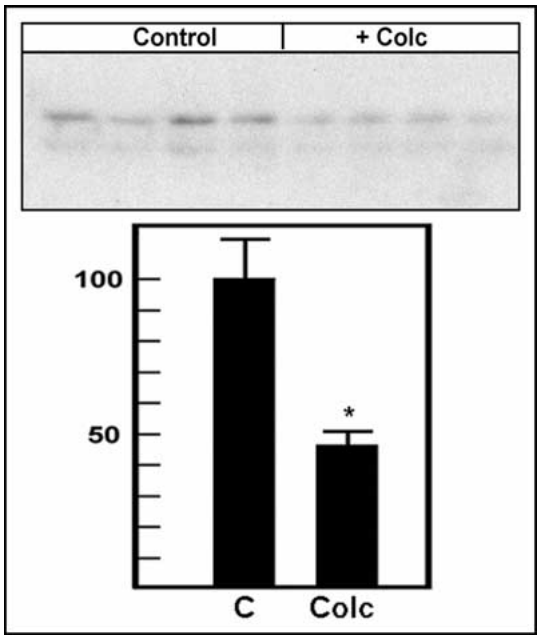

Fig. 6. Effects of colchicine treatment on PLM-b solubility. HEK cells were incubated under control conditions (*Control*, ***C***) or with 2 μM colchicine (*Colc*) for 4 h ($N = 4$). Cells were scraped in Zwittergent-containing buffer, centrifuged at 80,000 × *g* and insoluble pellets analyzed by SDS-PAGE and immunoblotting with anti-PLM-b antibodies. Autoradiogram, *upper panel*; quantified data, *lower panel*. Control, 100 (normalized area) ± 12.3; colchicine treatment, 46.5 ± 3.8; asterisk, $p = 0.017$ (Student's *t*-test).

and immunological properties that differed significantly from standard PLM, leading us to conclude that this b form of PLM represents a distinct conformational form of PLM. Based upon our findings, PLM-b can be biochemically distinguished from PLM in several ways; 1) it is a 15 kDa protein in cells, which reacts strongly and exclusively with anti-phospho-PLM antibodies; 2) it is the detergent-insoluble form of PLM; 3) relative to standard PLM, PLM-b migration by SDS-PAGE decreases when percent bis-acrylamide is increased; 4) it exhibits only minor increases in levels with Ad.PLM treatment, despite large fold-changes in standard PLM; and 5) it exhibits a distribution among cell lines and tissues that differs from that of standard PLM. Standard PLM is defined here as a collection of polypeptide forms that generally appear by SDS-PAGE analysis as a diffuse 15 kDa protein. Although we observed that the entire set of 3–5 polypeptide bands increases with PLM cDNA overexpression (Fig. 3), we made no attempt in this study to determine the reason for this microheterogeneity.

Immunoreactivity of PLM-b

If PLM-b represents another molecular form transcribed from PLM cDNA, as suggested by our data, the PLM C-terminus must exist in at least two stable

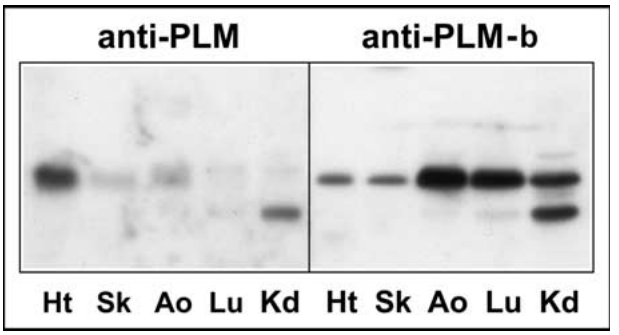

Fig. 7. Distinct tissue distributions of PLM and PLM-b. Tissue samples were dissected from a male Sprague-Dawley rat, diced, and homogenized in 2% SDS. Samples (80 mg) were applied to SDS-PAGE and transferred to PVDF membrane, then immunoblotted with either anti-PLM (*left panel*) or anti-PLM-b (*right panel*) antibodies. *Ht*, heart; *Sk*, hindleg skeletal muscle; *Ao*, aorta; *Lu*, lung; *Kd*, kidney.

conformations. The conformations are necessarily maintained even after SDS treatment and electrophoretic transfer, suggesting that covalent modifications are involved. Most IgG molecules raised against (Ser^{68}-P)PLM 57–70 did not react with PLM-b, but a minor fraction reacted very strongly. Isolation of antibodies to each of the two forms occurred in two different rabbit antisera tested (P-S and P-T) but not with antiserum raised to PLM 58–71 without phosphorylated residues present. As was the case for P-S antiserum, most P-T anti-PLM antibodies reacted with standard (detergent-soluble) PLM, but P-T also contained antibodies reacting only with form-b-like band(s) in the detergent-insoluble fraction (Fig. 5). These immunoreactive bands appeared to co-migrate with or just above PLM-b, as seen for P-S antiserum. Although these immunoreactive forms of PLM might represent PLM stably phosphorylated on Ser^{68} and Thr^{69}, further experiments will be needed to establish the identity of PLM-b as phosphorylated PLM. Immunoreactivity of the B8 monoclonal antibody with PLM-b represented further compelling evidence that it was a bona fide form of PLM.

