Identification of a Major Protein Kinase C-binding Protein and Substrate in Rat Embryo Fibroblasts

DECREASED EXPRESSION IN TRANSFORMED CELLS*

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We have used an interaction cloning strategy to isolate cDNAs for sequences that interact with protein kinase C (Chapline, C., Ramsay, K., Klauck, T., and J. Jaken, S. (1993) J. Biol. Chem. 268, 6858–6861). In this paper, we report a novel sequence, clone 72, isolated according to this method. Clone 72 has a 4.8-kilobase pair open reading frame; antibodies to clone 72 recognize a >200-kDa protein in cell and tissue extracts. Clone 72 message and protein are detected in a variety of tissues. Immunoprecipitation studies demonstrate that clone 72 is the major >200-kDa binding protein described previously in REF52 fibroblasts (Hyatt, S. L., Liao, L., Aderem, A., Nairn, A., and J. Jaken, S. (1994) Cell Growth & Differentiation 5, 495-502). Expression of clone 72 message and protein are decreased in progressively transformed REF52 cells. Since clone 72 is both a protein kinase C (PKC)-binding protein and substrate, decreased levels of clone 72 may influence both the subcellular location of endogenous PKCs as well as signaling events associated with clone 72 phosphorylation. Our results emphasize that the role of PKCs in carcinogenesis may involve several factors, including the quantity and location of the PKC isoforms and their downstream targets.

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† The abbreviations used are: PKC, protein kinase C; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s); MARCKS, myristoylated alanine-rich C kinase substrate.
solution, washed three times in 2 × SSC containing 0.1% SDS at room temperature, and three more times in 0.1 × SSC containing 0.1% SDS at 50°C. Films shown were exposed for 1–2 days.

Immunoblots—Growth and properties of REF52 cells have been described previously (19, 20). Cell lysates and tissue homogenates were prepared as described (16). Aliquots (20–50 µg of protein) were separated by SDS-polyacrylamide gel electrophoresis and blotted to nitrocellulose. Blots were probed with affinity-purified clone 72 antisera as described (16). The PKC overlay assay is described in Ref. 13.

Immunofluorescence—REF52 cells were grown on glass coverslips. Cells were fixed in formaldehyde, permeabilized in methanol, and incubated with first and second antibodies as described previously (21).

Phosphorylation of Clones 53 and 72 Expressed Sequences—Purified recombinant clone 72ORIG expressed sequence (40 and 80 µg, respectively) was incubated with PKC purified from rabbit brains (22) (4 and 8 µg, respectively) in the presence of calcium, phosphatidylserine, and radiolabeled ATP as described previously (22). The phosphorylated proteins were digested with V8 protease overnight at 37°C. Peptides were separated on a C8 reversed phase column with an acetonitrile gradient in the presence of 0.01% trifluoroacetic acid. Phosphopeptides were separated by reversed phase HPLC, and phosphopeptides were identified by liquid scintillation counting.

RESULTS

Isolation of Clone 72 Open Reading Frame—In order to identify PKC-binding proteins and substrates that mediate PKC action in cells, a lgt11 cDNA library prepared from REF52 cells was screened for PKC interacting proteins as described previously (19). Two clones isolated during this screen, clones 53 and 72, were overlapping (Fig. 1). Additional 5′ sequence was obtained by 5′-RACE using primers to the clone 53 sequence and by screening a rat brain library with a clone 53 cDNA probe. Both of these approaches led to identification of the apparent 5′ end of the coding sequence. An open reading frame with a start methionine and a consensus Kozak sequence begins at position 34. Additional 3′ sequence was obtained by PCR screening the REF52 library with clone 72 primers. A summary of the individual clones obtained with these methods is shown in Fig. 1. The nucleic acid and predicted amino acid sequences are shown in Fig. 2. Database searches indicated clone 72 had substantial homology to the recently reported clone 322 (23); however, clone 72 had an additional ~1000 bp of open reading frame in the 5′ region and ~400 bp less in the 3′ region due to the stop codon at position 4821. Several differences in primary sequence were also noted. No overall homologies to other nucleotides or amino acid sequences were detected. However, a search for domain similarities indicated significant homology with two well characterized PKC substrates, neuromodulin and MARCKS. Residues 286–319 have significant homology to the PKC phosphorylation site in neuromodulin (and MARCKS) (24–26). Two other peptides (148–178 and 502–532) also have significant homology to the PKC phosphorylation site in MARCKS. In addition to the phosphorylation domain homology, four peptides (residues 221–263, 317–359, 404–446, 614–656) have significant homology to an acidic domain in neuromodulin (residues 120–162 in rat neuromodulin). Repeats of these highly acidic peptides (more than 30% acidic residues) are likely to be important for clone 72 structure.

Expression of Recombinant Clone 72—The original clone 72 partial cDNA was expressed in bacteria as a recombinant His-tagged fusion protein. The recombinant protein had an apparent molecular mass of 97 kDa, which is significantly larger than the predicted molecular mass of 44 kDa (Fig. 3). In general, anomalously slow migration on denaturing gels is characteristic of PKC-binding proteins (data not shown), including MARCKS (16), and may reflect an extended, rod-like structure. Antiserum raised to the purified recombinant protein recognized the expressed sequence and furthermore recognized a high molecular mass (>200 kDa) protein in REF52 cell extracts (Fig. 3). The antisera immunoprecipitated a >200-kDa protein from REF52 cell extracts which reacted with affinity-purified clone 72 antibody on immunoblots (Fig. 4A). The clone 72 antibody immunoprecipitates also contained a >200-kDa PKC-binding protein detected by PKC overlays (Fig. 4B). Thus, the cDNA isolated according to PKC binding activity in the interaction cloning assay appears to code for a PKC-binding protein expressed in REF52 cells.

