Cytoplasmic targeting signals mediate delivery of phospholemman to the plasma membrane

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Submitted 9 March 2005; accepted in final form 13 December 2005

Lansbery, Kristan L., Lauren C. Burcea, Margareta L. Mendenhall, and Robert W. Mercer. Cytoplasmic targeting signals mediate delivery of phospholemman to the plasma membrane. Am J Physiol Cell Physiol 290: C1275–C1286, 2006. First published December 21, 2005; doi:10.1152/ajpcell.00110.2005.—The FXYD protein family consists of several small, single-span membrane proteins that exhibit a high degree of homology. The best-known members of the family include the γ-subunit of the Na⁺–K⁺-ATPase and phospholemman (PLM), a phosphoprotein of cardiac sarcolemma. Other members of the family include corticosteroid hormone-induced factor (CHIF), mammary tumor protein of 8 kDa (Mat-8), and related to ion channels (RIC). The exact physiological roles of the FXYD proteins remain unknown. To better characterize the function of the members of the FXYD protein family, we expressed several members of the family in Madin-Darby canine kidney (MDCK) cells. All of the FXYD proteins, with the exception of PLM, were primarily found in the basolateral plasma membrane. Surprisingly, PLM, a previously characterized plasma membrane protein, was found to colocalize with the endoplasmic reticulum marker protein disulﬁde isomerase. Treatment of MDCK cells expressing PLM with an agonist of PKC caused some of the PLM to be redistributed to the plasma membrane. Site-directed mutagenesis of residues within the cytoplasmic domain of PLM indicated that a negative charge at Ser69 is necessary to shift the localization of PLM to the plasma membrane. In addition, other regions of PLM necessary for either its endoplasmic reticulum or plasma membrane localization have been elucidated. In contrast to PLM, the plasma membrane localization of CHIF and RIC was not altered by mutation of potential cytoplasmic phosphorylation sites. Overall, these results suggest that phosphorylation of specific residues of PLM may direct PLM from an intracellular compartment to the plasma membrane.

THE BIOCHEMICAL MECHANISM accounting for the increase in cardiac contractility caused by β-adrenergic agonists is not fully defined. Phosphorylation reactions initiated by increased intracellular concentrations of cAMP are believed to mediate the effects of catecholamines on the heart, but the relevant target proteins have not been completely characterized. In the heart, a major substrate for cAMP-dependent kinase (PKA) and protein kinase C (PKC) is phospholemman (PLM). PLM is a plasma membrane protein found in cardiac (6, 41, 43), skeletal (55, 56), and smooth muscle (10), liver (13), and adrenal tumor cells (57). Activation of α- or β-adrenergic receptors in the myocardial sarcolemma results in increased phosphorylation of PLM (23, 32, 43); activation of β-adrenergic or vasopressin receptors in smooth muscle also increases phosphorylation (9, 10). In isolated guinea pig heart, administration of the β-adrenergic agonist, isoproterenol, results in phosphorylation of PLM, coinciding with an increase in contractility (42). Moreover, overexpression of PLM in adult rat myocytes affects contraction and Ca²⁺ homeostasis (48), apparently by modulating Na⁺/Ca²⁺ exchange (59). In these cells, PLM colocalizes with the Na⁺/Ca²⁺ exchanger in the sarcolemma and T tubules (59). However, the exact role of PLM in the regulation of cardiac contractility is not clear. Phosphorylation of PLM may allow it to associate with other protein(s) to influence or regulate their activity (31). Alternatively, it has been suggested that phosphorylation of PLM may influence the translocation of other proteins to the plasma membrane (53). PLM is a member of a gene family of small membrane proteins that contain an invariant motif of FXYD. These proteins are present mainly in tissues that perform transepithelial fluid and solute transport or that are electrically excitable (49). Currently, the family consists of seven members numbered according to their order of initial sequencing (Fig. 1): PLM (FXYD1), Na⁺–K⁺-ATPase γ-subunit (FXYD2), mammary tumor protein of 8-kDa (Mat-8; FXYD3), corticosteroid hormone-induced factor (CHIF; FXYD4), related to ion channel (RIC; FXYD5), phosphohippolim (FXYD6), and one family member (FXYD7) originally identified in the databases. When expressed in Xenopus oocytes, several members of the FXYD gene family induce ion channels. For example, PLM and Mat-8 induce hyperpolarization-activated Cl⁻ currents (34, 35), whereas CHIF evokes slowly activating, depolarization-induced K⁺ currents (1). The γ-subunit of the Na⁺–K⁺-ATPase induces large nonselective currents in Xenopus oocytes (33, 45). When reconstituted into planar lipid bilayers, recombinant PLM results in ionic conductances similar to those observed in oocytes expressing PLM, suggesting activity intrinsic to the protein (34). However, Xenopus oocytes have endogenous channels with properties strikingly similar to those induced by the FXYD proteins (26). Our studies suggest that γ is activating voltage-gated, large-diameter pores that are intrinsically present within the oocyte membrane (45). Influenza B virus NB protein, a single membrane spanning protein not related to the FXYD proteins, also activates an endogenous oocyte conductance by shifting its voltage dependence to less hyperpolarized potentials (47). Consequently, it is becoming increasingly evident that heterologous expression of membrane proteins in Xenopus oocytes often induces or modifies endogenous currents (51). Thus, these small, single membrane-spanning proteins may act in conjunction with endogenous protein partners. It is not clear whether the ability of the FXYD proteins to induce ion currents in Xenopus oocytes is of...
physiological relevance to mammalian cells. Moreover, the exact functional roles of the FXYD proteins in mammalian cells remain unknown.

