35H, a Sequence Isolated as a Protein Kinase C Binding Protein, Is a Novel Member of the Adducin Family*

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We recently cloned a partial cDNA (35H) for a protein kinase C (PKC) binding protein from a rat kidney cDNA library and demonstrated that it is a PKC substrate in vitro (Chapline, C., Ramsay, K., Klauck, T., and J aken, S. (1993) J. Biol. Chem. 268, 6858–6861). Additional library screening and 5′ rapid amplification of cDNA ends were used to obtain the complete open reading frame. Amino acid sequence analysis, DNA sequence analysis, and Northern analysis indicate that 35H is a unique cDNA related to α- and β-adducins. Antisera prepared to the 35H bacterial fusion protein recognized two polypeptides of 80 and 90 kDa on immunoblots of kidney homogenates and cultured renal proximal tubule epithelial cell extracts. The 35H-related proteins were similar to α- and β-adducins in that they were preferentially recovered in the Triton X-100-insoluble (cytoskeletal, CSK) fraction of cell extracts and were predominantly localized to cell borders. Phorbol esters stimulated phosphorylation of CSK 35H proteins, thus emphasizing that sequences isolated according to PKC binding activity in vitro are also PKC substrates in vivo. The phosphorylated forms of the 35H proteins were preferentially recovered in the soluble fraction, thus demonstrating that phosphorylation regulates their CSK association and, thereby, their function in regulating cytoskeletal assemblies.

We have isolated another PKC binding protein partial cDNA (clone 45) from a rat fibroblast library with substantial homology to α-adducin. Antiserum raised against this expressed sequence recognized a protein of 120 kDa, the reported size of α-adducin, on immunoblots of renal proximal tubule epithelial cell extracts. A 120-kDa protein that cross-reacts with the clone 45 (α-adducin) antisera coprecipitated with 35H immunocomplexes, indicating that α-adducin associates with 35H proteins in vivo. Taken together, these results indicate that 35H is a new, widely expressed form of adducin capable of forming heterodimers with α-adducin. We propose naming this adducin homologue γ-adducin.

Protein kinase Cs are a heterogeneous group of phospholipid-dependent kinases important for cell growth and differentiated functions (reviewed in Ref. 1). The family can be divided into three categories based on enzymatic properties. The conventional or Group A PKCs are calcium-dependent kinases whose activities are stimulated by diacylglycerol or phorbol esters. The novel or Group B PKCs are calcium-independent but still diacylglycerol-stimulatable. The atypical or Group C PKCs are calcium- and diacylglycerol-independent. Most cells express more than one type of PKC, which implies that PKCs have unique rather than overlapping functions.

Activation of Group A and B PKCs is regulated by receptor-mediated production of diacylglycerol through phospholipase C or D pathways (1). In many cases, activation correlates with PKC redistribution from soluble to particulate fractions. However, in other cases, evidence for activation in the absence of measurable translocation has been noted (2–5). The correlation between translocation and activation is further confused by the fact that PKC inhibitors such as staurosporine can also cause PKC redistribution (6, 7). Thus, redistribution does not necessarily reflect PKC activation under all conditions.

An alternative method for studying PKC activation is to monitor the phosphorylation state of PKC substrates. For example, phosphorylation of MARCKS, a major PKC substrate in many cells, has been useful for demonstrating PKC activation in response to a variety of physiological agonists (8). Our goal has been to identify a panel of high affinity substrates for PKCs that can be used as reporter systems to monitor PKC activity in response to external stimuli and in pathological processes. To this end, we developed a blot overlay assay to identify PKC binding proteins. In subsequent studies, we demonstrated that MARCKS and other PKC substrates interact with PKCs in this in vitro assay (9, 10) and suggested that, in general, binding proteins are also substrates. The overlay assay is based on a phospholipid-dependent, high affinity interaction between PKCs and recognition sites on other proteins (substrates) (11). With slight modifications, the assay can also be used in an interaction cloning strategy to isolate expressed cDNAs for PKC substrates (12).

