Calmodulin Binds to the Basolateral Targeting Signal of the Polymeric Immunoglobulin Receptor*

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The plasma membrane of polarized epithelial cells is divided into apical and basolateral surfaces, each having a distinct protein and lipid composition. Cells use two pathways to deliver proteins to the correct surface. First, newly made proteins are packaged in the trans-Golgi network (TGN) into vesicles that deliver them directly to either the apical or basolateral surface. Second, proteins can be delivered first to one surface and then endocytosed and transcytosed to the opposite surface. At least in the well polarized Madin-Darby canine kidney (MDCK) cell line, targeting to the basolateral surface generally requires a sorting signal located in the cytoplasmic domain of the protein. For a number of basolateral proteins it has been found that mutations in the cytoplasmic domain prevent TGN to basolateral targeting (1). The first basolateral signal to be identified is the 17-amino acid membrane proximal segment (residues 653–670) of the cytoplasmic domain of the polymeric immunoglobulin receptor (pIgR) (2). This protein is normally delivered from the TGN to the basolateral surface, and then endocytosed and transcytosed to the apical surface. Deletion of most of this 17-residue segment prevents TGN to basolateral delivery. Moreover, this segment can be transplanted to a heterologous reporter molecule, which is then re-targeted from the apical to the basolateral surface (2). In at least one other case, the low density lipoprotein receptor, autonomous basolateral sorting signals have been identified (3).

The pIgR basolateral targeting signal has been systematically analyzed by alanine scanning mutagenesis (4). Mutation of His-656, Arg-657, or Val-660 to Ala substantially diminishes, but does not eliminate, TGN to basolateral targeting. Mutation of other residues had little or no effect. The structure of a synthetic 17-residue peptide corresponding to this signal has been determined by two-dimensional nuclear magnetic resonance spectroscopy (4). This peptide tends to adopt a putative type 1 β-turn, encompassing residues 658–661, followed by a nascent helical structure. In MDCK cells polarized sorting takes place in both the TGN and after endocytosis. Mutations that diminish TGN to basolateral sorting and increase TGN to apical sorting have a similar effect on sorting in the endocytic pathway, decreasing recycling to the basolateral surface and increasing transcytosis to the apical surface (5). These data indicate that the basolateral targeting signal also functions as a signal to retrieve the pIgR from the endocytic pathway to the basolateral surface. Very similar results have been obtained with the two basolateral sorting signals of the low density lipoprotein receptor (6). This suggests that the same sorting machinery operates in both the TGN and the endocytic pathway, or that sorting in both pathways actually involves in regulation of pIgR transcytosis and/or signaling by pIgR.

We have identified a major calmodulin (CaM)-binding protein in rat liver endosomes using 125I-CaM overlays and CIRIT 93-22. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

These authors made equal contributions to this work.

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occurs in a common compartment. Transcytosis of plgR is regulated by several mechanisms. Phosphorylation of Ser664, which is part of the basolateral sorting signal, stimulates transcytosis. Mutation of Ser-664 to Ala (S664A) reduces transcytosis, while mutation to Asp (S664D), whose negative charge may mimic a phosphate, stimulates transcytosis (7). Phosphorylation apparently works by weakening the basolateral signal, since the S664D mutant exhibits decreased TGN to basolateral sorting, increased TGN to apical sorting (5), as well as decreased recycling and increased transcytosis after endocytosis.

Binding of the ligand, dimeric IgA (dlgA) to the plgR also stimulates transcytosis. Ligand binding stimulates transcytosis of the wild-type plgR, as well as S664D and S664A (8, 9). Ligand-dependent stimulation suggests that the plgR may transduce a signal to the cytoplasm. Indeed, we have recently found that dlgA binding causes tyrosine phosphorylation of a phosphatidylinositol-specific phospholipase C (PLC) (2). Transcytosis of plgR and other molecules is stimulated either by activation of protein kinase C (10) or by increase in intracellular free Ca2+ (11), so both arms of the phospholipase C signaling pathway may redundantly stimulate transcytosis. Delivery to the apical surface, either by transcytosis or directly from the TGN, is also stimulated by the heterotrimeric G protein, Gs (11, 12), as well as cAMP and protein kinase A (13, 14). Both the Gs and Gip subunits are stimulatory, at least for transcytotic apical delivery (11). Transcytosis in MDCK cells is stimulated by bradykinin, while in animals transcytosis is inhibited by bradykinin (3), so both arms of the G protein subunits are stimulatory, at least for transcytosis targeting signal, stimulates transcytosis. Mutation of Ser-664 to Ala (S664A) reduces transcytosis, while mutation to Asp (S664D) has also been described (28).

