p32 (gC1qBP) Is a General Protein Kinase C (PKC)-binding Protein

INTERACTION AND CELLULAR LOCALIZATION OF P32-PKC COMPLEXES IN RAT HEPATOCYTES*†

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The aim of this study was to identify cellular proteins that bind protein kinase C (PKC) and may influence its activity and its localization. A 32-kDa PKC-binding protein was purified to homogeneity from the Triton X-100-insoluble fraction obtained from hepatocytes homogenates. The protein was identified by NH₂-terminal amino acid sequencing as the previously described mature form of p32 (gC1qR). Recombinant p32 was expressed as a glutathione S-transferase fusion protein, affinity-purified, and tested for an in vitro interaction with PKC using an overlay assay approach. All PKC isoforms expressed in rat hepatocytes interacted in vitro with p32, but the binding dependence on PKC activators was different for each one. Whereas PKCδ only binds to p32 in the presence of PKC activators, PKCe and PKCd increase their binding when they are in the activated form. Other PKC isoforms such as β, ε, and θ bind equally well to p32 regardless of the presence of PKC activators, and PKCμ binds even better in their absence. It was also found that p32 is not a substrate for any of the PKC isoforms tested, but interestingly, its presence had a stimulatory effect (2-fold for PKCδ) on PKC activity. We also observed in vivo interaction between PKC and p32 by immunofluorescence and confocal microscopy. A time course of phorbol ester treatment of cultured rat hepatocytes (C9 cells) showed that PKCθ and p32 are constitutively associated in vivo, whereas PKCδ activation is required for its association with p32. Our data also showed that phorbol ester treatment induces a transient translocation of p32 from the cytoplasm to the cell nucleus. Together, these findings suggest that p32 may be a regulator of PKC location and function.

Protein kinase C (PKC) † comprises a large family of serine/threonine kinases that play key regulatory roles in growth, differentiation, cell survival, neurotransmission and carcinogenesis (1). Based on structural characteristics that determine differences in their lipid and calcium binding properties, PKC are divided into three groups: conventional, novel, and atypical PKC (2). These differences do not, however, adequately account for the isozyme specificity observed in vivo, because overlapping specificity of these kinases is commonly observed in vitro. On the other hand, the study of PKC function is complicated by the existence of many isoforms within the same cellular type that redistribute from soluble to membrane-bound intracellular compartments as a result of their activation (3–5).

Recent observations have revealed protein kinase-anchoring and scaffold proteins as essential elements in cell signaling (6–8). Given the dynamic nature of protein phosphorylation reactions, coordinated control of both kinases and phosphatases is often required. Cells seem to have solved this problem by utilizing anchoring proteins that bind to subcellular structures and localize their complement of enzymes/adaptor proteins close to their sites of action. Indeed, genetic, biochemical, and cell-based screens have revealed a heterogeneous group of PKC-binding proteins that can influence its subcellular localization, substrate availability, exposure to allosteric activators, and activation-dependent relocation within cells. This group includes the following: substrate-binding proteins that interact with PKC prior to phosphorylation (9); cytoskeletal/vesicle interacting proteins (10–12); AKAPs, named so as a result of their original discovery as cAMP-dependent (protein kinase A)-anchoring proteins (13); and a class of proteins referred to as RACKs (receptors for activated C-kinase) (14–16).

Using overlay assays, we have identified in rat hepatocytes several proteins that interact with PKC (16, 17) and purified some of them from the Triton X-100-insoluble fraction of these cells to apparent homogeneity (18). In the current work, we purified a 32-kDa PKC-interacting glycoprotein and identified it as the previously described receptor for the globular “heads” of complement component C1q (gC1q-R) (19). Originally, p32 was characterized as a component of the AS/SF2 splicing activity purified from HeLa cells (20). Subsequent studies have shown that the molecule interacts with several cellular and viral proteins that participate in mitochondrial function (21, 22), transcription, and splicing factor modulation (23, 24). Since the original study, it has become apparent that p32 is an evolutionarily conserved eukaryotic protein. Homologous genes have been identified in a number of eukaryotic species ranging from fungi to mammals. Recently, the interaction of p32 with methylrhodamine B isothiocyanate; BSA, bovine serum albumin; IPTG, isopropyl-1-thio-β-D-galactopyranoside; GST, glutathione S-transferase; TPA, 12-O-tetradecanoylphorbol-13-acetate.
PKCµ has been reported (25). In the B cell line SKW 6.4, p32 appears to function as a compartment-specific negative regulator of the kinase activity of PKCµ. Here we present evidence that p32 interacts in vitro with all PKC isoforms expressed in rat hepatocytes and stimulates PKC activity. We also show that PKCδ and p32 are constitutively associated in vivo and that treatment of intact cells with phorbol ester promotes p32-PKCδ in vivo interaction and a transient redistribution of p32 to the cell nucleus.