In our original report on interaction cloning of PKC substrates (12), we identified two PKC binding sequences: MARCKS-related protein (clone 35A) and a unique partial cDNA sequence with homology to adducins (clone 35H). Both were shown to be PKC substrates in vitro. In the present study, we report the complete coding sequence for 35H, identify it as a novel form of adducin, and establish that it is also a PKC binding protein.

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†Abbreviations used are: PKC, protein kinase C; CSK, cytoskeleton or cytoskeletal; MARCKS, myristoylated alanine-rich protein kinase C substrate; MSB, microtubule stabilization buffer; PDBu, phorbol 12,13-dibutyrate; RPTE, renal proximal tubule epithelial cells; PIPES, 1,4-piperazinediethanesulfonic acid; kb, kilobase pair(s); PS, phosphatidyliner.
substrate in vivo. These results underscore the utility of the PKC interaction cloning approach for identifying novel PKC substrates.

**Experimental Procedures**

**Materials**—Male Fisher 344 rats weighing 150–175 g were obtained from Taconic Farms (Germantown, NY). Collagen type I was purchased from Collaborative Biomedical Products (Bedford, MA). Phosphate-free Dulbecco's modified Eagle's medium, penicillin G (Formalin-fixed Staph A cells), and streptomycin were from Life Technologies, Inc. [3H]leucine, Ham's F12 medium, and fetal bovine serum were from Life Technologies, Inc. Immunoprecipitation reagents and alkaline phosphatase-conjugated goat anti-rabbit IgG was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Aqua-PolyMount was from Polysciences, Inc. (Warrington, PA).

**Cell Culture—Primary RTPE cultures were prepared from rat kidney proximal tubules and were grown on collagen type I coated plastic culture dishes as described (13, 14).**

**5′ Rapid Amplification of cDNA Ends (RACE)**—A partial cDNA containing the 3′ end of the 35H sequence was originally isolated from a rat kidney oligo(dT)-primed cDNA library by PKC interaction cloning (12). Since Northern analysis indicated substantial message expressed in brain, a rat brain random primed cDNA library (Clontech) was screened with the 35H partial cDNA clone in order to isolate more 5′ regions of the open reading frame. This screen produced a clone that overlapped the original kidney clone but included no additional 5′ sequence. The sequence of the brain clone was identical to that of the kidney clone. Two rounds of 5′ rapid amplification of cDNA ends from rat kidney mRNA were performed on 800 base pairs of additional 5′ sequence. These sequences were also isolated from a REF52 cell library using two rounds of 5′ polymerase chain reaction. A third round of 5′ polymerase chain reactions from either the kidney or the REF52 library or 5′ rapid amplification of cDNA ends reactions with kidney or REF52 mRNA did not yield products with additional 35H 5′ sequence. However, 5′ polymerase chain reaction from the rat brain library (Clontech) did produce an overlapping product, which included the 5′ end of the 35H sequence.

**Preparation of Anti-35H Antisera—**35H cDNA was inserted in frame into a pQE bacterial expression vector (QIAGEN; Chatsworth, CA) to produce a recombinant protein containing 6 histidine residues at the N terminus of the 35H sequence. These sequences demonstrate that rat 35H shares substantial homology with the 35H and the human 35H sequences indicates that species differences alone do not account for the difference between 35H and the human adducin sequences.

**Preparation of Clone 45 Fusion Protein and Antisera—**Clone 45 is another partial cDNA isolated from a REF52 cell library on the basis of PKC binding activity as described (12). Clone 45 is a 935-base pair fragment with 80% homology to human α-adducin, suggesting that clone 45 is the rat homologue of human α-adducin. Clone 45 cDNA was inserted into the pQE vector and expressed as the 6X His-tagged fusion protein as described above. Antisera were prepared to clone 45 fusion protein and immunopurified against the bacterially expressed fusion protein.