Several members of the FXYD family appear to associate with the Na⁺/K⁺-ATPase to modulate its activity (reviewed in Ref. 14). The Na⁺/K⁺-ATPase is a membrane-associated protein responsible for the high intracellular K⁺ and low intracellular Na⁺ concentrations characteristic of most animal cells. The enzyme consists of two noncovalently linked subunits: a 100-kDa multispanning membrane protein termed the α-subunit, and the β-subunit, a smaller glycosylated membrane protein. Multiple isoforms for both α- (α₁, α₂, α₃, α₄) and β- (β₁, β₂, β₃) subunits have been identified. These isoforms exhibit a tissue-specific and developmental pattern of expression that may be important in the maintenance and regulation of Na⁺/K⁺-ATPase activity (reviewed in Ref. 8). Although not integral parts of the Na⁺/K⁺-ATPase enzymatic complex, several FXYD proteins modulate Na⁺/K⁺-ATPase activity. For example, the γ-subunit can modify the voltage dependence of K⁺ activation (5) and influence the apparent affinity of the enzyme for Na⁺, K⁺, and ATP (2, 50). When expressed in Xenopus oocytes, PLM induces a slight decrease in the K⁺ affinity and a nearly twofold decrease in the Na⁺ affinity of the α₁β₁ and α₂β₁ isozymes (15). In addition, in native rat heart, it appears that PLM can associate with the α₁ but not the α₂ subunit (19). Moreover, PLM-deficient mice have reduced cardiac Na⁺/K⁺-ATPase activity (28). In contrast, CHIF when expressed in Xenopus oocytes with the Na⁺/K⁺-ATPase α₁ and β₁-subunits, increases the Na⁺ affinity and decreases the apparent K⁺ affinity of the enzyme (3). Finally, FXYD7 can interact with the α-isozymes containing the β₁ but not the β₂ isoform. Moreover, in oocytes, FXYD7 decreases the apparent K⁺ affinity of the α₁β₁ and α₂β₁ isozymes, but not α₁β₂. However, in the rat brain, FXYD7 appears to associate only with the α₁β isoforms (4).

To better characterize the function of the members of the FXYD protein family, we have expressed several of the family members in a mammalian cell line. To determine the subcellular localization of the different family members, we fused a myc epitope tag to the carboxy terminus of each of the FXYD proteins and expressed them in Madin-Darby canine kidney (MDCK) cells. All of the FXYD proteins, with the exception of PLM, were found primarily in the basolateral plasma membrane. Surprisingly, PLM, a previously characterized plasma membrane protein, was found to colocalize with the endoplasmic reticulum (ER) marker protein disulfide isomerase (PDI) in the MDCK cells. Treating MDCK cells expressing PLM with an agonist of PKC caused some of the PLM to be redistributed to the plasma membrane. Site-directed mutagenesis of residues within the cytoplasmic domain of PLM indicated that a negative charge at Ser69 is necessary to shift the localization of PLM to the plasma membrane. In addition, other regions of PLM necessary for either its ER or plasma membrane localization have been elucidated. These results suggest that phosphorylation of specific residues of PLM may direct PLM from an intracellular compartment to the plasma membrane. Understanding the regulation of PLM may provide insights into the physiological roles of PLM at the plasma membrane.