**Two-dimensional Electrophoresis and Western Blotting—**Two-dimensional electrophoresis was carried out as described (29). After electrophoresis, proteins were transferred to nitrocellulose filters at 20 V overnight at 4 °C. Filters were incubated in phosphate-buffered saline containing 5% powdered skim milk to block nonspecific sites. The nitrocellulose was then incubated with a 1:1000 dilution of the monoclonal ascites SC-166 directed against the cytoplasmic tail of the plgR in phosphate-buffered saline containing 0.05% Tween 20, followed by horseradish peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), and detection with 4-chloro-1-naphthol as described by Burnette (30).

**MDCK Clones Expressing plgR Constructs—**All nucleotide and amino acid positions are according to the original published rabbit plgR sequence (33). MDCK strain II cells expressing wild-type plgR (34), a mutant lacking a membrane-proximal 14-amino acid segment of the cytoplasmic tail (35), and a “tail-minus” construct lacking all but the two membrane-proximal amino acids of the cytoplasmic tail (2, 33) termed R655-STOP (4), have been described previously. An MDCK clone expressing plgR in which the cytoplasmic domain consists only of the membrane proximal 17-amino acid basolateral targeting signal (T670-STOP) (4), and point mutants in which individual residues in the plgR 17-amino acid basolateral targeting signal are replaced with alanine in the context of the entire cytoplasmic tail (H656A, R657A, N659A, and V660A) (5) have also been described. These mutants are indicated by the wild-type amino acid, followed by the position and the new amino acid (e.g. H656A designates a mutant where histidine at position 656 has been mutated to alanine). A construct encoding the full-length plgR molecule with a substitution of alanine for arginine at position 658 was constructed by ligating a DNA fragment encoding the C terminus (residues 661–755) of wild-type plgR (35) to a fragment encoding residues 1–660 derived from the R655A/T670-STOP mutant described previously (4) via a common Aval site at nucleotide position 707. The resulting cDNA was ligated into the BglII site of pcB6 (36) and transfected into MDCK strain II cells as described previously (4). Mutants in which serine 664 has been mutated to alanine (S664A) or aspartic acid (S664D) have also been described (7).

**Immunoprecipitation—**Immunoprecipitation of plgR was performed using protein G-Sepharose (Sigma) coupled to polyclonal sheep anti-CaM IgG, essentially as described (4).

**Calmodulin Affinity Chromatography—**For Calmodulin agarose binding assays, MDCK cells expressing wild-type or constructs were grown on 24-mm Transwell filters (0.4-μm pore size, Costar Corp., Cambridge, MA) metabolically labeled at 37 °C for 40 min on an 80-μl drop of Dulbecco’s modified Eagle’s medium lacking cysteine (obtained from the UCSF cell culture facility, supplemented with 5% dialyzed fetal bovine serum) containing 25 μCi of [35S]lysine (Du Pont NEN). The Transwell filters were washed three times with phosphate-buffered saline containing CaCl2 and Mg2+, and filters were cut out and placed in 1 ml of ice-cold solubilization buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, containing 1% Triton X-114 (Boehringer Mann-
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125I-CaM (prepared by the iodine monochloride method; Ref. 37), was incubated with crude membranes in HNMC for 1 h at 4°C in the presence or absence of 3 mM EGTA. BS4 was added to a final concentration of 0.5 mM, and the incubation was continued for 1 h. Samples were quenched with glycine, boiled in SDS, and immunoprecipitated and analyzed as above.