**Preparation of Cell Lysates and Kidney Homogenates for Immunoblot Analysis—**Cell lysates were prepared as described (24). Freshly isolated rat kidney cortex was homogenized in homogenization buffer (20 mM Tris-Cl, pH 7.4, 0.25 M sucrose, 5 mM EDTA, 1 mM dithiotreitol, 10 μM leupeptin, 10 μM aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Protein concentrations were determined according to the method of Bradford (15) using reagents from Bio-Rad. Immunoblot analysis was performed as described (24).

**Preparation of Triton X-100 Soluble and Cytoskeletal Fractions—**Cells were washed twice with microtubule stabilization buffer (MSB; 0.1% PIPES-NaOH, pH 6.9, 2 mM glycerol, 1 mM EDTA, and 1 mM magnesium acetate) (16). Soluble proteins were extracted with 0.2% Triton X-100 in MSB containing 10 μg/ml leupeptin and 10 μg/ml aprotinin for 4 min at room temperature. Triton X-100 insoluble proteins, referred to as the CSK fraction, were washed with MSB. For immunoprecipitation and immunoprecipitation, CSKs were scraped into MSB containing leupeptin and aprotinin and sonicated.

**Metabolic Labeling—**Cells grown on 35-mm dishes were washed with phosphate-free Dulbecco's modified Eagle's medium three times, labeled with 100 μCi/ml [3H]leucine (in phosphate-free Dulbecco's modified Eagle's medium with supplements as described under "Cell Culture") for 4 h at 37°C. During the final labeling period, cells were treated with PDBu for the indicated times. When PDBu treatment was longer than 4 h, the [3H]leucine was added during the final 4 h. After labeling, cells were washed twice with phosphate-buffered saline and scraped into 1 ml of radioimmunoprecipitation assay buffer, which was 50 mM Tris-Cl, pH 7.4, 0.15 M NaCl, 1 mM EDTA, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 50 mM sodium fluoride, and 1 mM sodium vanadate. For soluble and CSK preparations, cells were washed twice with MSB after labeling, and the soluble and CSK fractions were prepared in 0.8 ml of MSB as described above. Samples were adjusted to 1 × radioimmunoprecipitation assay buffer for immunoprecipitation.

**Immunoprecipitation of 35H—**[3H]labeled samples (1 ml), prepared as described above, were preincubated by incubating with 150 μl of formalin-fixed Staph A cells (10%, w/v) for 1 h. Preincubated supernatants were collected and incubated with 10 μl of anti-35H antiserum or preimmune serum for 1 h. Protein A-Sepharose (40 μl, 50%, w/v) was added, and the incubation continued for another hour. All incubations were performed at 4°C on a shaker. Immunocomplexes were collected by centrifugation and washed twice in radioimmunoprecipitation assay buffer twice in radioimmunoprecipitation assay buffer once, in low salt buffer (50 mM HEPES, pH 7.4, 0.15 M NaCl, 1 mM EDTA, and 0.1% Nonidet P-40), once in high salt buffer (50 mM HEPES, pH 7.4, 0.5% NaCl, 1 mM EDTA, and 0.1% Nonidet P-40), and once in low salt buffer again. Immunoprecipitated proteins were eluted from protein A-Sepharose by boiling for 5 min in Laemmli buffer (17) and then separated on 7.5% denaturing polyacrylamide gels. Immunoprecipitated proteins were visualized by autoradiography. In some experiments, immunoprecipitates from non-radioactive cells were collected in parallel and blotted to nitrocellulose.

**Immunocytofluorescence—**Cells were grown on 12-mm glass cover slips as described under "Cell Culture." Whole cell and CSK preparations were fixed with 3.7% formaldehyde in phosphate-buffered saline (whole cell) or in MSB (CSK) for 10 min at room temperature and permeabilized with absolute methanol for 6 min at 0–2°C (18). The coverslips were blocked with 1% bovine serum albumin in phosphate-buffered saline for 30 min and stained sequentially with 10 μg/ml affinity-purified anti-35H antibody for 1 h and with fluorescein-conjugated goat anti-rabbit IgG for 1 h. The coverslips were mounted with Aqua-PolyMount onto slides and observed under a Nikon fluorescence microscope with a 60 × objective lens.