MATERIALS AND METHODS

Antibodies. The α₁-subunit of the Na⁺/K⁺-ATPase was identified with an antibody to a synthetic peptide derived from the aminoterminal of the rat α₁-subunit (7). PDI was identified with a commercially available rabbit polyclonal antibody (StressGen Biotechnologies, Victoria, BC, Canada). T7 peptide antibody was purchased from Biodesign International (Saco, ME). The mouse monoclonal antibody against the myc epitope (17) was kindly provided by Maurine E. Linder (Washington University).

Cell culture. MDCK cells (clone G) were obtained from W. J. Nelson (Stanford University). The cells were maintained by being passaged at low density every 3 days. Following trypsinization, the cells were resuspended in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 200 μM l-glutamine, 0.25 μg/ml Fungizone, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were transfected with the indicated cDNAs subcloned into pcDNA3.1 (—) Myc-HisA (Invitrogen, Carlsbad, CA) such that all expressed polypeptides included a myc epitope tag at the carboxy terminus. PCR was used to create all constructs from the following cDNAs: PLM (accession no. NM019503), CHIF (L41254), Mat-8

C1276 CYTOPLASMIC TARGETING SIGNALS DIRECT PLM DELIVERY

Fig. 1. Alignment of FXYD family members. Alignment of the deduced amino acid sequences of the human and rat γ-subunits of the Na⁺/K⁺-ATPase, mouse phospholemman (PLM), rat corticosteroid hormone-induced factor (CHIF), mouse mammary tumor protein of 8 kDa (Mat-8), and protein related to ion channels (RIC). Invariant amino acids are shown shaded. A bar indicates the putative transmembrane domain.
containing 0.1% NGS, were applied for 1 h at room temperature. The antibodies (Molecular Probes, Eugene, OR) diluted 1:1,000 in HBS three washes in HBS, Alexa 488-, or Cy3-conjugated secondary antibody yielded identical results. MDCK cells were transfected using Lipofectamine (GIBCO-BRL, Rockville, MD) according to the supplier’s protocol. Stably transfected cells were selected for and grown in media supplemented with 800 μg/ml geneticin (GIBCO-BRL). Briefly, following transfection, the cells were split and plated at a high density. The cells were grown for 1 wk or more without being passaged; the medium was replaced approximately every 2 days. For PKA stimulation, confluent MDCK cells on coverslips were treated with 2 mM dibutyryl adenosine 3',5'-cyclic monophosphate (DbcAMP; Sigma, St. Louis, MO) in growth medium. At the indicated times, the treated cells were washed once in 150 mM NaCl and 25 mM HEPES, pH 7.4 (HBS), and processed for immunofluorescence. To activate PKC, cells were treated with 1 μM phorbol 12-myristate 13-acetate (PMA; Sigma) in growth medium. At the times indicated, the cells were washed once in HBS and processed for immunofluorescence.

**Immunofluorescence.** MDCK cells were plated at low density on collagen-coated coverslips and grown to confluence. Once confluent, the MDCK cells were washed once with HBS and fixed/permeabilized in 100% methanol at −20°C for 10 min. The coverslips were washed three times with 4°C HBS. Nonspecific sites were blocked for 1 h at 37°C in HBS containing 5% normal goat serum (NGS). Primary antibodies against the α-subunit of the Na+-K+-ATPase (α1 diluted 1:100) and myc (ascites fluid diluted 1:2,000) were diluted in HBS containing 1% NGS and applied to cells overnight at 4°C. Following three washes in HBS, Alexa 488-, or Cy3-conjugated secondary antibodies (Molecular Probes, Eugene, OR) diluted 1:1,000 in HBS containing 0.1% NGS, were applied for 1 h at room temperature. The samples were washed three times in HBS and mounted in ProLong Antifade (Molecular Probes). The samples were viewed with a Zeiss Axioskop microscope (×40 objective). Images were captured digitally with the microscope using Axiovision 2.0 software. Images were viewed and assembled using PhotoShop 6.0 and Illustrator 9.0 (Adobe).

**RESULTS**

PLM is localized to ER in MDCK cells. To study the expression of an FXYD protein in a polarized, mammalian cell background, we expressed PLM in MDCK epithelial cells, a frequently used renal cell culture model. To identify the PLM polypeptide, a myc epitope tag was fused to the cytoplasmic carboxy terminus of the protein sequence. As shown in Fig. 2, indirect immunofluorescence with an antibody to the myc epitope indicated that PLM is restricted to an intracellular compartment in the MDCK cells. Surprisingly, PLM colocalized with an ER chaperone protein, PDI, and exhibited little overlap with the Na+-K+-ATPase α-subunit at the plasma membrane. No fluorescence was observed in MDCK cells transfected with only the myc vector (data not shown). As shown, PLM with a T7 epitope tag also was restricted to intracellular compartments demonstrating that the myc sequence did not affect PLM localization. These results are in contrast to earlier studies, in which PLM has been characterized as the major plasma membrane substrate for PKA and PKC in heart cells (41). In addition, following activation of PKA or PKC, PLM has been identified in the plasma membrane of cardiac (6, 41, 43), skeletal (55, 56), smooth muscle (10), and liver (13) cells, as well as in adrenal tumor cells (57).