Tissue Distribution of Clone 72—The relative expression of clone 72 in various tissues was compared by Northern and Western blots. The major form of the detectable message was 6 kb and was abundantly expressed in testes, heart, skin, and brain (Fig. 5A). Smaller message sizes were also detected in skin, liver, and heart. Since identical results were obtained with blots probed with clone 72 and clone 53 cDNA probes (data not shown), these bands appear to be specific and most likely represent message species related to clone 72.

Immunoblots of rat tissues demonstrated a ladder of immunoreactive proteins >200 kDa in most tissues (Fig. 5B). Expression levels were highest in testes and brain. Smaller bands that may be related either to the smaller, related messages or to proteolysis were also detected in most tissues. Thus, clone 72 encodes a high molecular weight PKC-binding protein that is widely expressed in mammalian tissues.

Clone 72 Is a PKC Substrate—Clones 72ORIG and 53ORIG expressed sequences were purified and used in vitro kinase assays with PKC. After phosphorylation in the presence of radiolabeled ATP, the proteins were digested with V8 protease, peptides were separated by reversed phase HPLC, and phosphopeptides were identified by liquid scintillation counting.

Only one V8 peptide from clone 72ORIG contained phosphate; this was identified as 494R(35)E529 by automatic Edman degradation (underlined in Fig. 2). A synthetic peptide corresponding to 496V(33)E529 was also phosphorylated by PKC in vitro with a stoichiometry of 0.72. A second V8 peptide in 53ORIG was also phosphorylated. Further digestion with chymotrypsin indicated that the phosphorylation site was within the peptide 296R(13)D308 (underlined in Fig. 2). Both of these sequences were identified in the domain search as regions homologous to the phosphorylation motifs in neuromodulin and/or myristoylated alanine-rich C kinase substrates (see above). These results are consistent with previous studies that established a strong correlation between PKC-binding proteins and substrates (16).

Clone 72 Expression Is Transformation-sensitive—REF52 (REFA) cells are the parental cell line for progressively transformed SV40 derivatives, REF52, and REFD. REF52 cells...
are morphologically transformed, REFC cells are capable of growth in soft agar, and REFD cells are fully transformed as demonstrated by their ability to produce tumors in nude mice (12, 13, 16). Northern blots demonstrated the progressive loss of PKC-binding proteins in transformed cells. The primary sequence of clone 72 and the consensus sequence of the cDNA clones and PCR products were assembled from the contig map shown in Fig. 1. The translated sequence of the open reading frame (34-4824 bp) is also shown. Position of the peptides in clone 72 ORIG and 53 ORIG that were phosphorylated by PKC are underlined.

Fig. 2. Primary sequence of clone 72. Consensus sequence of the cDNA clones and PCR products was assembled from the contig map shown in Fig. 1. The translated sequence of the open reading frame (34-4824 bp) is also shown. Position of the peptides in clone 72 ORIG and 53 ORIG that were phosphorylated by PKC are underlined.
of clone 72 message with progressive transformation of REF52 cells (Fig. 6). Immunoblots also indicate decreased expression of clone 72 protein with progressive transformation of REF52 cells (Fig. 7A). Loss of immunoreactive clone 72 correlates with the progressive loss of the major >200-kDa PKC-binding protein with progressive transformation of REF52 cells (Fig. 7A). Decreased expression of clone 72 in transformed cells appears to be general rather than specific for SV40 transformation,
since clone 72 protein was not detected in ras-transformed REF52 cells.

**DISCUSSION**

We have used the PKC overlay assay to identify PKC-binding proteins in cell extracts and to isolate cDNAs for these binding proteins. Clone 72 is a novel sequence isolated from a REF52 cell expression library according to its PKC binding properties. Considerable effort was required to isolate the entire open reading frame of this large protein. Clone 72 may be identical to a recently reported sequence, clone 322, which appears to be missing approximately 1000 bp of coding sequence in the 5' end (23). Other differences between the reported clone 322 and clone 72 sequences may be attributed to differences between manual and automated sequencing. The translated sequence does not share significant homology with other proteins in the data bases.

Northern analysis with clone 53 and clone 72 cDNA probes indicate that clone 72 has a broad tissue distribution. These data were substantiated by immunoblot analysis of various tissues with affinity-purified antibody to the clone 72 expressed sequence. Previous studies with the related sequence, clone 322, did not detect a similar tissue distribution profile. The reasons for these discrepancies are not clear at this time. Both studies identified abundant expression of the 6-kb transcript in testes and skin, whereas abundant expression in heart was detected only in this study. The presence of additional mes-

2 I. Gelman, personal communication.
was phosphorylated by PKC. In agreement with this, we found that clone 72 was phosphorylated by PKC directly due to PKC. Taken together, these data show that phosphorylation of clone 72 in REF52 cells (data not shown), further studies are required to establish that the phosphorylation is directly due to PKC. Further studies are required to determine if the differential localization is due to differential expression of PKC-binding proteins in the normal and transformed cells. In principle, inappropriate localization could lead to promiscuous phosphorylation by PKCs and consequently contribute to disordered signaling and loss of growth control.

In summary, our results underscore that understanding the role of PKC signaling in transformation requires analysis not only of the expression of the PKC isozymes and their activation in response to exogenous stimuli, but also analysis of the expression of the downstream targets that mediate the effects of PKC activation on cellular functions, including growth, differentiation, and gene expression.

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