**Northern Blot Analysis—**Blots containing 2.5 μg of poly(A) mRNA from multiple tissues were purchased from Clontech. Random labeled cDNA probes were prepared with [32P]dCTP. Prehybridization conditions were 3 h at 42°C in 5 × SSPE and 2 × Denhardt's in the presence of 100 μg/ml denatured salmon sperm DNA, 2% SDS, and 50% formamide. Blots were hybridized for 48 h at 42°C in the prehybridization solution, washed three times in 2 × SSC containing 0.1% SDS at room temperature, and washed three more times in 0.1 SSC containing 0.1% SDS at 50°C. Films were exposed for 6–7 days.

**RESULTS**

**Relationship of 35H to Adducins—**The original clone 35H, which is a partial cDNA from a rat kidney library, had substantial homology to the 3′ end of human brain β-adducin in defined areas (12). However, since the sequences diverged significantly in other areas, the extent of the relationship to β-adducin could not be determined. Both library screening and 5′ rapid amplification of cDNA ends were used to obtain the entire 35H coding sequence. Alignments of the full-length 35H translated sequence with human α- and β-adducins demonstrate that although 35H shares substantial homology with α- and β-adducins, it is a unique sequence (Fig. 1). Overall, rat 35H is 50–60% homologous to human α- and β-adducins. In contrast, rat and human β-adducin sequences are nearly identical (90% homology with conservative substitutions except for the highly variable C terminus, which is truncated in the reported rat sequence (19)). The high degree of homology between the rat and human β-adducin sequences indicates that species differences alone do not account for the difference between 35H and the human adducin sequences.
In previous studies, alignments of human \( \alpha \)-adducin with human and rat \( \beta \)-adducins revealed that the adducins share a homologous N-terminal domain followed by a highly divergent region near the C terminus and a highly conserved polybasic region at the extreme C terminus (residues 696–726 in \( \beta \)-adducin) (19, 20). This overall pattern is also apparent in the 35H alignment. Within the relatively conserved N-terminal domains, divergent regions are separated by stretches with near identity and conservative substitutions (Fig. 1, shaded regions). In general, there is a good correlation in spacing of the conserved and divergent domains within \( \alpha \)-adducin, \( \beta \)-adducin, and 35H sequences. This alignment suggests that adducins are a family of proteins with constant domains separated at defined intervals by isoform-specific sequences, and furthermore that 35H is a newly identified member of the adducin family.

35H shares homology to \( \beta \)-but not \( \alpha \)-adducin in two of the variable regions (437–447 and 472–484; numbers refer to \( \beta \)-adducin residues shaded in Fig. 1). On the other hand, 35H shares homology to \( \alpha \)-adducin in other variable regions (8–15, 19–27, 85–92; numbers refer to \( \alpha \)-adducin residues shaded in Fig. 1). These results emphasize that 35H is a unique sequence distinct from previously identified forms of adducin. Both \( \alpha \)-adducin and 35H diverge significantly from the \( \beta \)-adducin putative calmodulin binding domain (446–454) (21). Diversity in this sequence suggests a potentially significant difference in calmodulin regulation of 35H and \( \beta \)-adducin functions. The conserved high positive charge density domains in the extreme C termini are identical with the exception of the change of a potential phosphorylatable Ser to Asn in 35H. 35H does not contain Tyr316 of \( \alpha \)-adducin or Arg529 of \( \beta \)-adducin, which have been identified as a potential tyrosine kinase phosphorylation site or a determinant of CAMP-dependent kinase phosphorylation, respectively (22). Mutations in these sites have been linked to hypertension.