**EXPERIMENTAL PROCEDURES**

**Materials and Antibodies**—Phorbol 12-myristate 13-acetate, histone H1-I-IIS, phosphatidylserine, Triton X-100 and 1,2-diolein were from Sigma. Protein A-Sepharose and Ro 31–8220 were from Calbiochem, and DEAE-cellulose (DE-52) was from Whatman. Goat anti-rabbit and TRITC-conjugated goat anti-mouse antibodies were from Zymed Laboratories Inc. Conjugated goat anti-rabbit and alkaline phosphatase.

**Detection of Carbohydrates in Purified p32**—Purified p32 was transferred to a nitrocellulose membrane with a model LF3000 protein sequencer (Beckman) using chemicals and software supplied by the manufacturer.

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**Cloning of p32 cDNA**—A clone encoding p32 (gC1qBP), derived from a mouse embryonic cDNA library, was found by searching the EST (expressed sequence tag) database. This plasmid was purchased from Research Genetics Inc., and the nucleotide sequence of its insert was determined by the chain termination method (28). For the construction of the GST-p32 fusion protein, the 618-bp fragment resulting from EcoRI (SI nuclease trimmed) and DraI digestion of the p32 cDNA was subcloned at the SmaI site of the pGEX2T vector (Amersham Bio- sciences, Inc.). The proper orientation of the subcloned insert was verified digested, and the insert in-frame insertion was verified by nucleotide sequencing. Expression of the GST-p32 fusion protein was done in the protease-deficient BL21 strain of Escherichia coli (Novagen, Madison, WI), and its affinity purification was performed as described by Smith and Corcoran (29).

**Partial Purification of PKC**—PKC was purified as described previously (30). In brief, hepatocytes were homogenized in ice-cold buffer (20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.5 mM Triton X-100, 50 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 0.1 mg/ml trypsin inhibitor), incubated at 4 °C for 30 min, and centrifuged. Portions of the supernatant were applied to DEAE-cellulose columns that had been equilibrated with column buffer (20 mM Tris-HCl, pH 7.5, and 50 mM 2-mercaptoethanol) at 4 °C. After washing with column buffer, total PKC was eluted with column buffer containing 0.25 M NaCl, 2 mM EDTA, and 0.1 mg/ml trypsin inhibitor. The eluate was concentrated with an Amicon device (YM-30 membrane) and used for overlay assays.

**Determination of PKC Binding Activity by an Overlay Assay**—PKC binding was determined as described previously (16). Briefly, sample protein was separated by SDS-PAGE (10% gels) and transferred to poly(vinylidene difluoride) membranes. Detection of PKC activity was carried out on poly(vinylidene difluoride) membranes and subjected to NH2-terminal sequence analysis. The sequence LHTEGDKAFVEFLTDEIKEE was obtained with a model LF3000 protein sequencer (Beckman) using chemicals and software supplied by the manufacturer.
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Cloning and Expression of p32 as GST Fusion Protein—p32 cDNA was obtained from a clone derived from a mouse embryonic cDNA library (Research Genetics Inc). As Fig. 2A shows, the nucleotide sequence of this cDNA contains a complete reading frame encoding a 279-residue protein that shows minor discrepancies with the one reported by Lynch et al. (33). Differences occur at residue 11 (Ala instead of Ser) and at residue 160 (Leu instead of Arg). Additionally, our sequence has three alamines (residues 14–16), instead of only two as reported by Lynch et al. (33) at the same position. The “mature” form of the protein corresponding to residues 74–279 presumably is generated by site-specific cleavage and removal of the highly basic 73-residue-long NH₂-terminal segment during post-translational processing.

The 618-bp fragment resulting from the EcoRI (SI nuclease trimmed) and DraI digestion of the plasmid containing p32 cDNA was subcloned in the pGEX2T vector. The resulting GST-fusion protein includes the 49.3-kDa marker in SDS-PAGE in accordance with the expected mass calculated from the GST moiety (27.5 kDa) and the encoded cDNA (22.3 kDa). The purified GST-p32 fusion protein was then used to produce a polyclonal antibody as described under “Experimental Procedures.” Fig. 2C shows the Western blots performed with pre-immune and immune sera. The antibody specifically recognized both recombinant GST-p32 fusion protein (lane 2) and p32 present in the Triton-insoluble fraction derived from rat hepatocyte homogenates (lane 3) but barely recognized the GST moiety of the fusion protein (lane 1). With pre-immune serum the fusion protein remained undetectable.