![Image](https://example.com/image.png)
PLM is the only known FXYD family member localized to the ER. To determine whether MDCK cells are an appropriate cell line to test the subcellular distribution of the FXYD proteins, several other FXYD proteins were expressed with a carboxy-terminal myc tag in MDCK cells. As shown in Fig. 1, all the FXYD proteins have a single transmembrane domain and share significant amino acid homology. PLM is the only member known to have a cleaved signal sequence, although the processing of RIC with its extended amino terminus (not shown in Fig. 1) has not been characterized. The amino terminus of the processed PLM polypeptide begins at Glu29 of the open reading frame (boxed glutamate residue shown in Fig. 1). As shown in Fig. 3, all of the characterized FXYD proteins were detected at the basolateral plasma membrane, except PLM. Expression of the γ-protein at the plasma membrane is consistent with its association with the Na⁺-K⁺-ATPase. The Na⁺-K⁺-ATPase is located at the basolateral surface of kidney epithelial cells and in polarized MDCK cells, where it establishes the transepithelial Na⁺ gradient that supports the vectorial transport of ions and solutes (20, 39).

CHIF is an epithelial cell-enriched protein that is induced by aldosterone in a tissue-specific manner. The exact function of CHIF remains unknown. CHIF mRNA is abundantly expressed in the distal colon and the medullary and papillary kidney (11). In response to aldosterone, CHIF mRNA is strongly upregulated in the distal colon, but expression in the kidney is relatively unaffected (12). As shown in Fig. 3, CHIF is found at the basolateral surface of the MDCK cells, identical to its localization in the principal cells of the collecting duct and distal colon surface cells (46). However, in contrast to our results, Shi et al. (46) observed some intracellular localization of CHIF in addition to the plasma membrane.

Fig. 3. PLM is the only FXYD family member located in the ER of transfected MDCK cells. Transfected MDCK cells expressing the indicated myc-tagged FXYD family member were processed for double-immunofluorescence microscopy using a monoclonal antibody to myc and a rabbit antibody to the α-subunit of the Na⁺-K⁺-ATPase. The myc antibody was detected using a Cy3-conjugated goat anti-mouse antibody and the α-subunit antibody was visualized with an Alexa 488-conjugated goat anti-rabbit antibody. To demonstrate overlap, the respective images were merged; yellow indicates regions of colocalization. Bar, 10 μm.
The exact functions and cellular location of Mat-8 or RIC have not been determined. Interestingly, both Mat-8 and RIC were originally identified as factors upregulated by oncogenes. Both proteins localized to the basolateral plasma membrane of MDCK cells (Fig. 3). Mat-8 RNA is expressed in primary human breast tumors and in breast tumor cell lines (36), and murine Mat-8 is expressed at high levels in the uterus, stomach, and colon. RIC gene expression has been detected in the heart, spleen, lung, testis, and skeletal muscle of normal adult rats (18). It is not clear whether these upregulated genes are required for the transformed state or whether these proteins are characteristic features of secretory epithelial cells and are simply maintained in the transformed state.

The carboxy terminus of PLM is sufficient for ER localization. The localization of PLM to the ER appears to be unique among the FXYD family. Because the primary amino acid structure of the FXYD proteins is conserved (Fig. 1), chimeras were used to determine the domain of PLM responsible for its intracellular localization. Moreover, because the cytoplasmic domain of PLM is more conserved among species than its amino-terminal, extracellular domain, we replaced or added portions of the PLM cytoplasmic domain to another FXYD family member, γ (Fig. 4A). As shown in Fig. 4B, when the cytoplasmic domain of γ was replaced with the cytoplasmic domain of PLM (γ1–47/PLM38–72), the chimera localized to the same intracellular compartment as wild-type PLM. To further delineate the region responsible for the intracellular localization of PLM, the last 15 amino acids of the PLM carboxy terminus were added to the full-length γ-protein (γ/PLM56–72). As shown in Fig. 4B, γ localization was shifted to the ER. Both constructions resided in the same intracellular compartment as the wild-type PLM protein implying that the carboxy terminus of PLM is sufficient to confer localization to the ER.