**Tissue Distribution**—Northern analysis was used to compare the tissue distribution of 35H with \( \alpha \)- and \( \beta \)-adducins. Previous studies have shown that \( \alpha \)-adducin probes hybridized to a 4.0-kb mRNA with wide tissue distribution, whereas \( \beta \)-adducin probes hybridized to an 8.1-kb mRNA in brain, which was not detectable in liver and kidney (20). Three smaller messages (3.0–4.2 kb) were detected in spleen, kidney, and heart (19, 20). In contrast, 35H probes hybridized to a 4.5-kb message in all rat and human tissues examined (Fig. 2), although message was less abundant in lung and liver. A second, less prominent band at 7.5 kb was also detected in spleen, kidney, and testis. In summary, differences in the primary amino acid sequence, message size, and tissue distribution clearly distinguish 35H from \( \alpha \) and \( \beta \)-adducins.

Expression of 35H Protein in Primary RPTE and Rat Kidney—Antiserum raised against the bacterially expressed 35H fusion protein was used to study 35H-related proteins in cultured RPTE and kidney extracts. Affinity-purified anti-35H antibody reacted strongly with an 80-kDa protein in cell lysates of primary growing RPTE (Fig. 3A). A weaker band at 90 kDa was also apparent. The 80- and 90-kDa immunoreactive proteins were also detected in kidney homogenates, although the 90-kDa band was more intense relative to RPTE. Neither the 80- nor the 90-kDa proteins were detected by preimmune serum (data not shown). The reason for the relative increase in 90-kDa 35H in whole kidney homogenates is not known; however, since kidney contains many cell types in addition to proximal tubules, it is possible that different kidney cell types express different forms of 35H. Thus, two immunoreactive forms of 35H are expressed in kidney, and the 80-kDa form is preferentially expressed in the cultured RPTE. Because of their cross-reactivity with affinity-purified antisera to the 35H fusion protein, they will be referred to as 35H proteins.

\( \alpha \)-Adducin Is a PKC Binding Protein—The interaction cloning strategy was also used to screen a rat fibroblast \( \lambda \)gt11 library for PKC binding proteins. One product of this screen is a partial cDNA (clone 45) with a predicted translated sequence 77% identical to the carboxyl terminus of human \( \alpha \)-adducin (Fig. 4). The sequences diverge significantly only in the C
and represents a co-immunoprecipitating protein (see below). As shown in Fig. 3. The 120-kDa band was not detected on blots. Bands correspond to immunoreactive proteins detected on blots. The 90-kDa band to a lesser extent (2–3-fold). The 80- and 90-kDa bands were detected (as quantitated by scanning densitometry), and into the 80- and 120-kDa bands approximately 6-fold (terminus) in proteins precipitated by 35H antisera was determined (Fig. 5). In the absence of PDBu-treatment, three phosphoproteins of approximately 80, 90, and 120 kDa were specifically immunoprecipitated. PDBu increased phosphate incorporation into proteins precipitated by 35H antiserum and transferred to nitrocellulose paper. Blots were stained with affinity-purified anti-35H antibody (A) or with affinity-purified anti-45 antibody (B) at a concentration of 1 μg/ml. Results are representative of more than five experiments.

Fig. 3. Immunoblot analysis of renal 35H and α-adducin (clone 45). Homogenates were prepared from primary growing renal proximal tubule epithelial cells (lane 1) and rat kidney tissue (lane 2). Aliquots (100 μg of protein) were separated on denaturing polyacrylamide gels and transferred to nitrocellulose paper. Blots were stained with affinity-purified anti-35H antibody (A) or with affinity-purified anti-45 antibody (B) at a concentration of 1 μg/ml. Results are representative of more than five experiments.

Terminus, the region where rat and human β-adducin sequences diverge from each other (19). Antisera prepared to the expressed bacterial fusion protein and immunopurified against the cognate protein recognized a protein of 120 kDa, the reported size of α-adducin, in homogenates of cultured renal cells or tissue (Fig. 3B). The sequence identity and size similarity indicate that clone 45 is the rat homologue of human α-adducin.