RESULTS

Identification of a Rat Liver Endosome CaM-binding Protein as pIgR—CaM has been suggested to play several possible roles in membrane traffic in mammalian cells. We and others have examined the effects of inhibitors of CaM, such as W13 and W7, on various steps in membrane traffic (24, 25). In MDCK cells expressing plgR, these drugs inhibited transcytosis of plgR and recycling of transferrin, and caused all material endocytosed from both surfaces of the cell to be delivered to exceptionally large, abnormal endosomal structures (25), suggesting a role for CaM in polarized sorting in early endosomes. However, the mechanism(s) by which CaM regulates membrane traffic events is poorly understood.

In order to address the mechanism(s) by which CaM regulates endosomal function, we were interested in identifying major CaM-binding proteins in endosomes. Previous studies showed a major CaM-binding protein of approximately 115 kDa could be demonstrated by 125I-CaM overlay of two-dimensional protein blots of an endosome-rich fraction from rat liver (38). We discovered that this protein can also be labeled by staining with a monoclonal antibody (SC166) against the polymeric immunoglobulin receptor (plgR) (Fig. 1). Similar results were obtained with a rabbit antiserum raised against rat SC (data not shown).

Characterization of CaM Binding to plgR—In order to further support the identification of plgR as the CaM-binding protein, and to map and characterize the CaM binding site on plgR, we developed a CaM-binding assay as described under “Materials and Methods.” MDCK cells expressing the plgR were metabolically labeled and solubilized with Triton X-114. Hydrophobic, integral membrane proteins partition into the detergent phase during Triton X-114 phase separation induced by warming to 37°C. Surprisingly, when such a preparation is added to CaM-agarose in the presence of Ca2+, followed by washing to remove nonspecific binding, plgR is the major protein detected after the CaM beads are eluted with SDS and subjected to SDS-PAGE and fluorography (Fig. 2A). This result is striking when one considers that plgR is only a minor protein in the starting material (Fig. 2A), and that there are numerous CaM-binding proteins that might be present in the Triton X-114-partitioned material. It is similarly striking that plgR is the major CaM-binding protein detected in the rat liver membrane fraction (Fig. 1).

Binding of plgR to CaM-agarose is inhibited by addition of EGTA (Fig. 2A), excess CaM (Fig. 2B), or the CaM antagonist W-13 (N-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide; data not shown), further supporting the specificity of binding. If CaM binding to plgR is physiologic, then we would expect the CaM binding site to be on the cytoplasmic domain of plgR. To test this, we used a plgR construct encoding the extracellular and transmembrane domains of plgR, but lacking virtually all of the cytoplasmic tail (plgR R655-STOP). As shown in Fig. 3, this “tail-minus” construct shows no detectable binding to CaM. We used other mutants to localize the site of CaM binding on the plgR cytoplasmic tail (Fig. 3). Remarkably, a mutant lacking the membrane-proximal 14-amino acid segment of the cytoplasmic tail required for basolateral targeting (Δ655-668) also fails to bind CaM. Conversely, a mutant in which the cytoplasmic domain consists only of the membrane-proximal 17-amino acid basolateral targeting signal (plgR T670-STOP) exhibits CaM binding indistinguishable from that of full-length plgR.
Chemical Cross-linking of CaM to pIgR—CaM binding to pIgR was also demonstrated using chemical cross-linking. Metabolically labeled, Triton X-114-solubilized and partitioned material was incubated with the non-reversible cross-linker, BS3, in the presence of CaM. Samples were boiled in SDS, and pIgR was immunoprecipitated and analyzed by SDS-PAGE. As shown in Fig. 4, a significant fraction of wild-type pIgR is converted to a slower migrating species. This species is not observed if CaM, cross-linker, or free Ca\(^{2+}\) are omitted from the assay. Furthermore, the apparent M\(_r\) of the cross-linked complex is consistent with that expected for a stoichiometric pIgR-CaM complex (≈125 kDa). Specificity of the cross-linking as a CaM binding assay is further supported by the fact that mutant pIgRs lacking either the entire cytoplasmic tail (pIgR R655-STOP) or 14 amino acids of the basolateral targeting signal (D\(_{655–668}\)) could not be cross-linked to CaM (Fig. 4). These results suggest that pIgR binds directly to CaM and that it can bind to soluble CaM as well as CaM-agarose.