Specific domains regulate subcellular localization of PLM. Addition of the PLM cytoplasmic tail to the γ-protein shifted its location from the plasma membrane to an intracellular compartment. To further examine the ability of this domain to act as an ER retention signal, we removed the last 15 residues of PLM to create a truncation mutant at Glu57 (PLM1–57). As shown in Fig. 5, the truncated PLM protein was delivered to the plasma membrane, consistent with the carboxy terminus containing an ER retention signal. Thus the carboxy-terminal tail of PLM is required for the localization of the protein in the ER. As shown in Fig. 1, both γ and PLM have regions of negatively charged amino acids. The carboxy terminus of γ has a stretch of three negatively charged residues, whereas PLM has four. To determine whether these negatively charged residues play a role in the trafficking of PLM, we made another PLM truncation mutant (PLM1–51). As shown in Fig. 5, when the aspartate- and glutamate-rich region of the cytoplasmic domain was excluded from PLM, a majority of the protein was retained in the ER. In contrast to wild-type PLM, a small amount of PLM1–51 was detected at the plasma membrane. This suggests that retention of PLM1–51 in the ER is a result of inefficient export from ER, rather than terminal misfolding or aggregation. Moreover, the PLM1–51 mutant removes a DXE motif thought to act as a signal for release of vesicular stomatitis virus G protein (VSVG) from the ER (40). Subsequent studies with VSVG demonstrated that the DXE motif was part of a larger motif, including an important tyrosine residue (44). These adjacent residues implicated in the rapid exit of VSVG from the ER do not surround the DXE motif in PLM, but similar to VSVG, the negative amino acids in PLM appear to be necessary for efficient release of PLM from the ER.
Stimulation of PKC, but not PKA, affects the subcellular localization of PLM. To determine whether phosphorylation of PLM is required for its localization to the plasma membrane in MDCK cells, we used indirect immunofluorescence to assay for changes in the localization of PLM after stimulation of either PKC or PKA. Confluent MDCK cells expressing the myc-tagged PLM were treated with the activators of PKC or PKA, PMA, or DbcAMP, respectively. The cells treated with DbcAMP showed no change in the intracellular localization of PLM even after 5 h (results not shown). In contrast, after treatment with PMA, the expression of PLM was detected at the plasma membrane in many, but not all, of the cells after 40 min (Fig. 6). After 60 min of PMA treatment, the MDCK cells appeared to depolarize, making longer time points impossible. To verify that sequences within the PLM carboxy terminus are responsible for the shift from the ER to the plasma membrane, PKC was activated in cells expressing the γ protein/PLM chimera (γ/PLM56–72). As shown in Fig. 7, after 40 min, the γ/PLM56–72 chimeric protein redistributes to the plasma membrane on PKC activation.

Localization of PLM phosphorylation-deficient mutant is not affected by stimulation of PKA or PKC. To determine whether the shift in PLM localization was attributable to phosphorylation of PLM or another protein, we generated a phosphorylation-defective mutant of PLM (SSSS 62, 63, 68, 69 GAAA; PLMGAAD) and expressed it in the MDCK cells. In vitro, canine PLM is phosphorylated by PKC at Ser63 and Ser68, and PKA phosphorylates PLM at Ser68 (54). We mutated Ser62 to glycine because this is conserved in other FXYD family members. There was no difference in the expression or localization of the PLMGAAD mutant and a PLMAAAA mutant (data not shown). The localization of the phosphorylation-defective mutant before treatment (time 0) was identical to that of the wild-type PLM protein (data not shown). When MDCK cells expressing PLMGAAD were treated with agonists to PKA or PKC, there was little localization of PLMGAAD to the plasma membrane (data not shown). Thus it appears that phosphorylation of PLM by PKC is required to shift the localization of PLM from the ER to the plasma membrane.

Phosphorylation of PLM is required for plasma membrane localization. To determine which residues are important for the change in PLM localization, we substituted negatively charged aspartates where PLM could be phosphorylated by PKC. Mutation of a phosphorylated amino acid to aspartic or glutamic acid often mimics the phosphorylation event (21). As shown in Fig. 8, when Ser62, Ser63, Ser68, and Ser69 were replaced with aspartates (PLMDDDD), the modified PLM was redirected to the plasma membrane. The ability of the PLMDDDD mutant to transport to the plasma membrane supports phosphorylation as an important mechanism for the proper trafficking of PLM.