Phosphorylation of 35H in Primary Growing RPTES—Previous studies demonstrated that the bacterially expressed 35H protein could be phosphorylated by purified PKC in vitro (12). To determine if 35H proteins were PKC substrates in intact cells, primary growing RPTES were prelabelled with orthophosphate, and the effect of PDBu on stimulating phosphate incorporation into proteins precipitated by 35H antiserum was determined (Fig. 5). In the absence of PDBu-treatment, three phosphoproteins of approximately 80, 90, and 120 kDa were specifically immunoprecipitated. PDBu increased phosphate incorporation into the 80- and 120-kDa bands approximately 6-fold (as quantitated by scanning densitometry), and into the 90-kDa band to a lesser extent (2–3-fold). The 80- and 90-kDa bands correspond to immunoreactive proteins detected on blots as shown in Fig. 3. The 120-kDa band was not detected on blots and represents a co-immunoprecipitating protein (see below).

Since β-adducin is known to form heterocomplexes with 120-kDa α-adducin (23), we hypothesized that the 120-kDa protein coprecipitating with 35H antibodies may be α-adducin (our clone 45). To determine if α-adducin was present in the 35H immunocomplexes, 35H immunoprecipitates of cell lysates were prepared and blotted to nitrocellulose. Antibodies to 35H recognized the 80- and 90-kDa 35H in the immunoprecipitates but did not recognize the 120-kDa phosphoprotein apparent on the autorads (Fig. 6A). In contrast, affinity-purified antibodies to clone 45 (α-adducin) fusion protein recognized a 120-kDa band in the 35H immunoprecipitates (Fig. 6B). Since antibody to recombinant clone 45 protein does not recognize recombinant clone 35H protein and vice versa (Fig. 7), these results strongly suggest that α-adducin is not directly precipitated with 35H antisera but that it coprecipitates with the 35H proteins.

Regulation of 35H Cytoskeletal Association by Phosphorylation—The distribution of 35H proteins between Triton X-100-soluble and insoluble (CSK) fractions in resting and PDBu-stimulated cultures was analyzed by immunoblotting. Densitometric analysis indicated that in resting cultures, approximately 80% of the total 80- and 90-kDa proteins were recovered in the CSK fraction (Fig. 8). Activation of PKC by PDBu treatment increased soluble 35H proteins approximately 2-fold. It is likely that the increase in soluble 35H is due to redistribution from the CSK pool, although a corresponding decrease in the CSK 80- and 90-kDa proteins was more difficult to detect. These results suggest that only a small fraction of the total CSK 35H is redistributed to the soluble fraction with PDBu stimulation. After 16 h of PDBu treatment, which partially down-modulates PKC in these cells (24), the distributions of both 80- and 90-kDa proteins between soluble and CSK fractions were similar to resting cultures.

PDBu-mediated changes in CSK 35H were more apparent by immunocytofluorescence than by immunoblot. In resting primary RPTES, 35H staining was concentrated in cell-cell junctions in detergent-extracted (CSK) preparations (Fig. 9). PDBu rapidly caused a decline in 35H staining concentrated at cell borders and an increase in staining in cytoplasmic dots (Fig. 9B and C). The effect of PDBu was attenuated with prolonged PDBu treatment, which down-modulates PKC (Fig. 9D). Parallel studies with whole cell preparations indicated that the intensity and distribution of 35H staining were similar in CSK and whole cell preparations of resting cells (data not shown), consistent with the nearly quantitative recovery of 35H in the CSK fraction on immunoblots (Fig. 8). In whole cells, PDBu caused an increase in diffuse cytosolic staining and a decrease in cell border staining (data not shown) indicative of a redistribution from CSK to soluble fractions.