In order to determine if CaM can bind to pIgR that is still embedded in the membrane, we performed cross-linking experiments using crude MDCK membranes containing unlabeled pIgR. Radiolabeled CaM is incorporated into a complex of 125 kDa in a Ca\(^{2+}\)-dependent manner with membranes containing wild-type pIgR, but not with pIgR \(\Delta655–668\) (Fig. 5) or cells not expressing pIgR (data not shown). These results attest to the likelihood of physiologic binding of pIgR to CaM in intact cells.

CaM Binding to pIgR Point Mutants Does Not Correlate with Their Basolateral Targeting Phenotype—CaM binding to the basolateral targeting signal suggested that it might play a role in pIgR targeting. In order to determine whether there is a correlation between CaM binding and basolateral targeting, we performed CaM-binding assays on many of our pIgR clones that contain alanine point mutations in the basolateral targeting signal. Typical data from these assays are shown in Fig. 6. One mutant that is not basolaterally targeted shows reduced binding to CaM (R657A), but two others that are not basolat-

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**Fig. 1.** Identification of pIgR as the major CaM-binding protein on blots of two-dimensional gels from rat liver endosomes. An RRC endosome fraction from rat liver was subjected to two-dimensional electrophoresis and stained with Coomassie Blue (A), or transferred to nitrocellulose filters and probed with \(^{125}\)I-CaM (B) or a monoclonal antibody (SC 166) against pIgR (C). Numbers indicate major polypeptides: 1, unidentified protein found in RRC, MVB, and CURL; 2, 5'-nucleotidase; 3, group of polypeptides, including annexin VI, present in all endosome and plasma membrane fractions analyzed; 4, pIgR. The M\(_r\) and pH positions are indicated.

**Fig. 2.** Binding of pIgR to CaM-agarose. CaM-agarose beads were incubated under various conditions with Triton X-114 preparations of metabolically labeled proteins from pIgR-transfected MDCK cells as described under "Materials and Methods." After washing, the beads were boiled in SDS sample buffer and the eluate was analyzed by SDS-PAGE. A, Ca\(^{2+}\) dependence of CaM binding. The \(^{35}\)S-labeled proteins in the Triton X-114 fraction added to CaM beads are shown (starting material). The amount of pIgR in the starting material was analyzed by immunoprecipitation and SDS-PAGE (IP). The total \(^{35}\)S-labeled proteins bound to CaM-agarose after washing in buffer containing Ca\(^{2+}\) or EGTA are shown. Note that pIgR is the major protein detected on the CaM-agarose beads. Molecular weight standards are shown at left. B, proteins bound to CaM-agarose in the absence or presence of 1 \(\mu\)M free CaM, as indicated.
erally targeted (H656A and V660A) exhibit CaM binding that is comparable to wild-type pIgR. R658A, which is basolaterally targeted as accurately as the wild-type pIgR, does not bind well to CaM, while another mutant that is basolaterally targeted (N659A) does bind CaM. Thus, CaM binding does not correlate with the basolateral targeting phenotype of pIgR mutants.

CaM binding of several proteins is known to be modulated by phosphorylation (39). The pIgR has a major phosphorylation site at Ser-664 in the basolateral targeting signal, and phosphorylation of this residue stimulates transcytosis of pIgR (7, 8), presumably by inactivating the basolateraltargeting signal (5). We therefore assayed the ability of a non-phosphorylatable mutant, S664A, and a mutant that mimics the presence of a phosphate residue, S664D, to bind to CaM. Both of these mutants bind to CaM as well as wild-type pIgR (data not shown).

Thus phosphorylation of Ser-664 probably does not alter CaM binding.