To determine the requirements for localization of PLM to the plasma membrane, we altered the position and number of negative charges on the PLM cytoplasmic tail. As shown in Fig. 8A, an aspartate at Ser69 is necessary to localize PLM to the plasma membrane because PLMDDDA and PLMADDA are localized in the ER, and PLMDDDD is at the plasma membrane. Moreover, it appears that three negative charges are required for plasma membrane localization as PLMGAAD and PLMADD are confined to intracellular compartments (Fig. 8A). As shown in Fig. 8B, the replacement of the myc epitope with the T7 epitope tag in PLMGAAD and PLMADD did not influence the localization of PLM. These results, demonstrating the role of phosphorylation in regulating the subcellular localization of PLM, are tabulated in Table 1.

Phosphorylation vs. ER retention. The final three arginines at the carboxy terminus of PLM resemble the RKR motif implicated in the ER retention of potassium channel subunits (58). Alanine substitutions for any of the residues of the inwardly rectifying K+ channel 6.2 RKR motif resulted in abnormal surface expression of the channel subunit (58). To determine whether the carboxy-terminal RRR motif influences the ER retention of PLM, we created a truncation mutant lacking the last three amino acids, PLM1–69. As shown in Fig. 9, when the carboxy-terminal RRR motif is removed, the truncated PLM is delivered to the plasma membrane. Therefore, the RRR motif is required to retain PLM in the ER. Because it appears that phosphorylation is a factor in the subcellular localization of PLM, we generated a phosphorylation-deficient PLM mutant lacking the same three carboxy-terminal arginines, PLMGAAD1–69. Interestingly, the phosphorylation-deficient truncation mutant is localized in the ER (Fig. 9; PLMGAAD1–69). However, when the RRR motif was removed from a mutant that previously localized in the ER, PLMGAAD1–69 mutant localized both to an...
intracellular, punctate compartment and to the plasma membrane (Fig. 9; PLM_GAADI-69). Thus retention of PLM in the ER by the RRR motif appears to be countered by phosphorylation. In truncation mutants lacking the RRR motif, phosphorylation of PLM is still required for plasma membrane localization; however, one negative charge is sufficient for some of the PLM to be delivered to the plasma membrane. Therefore, it appears that the RRR motif is acting as a signal that increases the number of phosphorylation events required for PLM plasma membrane localization.

To determine which cytoplasmic motifs determine delivery of another FXYD protein to the plasma membrane, we analyzed the trafficking of CHIF. As shown in Fig. 1, CHIF does not contain a RRR motif, but it does have several serine residues that may be phosphorylated and involved in trafficking. To test whether activation of PKC or PKA could influence the subcellular localization of CHIF, we treated confluent MDCK cells expressing CHIF with agonists of PKA or PKC. There was no change in the subcellular localization with either treatment (data not shown). Kinases other than PKA and PKC may act on PLM (29, 37) and other FXYD family members. To determine whether phosphorylation of CHIF could affect its subcellular distribution, mutagenesis was performed on the carboxy terminus of CHIF (Table 2). Our analysis focused on serines conserved in CHIF with PLM and/or Mat-8, Ser84, and Ser63. Regardless of the mutations, CHIF was always found at the plasma membrane (data not shown).

**RXR domain at the carboxy terminus of RIC does not influence plasma membrane localization.** The carboxy-terminal RXR domain in PLM appears sufficient and necessary for ER retention of PLM. However, another FXYD family member, RIC, has an RXR domain at its carboxy terminus, but is able to efficiently exit the ER and traffic to the plasma membrane. Because the cytoplasmic domain of PLM is heavily

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**Fig. 6. Activation of PKC changes the subcellular localization of PLM to the plasma membrane.** MDCK cells expressing PLM as a myc-tagged fusion protein were removed from culture before treatment (time 0) or after the addition of 1 mM PMA at a specified time (5, 20, 40, or 60 min). After treatment, the cells were fixed and processed for immunofluorescence. PLM was detected using a monoclonal antibody to myc and visualized using a Cy3-conjugated goat anti-mouse antibody. The plasma membrane was detected using a polyclonal antibody to the α-subunit of the Na⁺-K⁺-ATPase. The α-subunit antibody was visualized with an Alexa 488-conjugated goat anti-rabbit antibody. To demonstrate colocalization, the respective images were merged; yellow indicates regions of overlap. After 20 min of treatment, a significant amount of PLM was detected at the plasma membrane. Bar, 5 μm.
phosphorylated in vivo (41, 43), we wanted to determine whether phosphorylation plays a role in the ER retention of other FXYD proteins with an RXR motif. We tested for an effect on the subcellular localization of RIC when Ser170 was changed to either a negatively charged aspartate or a neutral glycine (Fig. 10). RIC did not move off the plasma membrane when a charge was placed on the only serine in the cytoplasmic domain of RIC. Because neither mutation altered the localization of RIC to the plasma membrane, the RXR motif is not uniformly capable of retaining FXYD proteins in the ER.