The increase in soluble 35H (Fig. 8) and the decrease and reorganization of CSK 35H (Fig. 9) observed after PDBu treat-
ment suggested that phosphorylation may regulate the distribution of 35H proteins between soluble and CSK compartment. To test the hypothesis, we immunoprecipitated 35H proteins from soluble and CSK fraction of 32P-labeled primary growing RPTE (Fig. 10). Basal phosphorylation levels of 35H proteins in both soluble and CSK fractions were low. PDBu stimulated phosphorylation of 80- and 90-kDa 35H proteins; these phosphoproteins were preferentially recovered in the soluble fraction. Increased phosphorylation of the coprecipitating 120-kDa protein was also limited to the soluble pool. PDBu did not stimulate phosphate incorporation into the CSK pool of these proteins. Thus, although 35H protein mass was preferentially recovered in the CSK fraction as demonstrated by immunoblots (Fig. 8), phosphorylated forms were preferentially recovered in the soluble fraction (Fig. 10). Phosphorylation of 80- and 90-kDa 35H was attenuated after 4–14 h of PDBu treatment, which correlates with the time course for PKC down-modulation in these cells (25). To ensure proper quantitation of blots, we compared antibody recognition of native and phosphorylated 35H. 35H bacterial fusion protein was phosphorylated with purified PKC in vitro (12). 35H antibody detected both native and phosphorylated forms with the same intensity on immunoblots (data not shown). Thus, the increase in soluble 35H proteins in response to PDBu treatment cannot be attributed to increased antibody recognition of the phosphorylated form. Furthermore, we verified that 35H proteins were efficiently immunoprecipitated from both the soluble and CSK fractions (Fig. 11). Thus, pre-
isolated according to PKC binding activity are substrates for in vitro phosphorylation. Our original goal was to determine if sequences isolated according to PKC binding activity are substrates for in vitro phosphorylation.

Several lines of evidence demonstrate that 35H is related to previously reported adducins. Like \( \alpha \) - and \( \beta \)-adducins, 35H proteins are insoluble in Triton X-100 and concentrated at cell-cell junctions, and they are PKC substrates (23, 26). As previously reported for \( \alpha \)-adducin (26), phosphorylation of 35H proteins correlated with their redistribution from CSK to soluble fractions. Furthermore, \( \alpha \)-adducin (clone 45) co-immunoprecipitates with 35H, indicating that 35H proteins associate with \( \alpha \)-adducin. Other evidence emphasizes that despite the similarity to adducins, 35H is a unique sequence. The alignment shown in Fig. 1 indicates that 35H diverges significantly from \( \alpha \) - and \( \beta \)-adducins in discrete regions. Northern analysis demonstrates that the size and tissue distribution of 35H message are distinct from \( \alpha \) - and \( \beta \)-adducins. Taken together, these data demonstrate that 35H is a new member of the adducin family. We propose renaming it as \( \gamma \)-adducin. Since \( \alpha \) - and \( \beta \)-adducins are known to form heterodimers, the structure and organization of adducins in cells and tissues that express \( \alpha \)-adducin but not \( \beta \)-adducin (e.g. kidney (20)) have been questioned. It seems likely that 35H proteins (or other as yet unidentified adducins) could fulfill the role of \( \beta \)-adducin in forming heterodimers with \( \alpha \)-adducin in these tissues.

**FIG. 11. Immuno precipitation of soluble and cytoskeletal 35H.** Soluble (SOL) and cytoskeletal (CSK) fractions were prepared from primary growing RPTE. Fractions were immunoprecipitated with pre-immune serum (p.i.) or 35H antiserum. Immune complexes and proportional aliquots of starting material (total) were separated on denaturing polyacrylamide gels, transferred to nitrocellulose, and stained with affinity-purified anti-35H antibody. Similar results were obtained in one additional experiment.

**Fig. 12.** Phosphorylation regulates ternary complex formation among PKCs, binding proteins, and phospholipid surfaces. PKC binding to substrates is dependent on the interactions of both PKC and the substrate with an anionic phospholipid surface such as PS (solid ribbon). PKC interactions with PS are mediated through PS binding sequences within the regulatory (Reg) domain, whereas substrate interactions with PS are mediated through electrostatic interactions with high positive charge density sequences (++) . The model is drawn to indicate that the substrates also interact with the catalytic (Cat) domain of PKC (in order for phosphorylation to occur). Phosphorylation coordinately attenuates the interaction between the substrate and both PS and PKC. In the case of MARCKS and 35H, decreased PS binding in vitro correlates with redistribution of the phosphorylated proteins from particulate to soluble fractions in vivo.

