Interaction Cloning of Protein Kinase C Substrates

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We have previously used an overlay assay technique to detect proteins that interact with protein kinase C (PKC) (Hyatt, S. L., Klauck, T., and Jaken, S. (1990) Mol. Carcinogenesis 3, 45-53). In some cases, binding proteins were also identified as substrates. Therefore, we used the overlay assay approach to screen a rat kidney Xgt11 cDNA library to isolate and identify ad- toylated alanine-rich C kinase substrate (MARCKS)-serine (PS) and are substrates for PKC. Phosphorylation that will be useful in identifying isozyme-specific additional PKC substrates. Two clones have now been characterized. 35A is the rat homologue of the myristo- toylated alanine-rich C kinase substrate (MARCKS)-related F52 cDNA, whereas 35H is a partial cDNA with substantial homology to the 3' end of 3'-adducin. Both cDNAs encode proteins that bind phosphatidylserine (PS) and are substrates for PKC. Phosphorylation decreased both PS and PKC binding activities. Both proteins contain high density positive charge do- mains similar to that found in the major PKC substrate MARCKS. These results demonstrate that PKC inter- actions with certain substrate proteins are of suffi- ciently high affinity to facilitate their isolation via interaction cloning.

Protein kinase C (PKC)1 is a family of phospholipid-de- pendent kinases that are involved in regulation of growth, differentiation, and carcinogenesis (1, 2). Group A or conven- tional PKCs are calcium-dependent, whereas Group B or novel PKCs are calcium-independent (3). The reasons for PKC heterogeneity are not yet clearly identified, although differences in activation requirements, substrate specificities, subcellular locations, and down-modulation have all been noted. Our goal has been to identify targets of PKC phos- phorylation that will be useful in identifying isozyme-specific functions.

In normal REF52 fibroblasts, α-PKC is concentrated in the cytoskeleton at sites of cell-substratum adhesion known as focal contacts (4). α-PKC is also found in focal contacts of primary rat renal proximal tubule epithelial cells, and in addition, in cell-cell adhesion sites (5). We reasoned that α- PKC may be targeted to these specific locations via interac- tions with other proteins, and we have used an overlay assay approach to identify PKC-binding proteins in these cells (6, 7). Two lines of evidence demonstrated that the proteins identified by the assay are biologically interesting. First, the two major binding proteins in REF52 cells (Mr, 71 and >200 kDa) are not detected in SV40-transformed REFS2 cells (6). SV40-REF52 cells form less stable substrate attachment sites (known as close contacts), which do not contain α-PKC (6). Thus, there is a correlation between loss of α-PKC-binding proteins and loss of targeting to the cytoskeleton. Second, comparison of the properties of REF52 cell PKC-binding proteins and PKC substrates indicated that two of the major binding proteins are also substrates.2 (a) The two major binding proteins comigrated with the major PKC substrates on two-dimensional gels; (b) both of the binding proteins and the substrates were heat soluble; and (c) both of the binding proteins and substrates were not detected in extracts of SV40- REF52 cells. In other work, we have demonstrated that the major PKC substrate, MARCKS, is also detected as a PKC- binding protein in this assay.3 These results indicated that at least some of the binding proteins are also PKC substrates.

Based on these data, which demonstrated the potential utility of the assay, we used the overlay assay to screen an expression library in order to identify additional PKC-binding proteins and substrates.

Experimental Procedures

Materials—The rat kidney Xgt11 library was purchased from Clon- tech (Palo Alto, CA). pBluescript II SK was from Stratagene (La Jolla, CA). pQEIV was from Qiagen (Chatsworth, CA). PKC antibod- ies were from Upstate Biotechnologies (Lake Placid, NY). [35S]PS (50 mCi/mmol) was from Amerham Corp. Sequence homologies were identified in the GenBank data bank using software from Intelli- genetics.