DISCUSSION

Subcellular localization of PLM is regulated by phosphorylation. Previous studies provide convincing evidence that PLM is a phosphorylated protein residing in the plasma membrane of several cell types. However, the localization of PLM in unstimulated cells has not been well characterized. Our results provide evidence that the localization of PLM in either the ER or plasma membrane is dependent on its state of phosphorylation. We show that negative charges in the cytoplasmic domain, mimicking phosphorylation, permit release of PLM from the ER to the plasma membrane. The carboxy-terminal, cytoplasmic domain of PLM appears to be composed of three regions that mediate this subcellular switch. The last three carboxy-terminal amino acids, RRR, act to retain PLM in the ER.
the plasma membrane, whereas the PLMGAAD1–69 mutant remained in an intracellular compartment. Bar, 10 μm.

### Table 1. Mutations in cytoplasmic domain of PLM and their subcellular localization when expressed in MDCK cells

<table>
<thead>
<tr>
<th>Name</th>
<th>Carboxy-Terminal Sequence</th>
<th>Localization</th>
</tr>
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<tbody>
<tr>
<td>PLM WT</td>
<td>KRCRCKFNQQQRGPEDEEGFFSIRRLSSRRR</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>GAAA</td>
<td>KRCRCKFNQQQRGPEDEEGFFGAIIRLAAARR</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>1–51</td>
<td>KRCRCKFNQQQRGPEDEEGFF</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>DDDD</td>
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<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ADDA</td>
<td>KRCRCKFNQQQRGPEDEEGFFADIRRLDARRR</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>DDDD</td>
<td>KRCRCKFNQQQRGPEDEEGFFDDIRRLDARRR</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>GADD</td>
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<tr>
<td>GAAD</td>
<td>KRCRCKFNQQQRGPEDEEGFFGAIIRLDDARRR</td>
<td>Endoplasmic reticulum</td>
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<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>GAAA1-69</td>
<td>KRCRCKFNQQQRGPEDEEGFFGAIIRLAA</td>
<td>Endoplasmic reticulum</td>
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MDCK, Madin-Darby canine kidney; PLM, phospholemman; WT, wild type. The sequences of PLM mutations, in the order of their appearance in the study, are shown in the context of the entire cytoplasmic domain of PLM. Sites of potential phosphorylation are highlighted in gray, and the mutated amino acids are set in bold type.

the ER. In contrast, the putative phosphorylation sites (SSSS; 62, 63, 68, 69) act as a regulatory signal capable of overriding the ER retention signal. Finally, the stretch of negative amino acids, 51–57, also facilitates the exit of PLM from the ER to the plasma membrane.

The cytoplasmic domain of PLM from the canine, rat, human, and mouse is almost completely conserved, with the exception that mouse PLM has a serine at position 69 instead of a threonine. However, this is a conserved change because this threonine fits the phosphorylation consensus sequence for PKC (25). Although it is curious that threonine phosphorylation has not been observed in vitro from peptides derived from canine PLM (54), recent results using antibodies raised against PLM phosphopeptides suggest that T69 is phosphorylated (24).

In Xenopus oocytes, coexpression of PKA and PLM increased the amplitude of the ion current observed and the amount of PLM present at the plasma membrane (38). When PKA was coexpressed with a phosphorylation-defective PLM mutant (SSST 62, 63, 68, 69 AAAA), there was no increase in the amplitude of the induced ion current or the amount of PLM at the membrane. Therefore, phosphorylation by PKA appears to have an effect on the membrane localization of PLM. Even in the absence of PKA, the same study showed a PLM mutant (S68A) in the oocyte membrane in greater amounts than wild-type PLM. Coexpression of PKA and the same mutant (S68A) further increased the amount of PLM in the oocyte membrane. In contrast, PLM SSST 62, 63, 68, and 69 AAAA was not visible in plasma membranes, and coexpression with PKA did not increase the amplitude of ion current induced or the amount at the plasma membrane. These results suggest that Ser68 plays a role in directing PLM to the oocyte membrane; however, other serine residues also influence PLM localization.