DISCUSSION

CaM binds to and regulates the function of a wide variety of proteins. In most cases this binding occurs only when CaM is "activated" after binding Ca$^{2+}$. Because the affinity of CaM for Ca$^{2+}$ ($10^{-6}$ M) is above the resting [Ca$^{2+}$] ($10^{-7}$ M), CaM is a sensor for transient increases in [Ca$^{2+}$], binding to target proteins only when [Ca$^{2+}$] is elevated (39). CaM has been suggested to play several possible roles in membrane traffic in mammalian cells. Endocytosis in neurons is dependent on the phosphorylation state of dynamin (40), which in turn is a substrate for calcineurin (phosphatase 2B) (41), a phosphatase that is regulated by CaM binding. Cyclosporin A, an immune
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**Calmodulin Binds to pIgR Point Mutants Does Not Correlate with Their Basolateral Targeting Phenotype.**

Calmodulin (CaM) binds to pIgR point mutants, in which alanine is substituted at the indicated positions in the context of full-length pIgR, as analyzed as for Fig. 2A. Lanes showing an immunoprecipitation of pIgR from the starting material (IP) and 35S proteins bound to CaM-agarose in the presence of Ca2+ or EGTA are indicated. The percentage of each construct bound to CaM-agarose, relative to wild-type, and basolateral targeting phenotype (*, determined previously; Ref. 4) of each mutant: (+) normal basolateral targeting; (−), disrupted basolateral targeting, are indicated at right. Values represent the mean of two experiments (see “Materials and Methods” for details).

| 17-amino acid basolateral targeting signal | CaM-agarose binding assay | % bound to CaM relative to wild-type | basolateral targeting * |
|------------------------------------------|--------------------------|-------------------------------------|------------------------|
| Wild-Type                                | RARHHRRRDVRVSIGSYR       | (100)                               | +                      |
| H656A                                    | RARHHRRRDVRVSIGSYK       | 130                                 | −                      |
| R657A                                    | RARHHRHDVRVSIGSYK        | 11                                  | −                      |
| R658A                                    | RARHHRHDVRVSIGSYK        | 10                                  | +                      |
| N659A                                    | RARHHRHDVRVSIGSYK        | 83                                  | +                      |
| V660A                                    | RARHHRNDVRVSIGSYK        | 86                                  | −                      |

**Signaling by dIgA Binding to the pIgR or Other Extracellular Signals.**

Suppressor that functions by binding calcineurin, inhibits Ca2+/CaM-dependent secretion from pancreatic acinar cells, thereby implicating calmodulin/calcineurin in regulation of this secretion (42). More generally, [Ca2+]i, is clearly important in many intracellular membrane traffic events, including both regulated exocytosis of granules (43) and synaptic vesicles (44), as well as classically “constitutive” processes, such as endocytic events at Golgi transport (45) and nuclear envelope fusion (46) (at least in in vitro systems). It is not known if [Ca2+]i acts on CaM and/or some other target in these events.

We have found that CaM binds to the basolateral targeting signal of the pIgR, making CaM the first identified protein shown to bind specifically to a basolateral sorting signal. Binding is strictly dependent on Ca2+. The pIgR is the major CaM-binding protein detected in membranes of a highly purified rat liver endosome fraction, and is also the major CaM-binding protein detected in a Triton X-114 detergent phase extract from total metabolically labeled MDCK cells that are transfected with pIgR. These striking results suggest that the interaction of CaM and pIgR is of high specificity. We also detected the CaM-pIgR interaction by cross-linking, both in detergent extracts and, more significantly, in non-solubilized crude membranes prepared from MDCK cells. That CaM can bind to pIgR that is still in its native membrane makes it quite likely that under the appropriate in vivo conditions of elevated [Ca2+]i, CaM should bind to pIgR. Capturing the interaction of CaM with pIgR in intact cells may be quite different, as elevations of [Ca2+]i are generally very transient and spatially localized (47).