Library Screening and Isolation of Clones—cDNA clones for PKC- binding proteins were isolated by screening a rat kidney Xgt11 library using a PKC overlay assay similar to the method of Wolf and Sayhoun (9) with modifications (7). IPTG-induced proteins were immobilized on nitrocellulose lifts. Briefly, the nitrocellulose was blocked with 5% nonfat milk, and then incubated with 10 pg/ml PKC partially purified from rabbit brain (10). Incubations were at room temperature in 50 m M Tris-C1 (pH 7.4) containing 0.5 M sodium chloride (TBS) with the following additions: 10 mg/ml bovine serum albumin, 20 ng/ ml PS, 1 mM EGTA, 1.2 mM calcium, 10 ng/ml leupeptin, and 1 ng/ ml aprotinin. After washing, bound PKC was fixed by incubating with 0.5% formaldehyde. Excess formaldehyde was inactivated by washing with 2% glycine. After washing with phosphate-buffered saline, the nitrocellulose sheets were incubated with anti-α-PKC monoclonal antibody M6 (11) diluted in TBS containing 10 mg/ml bovine serum albumin. Positive clones were identified after incubat- ing with alkaline phosphatase-conjugated second antibody and color development. Positive clones were plaque-purified, and the α DNA was recovered. The inserts were excised with EcoRI and gel-purified. The clones were inserted into pBluescript II SK for sequencing. Sequencing was performed on a model 370A automated sequenator (Applied Biosystems). Inserts were excised from SK and subcloned into pQE Type IV in the appropriate reading frames to produce poly(His) fusion proteins. Where indicated, the poly(His) fusion proteins were purified by nickel affinity chromatography in 8 M urea buffer with 10 mM Tris-C1 and 100 mM phosphate (pH 8.0) accord- ing to the manufacturer's instructions (Qiagen, Chatsworth, CA).

1 The abbreviations used are: PKC, protein kinase C; PS, phosphatidylserine; MARCKS, myristoylated alanine-rich C kinase substrate; bp, base pair(s); IPTG, isopropyl-thio-β-D-galactopyranoside.

2 S. Hyatt and S. Jaken, manuscript in preparation.

3 S. Hyatt, A. Aderem, A. Nairn, and S. Jaken, S., submitted for publication.
Phosphorylation Studies — α-PKC was produced in Sf9 cells infected with recombinant α-PKC baculovirus (12). α-PKC was partially purified from cell homogenates following chromatography on DEAE-Sepharose and phenyl-TSK essentially as described (12). Samples were incubated in phosphorylation assay buffer (12), which was 50 mM Tris-Cl (pH 7.4) containing 1 mM dithiothreitol, 100 μg/ml phosphatidylserine, 5 mM magnesium chloride, 1 mM EGTA, and 1.2 mM calcium in the presence or absence of 25 μM ATP.

Phosphatidyliner Overlay Assay—Native or phosphorylated 35A and 35H fusion proteins were blotted to nitrocellulose. The nitrocellulose was overlaid with 20 pg/ml [14C]PS (specific activity 2-4 mCi/pmol) diluted in 50 mM Tris-Cl (pH 7.4) containing 0.5 M sodium chloride, 10 mg/ml bovine serum albumin, 1 mM EGTA, and 1.2 mM calcium for 1 h. Blots were washed briefly in phosphate-buffered saline, dried, and exposed to film for 2–4 days. Calcium requirements for PS binding to 35A and 35H have not been studied in detail. In related experiments, however, we have determined that PS binding to MARCKS does not require calcium.

RESULTS

Nitrocellulose lifts from a rat kidney λgt11 expression library were incubated with partially purified PKC in the presence of the PKC cofactors phosphatidyliner (PS) and calcium. After washing, bound PKC was detected with anti-α-PKC specific monoclonal antibodies. Out of 150,000 colonies screened, nine positive clones were detected. Upon subsequent screening, four were found to directly interact with the PKC antibodies and, therefore, do not represent binding proteins. Two apparent binding protein clones, 35A and 35H, were chosen for further analysis.

To demonstrate that the clones isolated actually code for PKC-binding proteins, clones 35A and 35H were expressed as bacterial fusion proteins. In the absence of IPTG induction, PKC binding to a small number of bacterial cell proteins was noted (Fig. 1). In the presence of IPTG, binding protein activities were induced. The induced proteins did not directly react with the anti-PKC antibodies (Fig. 1). Thus, although there is some background due to PKC interactions with endogenous bacterial cell proteins, this did not prevent identification and isolation of PKC-binding protein clones.