In Vitro Analysis of p32 Association with PKC Isoforms—Rat hepatocytes express seven PKC isoenzymes (18) that are co-eluted by DEA-CE-cellulose chromatography: two conventional isoforms (α and βII), four novel isoforms (δ, ε, η, and μ), and one atypical isoform (ζ). To explore which PKC isozyme binds to p32, we made use of this partly purified PKC extract as a probe in an overlay assay. Fig. 3A shows the Western blot analysis of the PKC preparation used, indicating that similar relative amounts of each PKC isoform were present in the overlay assay. Moreover, Fig. 3B (upper and lower) clearly indicate that all of the PKC isozymes tested bind to purified recombinant GST-p32. The binding is not due to secondary antibody interactions, and it is specific for p32 because no PKC became associated to other proteins such as BSA or GST alone under the conditions employed (see upper portion of Fig. 3B). The typical PKC substrate, histone H1, which associates with all PKC isoforms, served as the positive control (Fig. 3B, top). The data also indicate that p32 does not interact with all PKC isoforms in the same way, because dependence of the binding on PKC cofactors (phosphatidylserine, 1,2-diolein, and calcium) differs for each PKC isoform. Quantification of binding data, presented in Fig. 3C, demonstrates that whereas PKCζ is capable of binding p32 only in its activated conformation, other isozymes like PKC α and ζ, although interacting better with p32 in a cofactor-dependent manner, can also bind to p32 in their absence. On the other hand, the association between p32 and PKC β, ε, and η is equally efficient regardless of the presence of PKC activators, but it seems that PKCµ can bind p32 with great affinity in their absence. In summary, independency of binding to p32 on PKC activators ranges as follows: PKCµ >> β, ε, η > α > ζ >> δ.
P32 Is Not Phosphorylated by PKC and Increases PKC Kinase Activity in Vitro—To explore whether p32 is an in vitro substrate for several PKC isoforms, we used PKC isozyme-specific immune complexes to measure their phosphorylation activity toward GST-p32 in comparison with a typical PKC substrate, histone H1 III-S. Fig. 4A shows a representative...
autoradiography with its corresponding dried and Coomassie Blue-stained gel. As can be observed, whereas histone H1 was efficiently phosphorylated (only that phosphorylated by PKC is shown, but identical results were obtained with the other immunoprecipitated PKC isozymes), p32 was not phosphorylated by any PKC isoform tested, because the band corresponding to GST-p32 present in the Coomassie Blue-stained gel is absent at the same position in the autoradiography. It can also be seen that other proteins that co-immunoprecipitate with PKC were also phosphorylated. We analyzed the immunoprecipitates with anti-PKC antibodies (results not shown) and observed that the two more prominent bands observed in the autoradiography at 55 kDa corresponded to the catalytic fragment of PKC. The identity of the other faint bands is unknown.

Fig. 3. Overlay assay to determine binding of PKC isozymes to purified p32 (gC1qBP). A, Western blot of the PKC preparation used as probe in overlay assays. Samples (70 μg) of partially purified PKC by DEAE-cellulose chromatography from rat hepatocytes homogenates were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. Immunoblot analysis was performed with polyclonal isozyme-specific anti-PKC antibodies and developed with anti-rabbit IgG-alkaline phosphatase-conjugated secondary antibody. Positions of Mr standards are indicated. B, determination of PKC isoform binding to p32 by overlay assay. Samples (12 μg) of purified recombinant GST-p32, GST, or BSA (negative controls) or histone H1-IIIS (positive control) were subjected to SDS-PAGE (12% gels) and blotted onto nitrocellulose. PKC binding was determined by incubating the membranes in the absence or presence of partially purified PKC and in the absence or presence of cofactors (20 μg/ml phosphatidylserine, 0.8 μg/ml 1,2-diolein, and 0.5 mM CaCl₂) for 1 h. The mixture was removed, membranes were washed, and bound PKC was detected with isozyme-specific anti-PKC antibodies (which recognize all isoforms) as indicated on the figure. Membranes were developed with an alkaline phosphatase-conjugated secondary antibody. Positions of Mr standards are indicated; and the GST-p32 protein revealed by the PKC bound to it is also indicated with an arrow. Data are representative of four independent experiments. H-H1, histone H1-IIIS. C, quantification of PKC binding to p32 in the absence or presence of PKC cofactors. Band intensities were quantified by densitometric scanning, and data obtained in the presence of cofactors were taken as 100% for each PKC isozyme to normalize the results of different experiments. Values plotted are means ± S.E. for at least three independent experiments.
formed the assay in the absence or presence of p32 and in the absence or presence of the selective and potent PKC inhibitor Ro 31–8220. The upper portion of Fig. 4B shows a representative example of the results obtained with PKCθ and the lower portion those with PKCδ. We selected these isoforms on the basis of the strong in vitro interaction observed with p32 in a cofactor-dependent or independent manner, respectively, but similar results were obtained with the other five PKC isoforms expressed in rat hepatocytes (not shown). In Fig. 4C are presented the quantitative data of several experiments. As can be
observed, whereas PKCθ showed 1.5-fold activation in the presence of p32, PKCδ was activated 2-fold (indicated with a black arrow in Fig. 4B), because histone H1 phosphorylation increased in the presence of p32 in comparison with the obtained in its absence (see empty arrows) but not when the specific PKC inhibitor Ro 31–8220 was added. As indicated above, when we measured the activity of the other PKC isoforms under the same conditions, identical results were obtained, even if other substrates such as aldolase (in the case of PKCμ) or a peptide derived from myelin basic protein were used (data not shown).