Using a series of mutant pIgRs expressed in MDCK cells, we mapped the CaM binding site to the membrane-proximal 17-amino acids of the cytoplasmic domain of the pIgR. This same segment has previously been shown to be necessary and sufficient to direct the pIgR to the basolateral surface from either the TGN or the endocytic pathway (2). However, an analysis of Ala point mutants in this segment indicates that there is not a precise correlation between the residues needed for basolateral sorting and those needed for CaM binding. The sequence of this 17-residue segment is generally comparable with other known CaM binding sites. CaM often binds to a sequence with a preponderance of basic residues near the N terminus, and hydrophobic residues more concentrated near the C terminus (39). This is largely true of this 17-residue segment of the pIgR. It is remarkable that this short region of the cytoplasmic domain of the pIgR therefore has at least two separable functions, basolateral targeting and CaM binding. This segment also has a third function, as a rather weak signal for endocytosis, centered around tyrosine 668 (48). Based on our knowledge of how sorting occurs in other systems, it seems likely that a specific protein complex recognizes the basolateral sorting signal. This complex would be functionally and perhaps structurally homologous to the adaptor proteins found in clathrin-coated vesicles, and to the coatamer involved in the early portion of the biosynthetic pathway. A likely candidate for a component of the coat involved in basolateral sorting is the p200 protein found in the TGN (49). Such complexes presumably recognize specific signals on the cytoplasmic domains of integral membrane proteins. In the case of the AP2/HAD2 adaptor found in the plasma membrane, the signal contains a type 1 β-turn (50–53), perhaps somewhat similar to the type 1 β-turn that is an essential part of the basolateral signal of the pIgR (4). In contrast, CaM generally does not function as an adaptor, i.e., it does not recognize a feature on one protein, and then bind to another protein. Rather, when CaM binds to a site on a protein, a common consequence is that it simply masks that site, preventing it from interacting with anything else (39). We speculate that one possible role of CaM is to sequester the basolateral signal on the pIgR. In the absence of elevated [Ca2+]i, CaM would not bind to the pIgR. The hypothetical complex that recognizes the basolateral signal would bind to the signal and direct the pIgR to the basolateral surface. However, when [Ca2+]i is elevated, perhaps due to the binding of dIgA to the pIgR or to another hormone signaling event, CaM would bind to pIgR and thereby prevent the hypothetical complex from binding. By masking the basolateral signal, the pIgR would be allowed to be transcytosed to the apical surface.

In pancreatic epithelial cells, the elevation of [Ca2+]i, in response to extracellular signals is greatest in the most apical region of the cell (54). With strong stimulation a wave of elevated [Ca2+]i, can then propagate through the cell. It seems likely that in MDCK cells elevation of [Ca2+]i would similarly be concentrated in the most apical region of the cytoplasm. As described in the Introduction, the last known stage of pIgR transcytosis (i.e., step 3) is movement from the apical recycling compartment (located immediately beneath the apical plasma membrane) to the apical surface. It is possible that CaM binds primarily to the pIgR in this compartment and is involved in stimulating this step of transcytosis. Consistent with this hypothesis, we have observed that this is the step of transcytosis that is stimulated by dIgA binding (22), as well as by artificially raising [Ca2+]i. This localized increase in [Ca2+]i could avoid the binding of CaM to pIgR in other locations, e.g., the TGN, which might lead to inappropriate delivery of pIgR to the apical surface. Exocytosis of synaptic vesicles at nerve terminals represents an extreme example of how a highly localized increase in [Ca2+]i leads to membrane traffic at a precise location.
signals leads to activation of several second messenger pathways, which may redundantly stimulate transcytosis. In particular, artificial elevation of \([\text{Ca}^{2+}]\) stimulates apical transcytosis of several molecules (e.g. transferrin), although these effects are in general smaller than those observed with pIgR. The molecular mechanisms of these effects are unknown. We suggest that the specific binding of CaM to pIgR provides an additional regulatory mechanism, which amplifies the stimulation of transcytosis of pIgR.

CaM binding to the pIgR could have another, non-mutually exclusive role in regulation of pIgR traffic. When IgA binds to pIgR, the pIgR initiates a signaling cascade involving tyrosine phosphorylation. Preliminary data indicate that mutations in the 17-residue basolateral targeting signal of the pIgR prevent this signaling, suggesting that this segment has an additional role in signal transduction. Perhaps CaM binding to the pIgR serves to sequester this segment and thereby is part of a negative feedback loop that shuts off signaling by the pIgR.

Taken together our data indicate that the CaM binds to a portion of the pIgR that has multiple functions in regulation of polarized traffic. It will be interesting to learn if other receptors bind to CaM and if this is involved in regulation of their traffic by \([\text{Ca}^{2+}]\).

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