**DISCUSSION**

We have used a PKC overlay assay to detect PKC binding proteins and demonstrated a close correlation between PKC binding proteins (detected by blot overlay) and PKC substrates (detected by in vitro and in vivo phosphorylation assays) (9–11). We have also used the overlay assay in an interaction cloning strategy to isolate cDNAs for PKC binding proteins (12). Many, if not all, of the expressed cDNAs are PKC substrates in vitro. In the present studies, we have focused on clone 35H to determine if proteins corresponding to the cDNAs isolated according to PKC binding activity are substrates in vivo. Antibodies prepared to 35H recognize 80- and 90-kDa PKC substrates in RPTE cells, thus providing additional evidence that PKC binding proteins are PKC substrates. Furthermore, PKC phosphorylation regulated the CSK association of 35H (and clone 45) proteins. Thus, phosphorylation has demonstrable consequences on the function of these proteins, which, by analogy to other adducins, involves the regulation of actin assembly in the subcortical CSK.

Several known PKC substrates were isolated in our initial library screens for PKC binding proteins including MARCKS-related protein (clone 35A), \( \alpha \)-adducin (clone 45), annexin I, and annexin II. The fact that 35H, a unique sequence isolated according to PKC binding activity, also appears to be a PKC substrate in vivo further confirms this conclusion. Additional studies are in progress to determine if PKC is directly responsible for the PDBu-stimulated 35H phosphorylation and if other kinases also participate in 35H phosphorylation. In other studies, we have determined that two other unique binding protein sequences (clones 72 and 35F) are PKC substrates in vitro and in vivo (data not shown). MARCKS, a major PKC substrate in many cells, is also a PKC binding protein (9). Thus, there is a strong correlation between binding proteins detected in the overlay assay and PKC substrates. It should be noted that in addition to their roles as downstream targets and effectors of PKC activation, the apparent high affinity between PKC and the binding proteins also allows for the possibility that these sequences target PKCs to specific subcellular addresses in vivo, although we do not yet have direct evidence for this.

Each of the binding proteins we have tested binds phosphatidylserine (PS), and phosphorylation coordinately decreases PS
and PKC binding (9, 11, 12). Thus, PS bridging is an important component of PKC binding, and as described previously, it is also an important component for efficient trans-phosphorylation (27). Thus, in our working model (Fig. 12), we propose that PKC and binding proteins are brought into close proximity due to their common affinity for PS. Since not all PS binding proteins are PKC binding proteins, additional protein-protein interactions appear to be required for formation of the high affinity ternary complex (28). The model is drawn to indicate contact sites with the catalytic domain since the binding proteins are also substrates. PKC interactions with PS have been mapped to the regulatory domain, whereas MARCKS interactions with PS are due to electrostatic interactions with the high positive charge density domain containing the PKC phosphorylation site (8, 29, 30).

In addition to regulating PKC binding and phosphorylation, PS binding also regulates the association of MARCKS with cellular membranes (29, 31). In MARCKS and 35H2, phosphorylated serine residues are located within high positive charge density domains that support electrostatic interactions with the negatively charged lipid surface. Thus, phosphorylation results in a coordinate loss of both PKC and PS binding. Dissociation of PKC from the binding protein represents a means for regulating the association of PKC with its binding proteins/substrates (Fig. 12). Loss of PS binding correlates with redistribution of binding proteins (such as MARCKS and adducins) from particulate to soluble fractions. This redistribution may be the means by which PKC alters the functions of the CSK-associated proteins and exerts the profound changes in cell shape elicited by phorbol ester treatment.

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