**Phosphorylation as a mechanism for release from ER.** Phosphorylation and dephosphorylation of proteins is an important mechanism for regulating cellular processes. In addition, phosphorylation events may regulate the cellular trafficking of

### Table 2. Mutations in cytoplasmic domain of CHIF and their subcellular localization when expressed in MDCK cells

<table>
<thead>
<tr>
<th>CHIF Mutant</th>
<th>Carboxy-Terminal Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHIF WT</td>
<td>GKCKRKNHTPSSLPEKVTPLITPOSAS</td>
</tr>
<tr>
<td>SS 84,86 DN</td>
<td>GKCKRKNHTPSSLPEKVTPLITPDANT</td>
</tr>
<tr>
<td>SS 84,86 AN</td>
<td>GKCKRKNHTPSSLPEKVTPLITPDANT</td>
</tr>
<tr>
<td>SS 71,72 RT</td>
<td>GKCKRKNHTRPLPEKVTPLITPOSAS</td>
</tr>
<tr>
<td>SS 71,72 RD</td>
<td>GKCKRKNHTRPLPEKVTPLITPOSAS</td>
</tr>
<tr>
<td>SS 71,72 AA</td>
<td>GKCKRKNHTRPLPEKVTPLITPOSAS</td>
</tr>
<tr>
<td>SSSS 71,72,84 RTDN</td>
<td>GKCKRKNHTRPLPEKVTPLITPDANT</td>
</tr>
<tr>
<td>SSSS 71,72,84 RTAN</td>
<td>GKCKRKNHTRPLPEKVTPLITPDANT</td>
</tr>
<tr>
<td>TTSS 77,81,84,86,87 HANA</td>
<td>GKCKRKNHTRPLPEKVTPLITPDANT</td>
</tr>
</tbody>
</table>

CHIF, corticosteroid hormone-induced factor. The sequences of CHIF mutants are shown in the context of the entire cytoplasmic domain. All CHIF mutants and their respective carboxy-terminal sequences were localized in the plasma membrane. The mutated amino acids are set in boldface type.
proteins. In addition to PLM, the involvement of phosphorylation for trafficking from the ER has been proposed for another protein with a highly charged cytoplasmic tail, the lip35 isoform of the major histocompatibility complexity class II-associated invariant chain (ii). In B-cell lines, the ER retention of lip35 is ineffective and a fraction of lip35 is transported through the Golgi complex associated with class II molecules in a phosphorylation-dependent manner (27). Mutations altering the charge of Ser6 or Ser8 in the cytoplasmic tail of lip35 in a phosphorylation-dependent manner (27). Mutations altering the charge of Ser6 or Ser8 in the cytoplasmic tail of lip35 (M1HRRRSRSCREDQKPV) to an alanine prevented detection of the charge of Ser6 or Ser8 in the cytoplasmic tail of lip35 in a phosphorylation-dependent manner (27). Mutations alteration of lip35 is ineffective and a fraction of lip35 is transported II-associated invariant chain (Ii). In B-cell lines, the ER retention of lip35 isoform of the major histocompatibility complex class

Potential physiological implications of multiple PLM phosphorylation events. Previous results have identified phosphorylated PLM in purified canine cardiac sarcoplasmic vesicles after stimulation of the β-adrenergic receptor with isoproterenol (42). Our findings are consistent with these observations. Furthermore, the variety of combinations with which PLM may be phosphorylated suggests a role for PLM in the diverse responses observed between tissue types after adrenaline stimulation. Our study is the first to demonstrate that phosphorylation of Ser69 is important for PLM localization to the plasma membrane. Moreover, the association of PLM to other proteins at the plasma membrane or ER may be influenced by its pattern of phosphorylation. There is evidence to suggest that the pattern of PLM phosphorylation is both important and variable for its cellular function. In isolated skeletal muscle, insulin regulates the phosphorylation of both Ser63 and Ser68, whereas adrenaline regulates phosphorylation of Ser68 (54).

The increased transport of phosphorylated PLM to the plasma membrane may reflect its ability to override retention signals to exit the ER. Alternatively, phosphorylation of PLM may allow it to interact with another factor and traffic to the plasma membrane. This model is consistent with the identification of a 14-3-3 protein associating with lip35 in a phosphorylation-dependent manner to mediate its exit from the ER (27). Understanding of the regulation and role of PLM phosphorylation may provide insights into the functions of PLM and its putative protein partners.

AJP-Cell Physiol • VOL 290 • MAY 2006 • www.ajpcell.org
GRANTS
This work was supported by National Institutes of Health Grants GM-39746 and DK-064704.

REFERENCES