Clone 35A is a 1.5-kilobase pair sequence containing an open reading frame of 594 bp. DNA sequence analysis of clone 35A demonstrated >90% homology to mouse F52 (Mac-MARCKS), which is a recently reported sequence that shares significant homology to the major PKC substrate, MARCKS (13–15). The translated 35A sequence was 93% homologous to mouse F52 protein (Fig. 2). Both mouse F52 and 35A are homologous to MARCKS at the NH2-terminal myristoylation site and the internal PKC phosphorylation sites (16–19). The homology between mouse F52 and rat 35A included the entire F52 coding region and continued into the 3' non-coding region (data not shown). Sequence homology in the first 400 bases of the 3' non-coding region was 92%. The strong homology in both coding and non-coding regions indicates that clone 35A is the rat homologue of mouse F52.

Clone 35H is a 767-bp sequence containing the 35H coding region and continued into the 3' non-coding region (data not shown). Sequence homology in the first 400 bases of the 3' non-coding region was 92%. The strong homology in both coding and non-coding regions indicates that clone 35H is the rat homologue of mouse F52.

Fig. 1. Clones 35A and 35H encode PKC-binding proteins. Escherichia coli containing either the 35A or 35H plasmids were grown with or without IPTG for 4 h. Cell lysates were collected, and proteins were separated on denaturing polyacylamide gels and transferred to nitrocellulose. Parallel lanes were incubated either with the anti-α-PKC antibody M6 alone or with PKC and then M6 essentially as described for the overlay assay protocol for library screening. Asterisk (*) indicates the inducible PKC-binding proteins.
phorylation sites in the basic COOH-terminal domain of β-adducin was not homologous to the intervening P-adducin sequence except for serine 718. Sequence homology between P-adducin and ε-adducin, which is homologous, both have very high positive charge densities (+13/25 amino acids for MARCKS, F52, 35A, and +12/32 amino acids for β-adducin and 35H). The presence of high positive charge density regions in MARCKS, 35A, and 35H suggests that these regions are important in mediating PKC binding activity.

Previous work has demonstrated that PKC-binding proteins are also phosphatidylinositol (PS)-binding proteins; however, not all PS-binding proteins bind PKC efficiently (7, 9, 23). These results emphasize the potential importance of phospholipid bridging in mediating PKC interactions with other proteins but also suggest that protein-protein interactions may provide additional stabilization. [3H]PS overlays were used to determine if the proteins expressed by clones 35A and 35H bound PS. Binding was detectable with as little as 30 ng (~1 pmol) of protein (Fig. 5). Phosphorylation decreased both PS binding and PKC binding (Fig. 5A and B). Thus, the PKC interactions are strictly regulated by phosphorylation. These results also suggest that the PS binding and PKC phosphorylation sites are in close proximity.

**DISCUSSION**

Two PKC substrates have been cloned using an overlay assay to screen an expression library for PKC-binding proteins. Thus, the overlay assay represents a novel approach for cloning and identifying PKC substrates. Comparison of the sequences of the isolated clones with MARCKS, which is also a PKC-binding protein, suggests that highly basic domains are common among PKC-binding proteins. In MARCKS, this basic domain has been associated with several functions including actin, calmodulin, and phosphatidylinositol binding (19, 24–26). It is likely that these domains function in electrostatic interactions with PS, and that PS binding to PKC is an important component of the interactions detected by this method, as originally suggested (9). However, since the assay is performed in the presence of 0.5 M sodium chloride and since not all PS-binding proteins are good PKC-binding proteins (7), protein-protein interactions may also play a role. It is relevant to point out that several PKC substrates have been shown to interact directly with PS (27, 28). Overall, the data suggest that PS-dependent protein-protein interactions are important in determining PKC phosphorylation targets. Using a similar assay system, others have described a distinct domain in annexin I and p65 that appears to interact with activated PKCs (29, 30). The substrates that we have cloned with this overlay assay approach are distinct from the low molecular mass (~30 kDa) receptors for activated C kinase (RACKS) recently described (23). Thus, it is possible that

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several types of PKC-binding proteins with distinct functional domains will eventually be identified.

An important factor in considering what may be the primary targets for PKC phosphorylation in vivo is the subcellular localization of PKCs. In principle, location could regulate accessibility to substrate proteins (discussed in Ref. 31). Although it is interesting to note that α-PKC colocalizes with vinculin in focal contacts of REF52 cells (4, 6), and with 35H in cell-cell junctions of renal proximal tubule epithelial cells, we do not yet know if α-PKC interactions with these proteins target α-PKC to these locations. Additional studies describing the interactions between the phosphorylated and unphosphorylated forms of 35A and 35H with PKC in vitro and in vivo are required to fully address this problem.

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