Unique Detergent Insolubility and Attachment to Cytoskeleton

A major distinction between PLM and PLM-b was the difference in their solubility in nonionic detergents. Solubility in nonionic detergents is a hallmark of PLM biochemistry, as CHAPS and Triton X-100 have previously been used to solubilize and purify the protein (Chen et al., 1998). Indeed, the bulk of PLM rapidly overexpressed by Ad.PLM was quantitatively recovered in the detergent-soluble fraction (Fig. 4), whereas PLM-b was quantitatively recovered in detergent-insoluble fractions.

Because protein insolubility in nonionic detergents is commonly assumed to indicate an association with

the cytoskeleton, we tested whether treatment of cells with colchicine, a microtubule-disrupting agent, might increase detergent insolubility of PLM-b. Treatment of HEK cells with as little as 2 μM colchicine for 4 h led to an approximate doubling of the amount of PLM-b in detergent extracts (Fig. 6), consistent with this hypothesis. Future studies may be directed at defining the exact protein component(s) responsible for the cytoskeleton attachment of PLM-b.

Unique Tissue Distribution

Because immunoblot analysis of HEK for PLM-b showed measurable levels of PLM-b but not of standard PLM, we tested whether there might be a similar discrepancy among a panel of rat tissue samples. We found that tissues previously known to contain PLM (heart, aorta, brain) also contain the cytoskeleton-bound form of the protein, but that this bound form appeared to be the predominant form of PLM in some tissues examined, such as stomach, bladder, and kidney. No attempt was made in this study to discern the cell types in which PLM-b expression occurs. PLM-b migrated electrophoretically just above the diffuse bands of standard PLM in gels with 1.5% bisacrylamide, and this type of migration was seen in each tissue in which PLM-b predominates (*data not shown*). The relative levels of PLM and PLM-b among tissues cannot be discerned from data such as are shown in Fig. 7 because the reactivity of the two types of antibodies need not be similar. Indeed, the anti-PLM-b immunoreactivity appears particularly strong, perhaps because of the strongly antigenic properties of the unique epitopes recognized. This relative reactivity explains why anti-PLM-b immunoreactivity was detected in uninfected HEK cells, while anti-PLM immunoreactivity was not detected.

Lower Rate of PLM-b Overexpression with Ad.PLM Treatment

The level of PLM-b does not increase in response to Ad.PLM treatment as does standard PLM. PLM levels rose 20-fold over control levels; PLM-b levels approximately doubled. In fact, changes in PLM-b levels of overexpression were somewhat variable, varying from undetectable to a 2-fold increase, depending upon the experiment. An increase in PLM-b levels may require acquisition of the unique antigenic determinants of PLM-b over time that depend upon other cellular factors.

A Speculative Model of PLM-b Biochemistry

We suggest that PLM-b is bona fide PLM that contains a modified or otherwise altered C-terminus that results in detergent insolubility and altered immunoreactivity. We were unable to show that PLM-b represents phospho-PLM, in that addition of isoproterenol (Fig. 1) or forskolin (*data not shown*) did not increase levels of PLM-b in cells tested. A model for PLM-b (Fig. 8) that includes endogenous phosphate, while consistent with the results of our immunological approach, remains speculative. Nevertheless, the data suggest that a subset of PLM exists with an altered C-terminus, which is in association with cytoskeleton. Interestingly, PKA causes active Na pumps to arise from a cytoskeleton-attached pool of protein (Carranza et al., 1998; Gonin et al., 2001). Further studies are underway to characterize the biochemical mechanism by which PLM-b remains tightly bound.

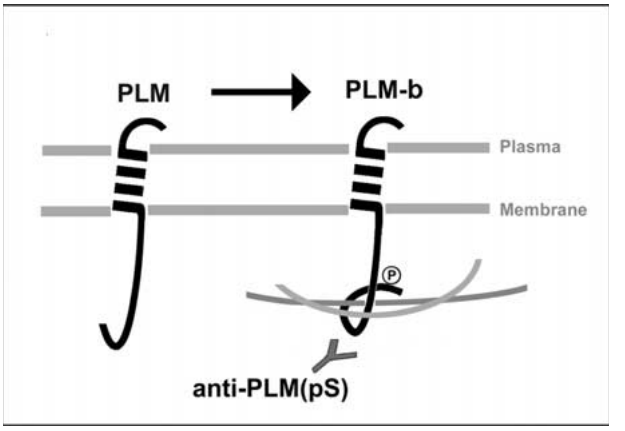

Fig. 8. Hypothetical model of PLM-b membrane biology. An altered conformation of the PLM C-terminus leads to a change in exposed epitopes, resulting in unique immunoreactivity for anti-PLM and anti-PLM-b antibodies. The N-terminus remains unaltered so that B8 antibodies detect both forms. PLM-b is bound to the cytoskeleton leading to its detergent insolubility. Some of our data are consistent with PLM-b containing phosphate on its C-terminus.

Much remains to be learned about the role of PLM and its phosphorylation in the cell. Whereas the discovery of its function in Na,K-ATPase regulation represents an important advance, the role of PLM regulation by phosphorylation has remained largely unexplored, perhaps due in part to an assumption that its regulation would be similar to that of phospholamban. The studies reported here may serve to point a new direction for future studies whereby PLM may play a role in sarcolemma-cytoskeleton communication.

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