**P32 Associates in Vivo with PKCδ and PKCζ—**Although it is clear that p32 binds all classes of PKC isoforms in vitro, it remained important to determine which isoform interacts with p32 inside the cells. Therefore, we examined in cultured rat hepatocytes (clone C9), by immunofluorescence and confocal laser microscopy, the possible subcellular co-localization of p32 with the previously selected PKCζ and PKCδ isoforms. We also studied the effect of PKC activation on this localization using 1 μM TPA (an optimal concentration reported for PKC activation on these cells) (34). Figs. 5 and 6 show representative experiments of the time course of treatment of subconfluent C9 cells with TPA and the dual immunostaining of p32 and PKCδ (Fig. 5) or p32 and PKCζ (Fig. 6) with specific antibodies, respectively. In untreated cells, p32 and PKCζ appear distributed throughout the cytoplasm, whereas PKCζ locates predominantly at the cytoplasm but also at the cell nucleus. Monolayers treated with TPA did not show a clear PKC redistribution as a result of its activation at the time points presented, although in some cells both PKC δ and θ concentrate at certain long and thin cell elongations. However, the cells treated with TPA changed their morphological appearance, as they lost their polygonal shape and retracted from each other (see Figs. 5 and 6). Furthermore, the addition of TPA induced a transient translocation of p32 to the cell nucleus, visualized 5 min after TPA treatment (insert and arrowheads in Fig. 5, arrows in Fig. 6), as 15 min after its addition, p32 is no longer found at the nuclei. Consistent with the data obtained in vitro, the immunofluorescence analysis shows a constitutive co-localization of p32 and PKCζ mainly at the perinuclear region, but also when p32 is at the cell nuclei (Fig. 5, merged images (Merge), with or without the addition of TPA). In contrast, the images in Fig. 6 indicate that PKCζ activation is required for its association with p32. Their co-localization increases with the time of TPA treatment, and it is also found mostly in the perinuclear region (Fig. 6, arrows in merged images). Taken together, the results presented here indicate that p32 and PKC θ and δ interact together in vivo.

**DISCUSSION**

The coordinated interaction of kinases, phosphatases, and other regulatory molecules with scaffolding proteins is emerging as a major theme in intracellular signaling networks (6–8). There are now an increasing number of PKC-binding proteins believed to play a role in directing the location and function of individual PKC isoforms to particular subcellular locations within cells (9–18). In this study, we have purified a PKC-binding protein with an apparent molecular mass of 32 kDa and identified it as the previously described p32 subunit of the pre-mRNA splicing factor SF2 (20) or the receptor of the globular heads of the complement factor CIq (19). As mentioned previously, recent evidence indicates that the molecule is a multifunctional protein with affinity for diverse ligands, which include thrombin, vitronectin, kininogen, a mitochondrial marker protein, and several viral proteins involved in viral gene expression (21–24). Very recently, Storz et al. (25) reported for the first time the interaction of this protein with PKC, demonstrating that p32 associates in vivo with PKCμ at the mitochondrial membranes of the B cell line SKW 6.4. Here, we provide evidence that p32 interacts differentially with several PKC isoforms both in vitro and in vivo. Using an overlay assay approach, we have demonstrated that although p32 is able to bind specifically to all PKC isozymes expressed in rat hepatocytes (α, β, δ, ε, θ, μ, and ζ), their binding affinities depend on the presence of PKC activators (Fig. 3). These results suggest, therefore, that there may be isoyzme-specific domains in PKC that are better exposed in a specific conformation that permits the interaction with p32.

We have also shown that although p32 binds to the kinase, it does not serve as a substrate for any PKC isoform tested (Fig. 4A). Interestingly, we demonstrated that p32 stimulates PKC activity in vitro, suggesting that it may also regulate positively the enzyme in vivo (Fig. 4, B and C). In this respect, our data are in agreement with those of Storz et al. (25), who report that PKCμ does not phosphorylate p32, but are in contrast with their finding that p32 inhibits aldolase phosphorylation by PKCμ. It is important to consider that the in vitro studies of Storz et al. (25) suggest that the presence of p32 does not inhibit the kinase activity.
intrinsically, because autophosphorylation of PKC\(_\mu\) not only was unaffected but was slightly increased in the presence of p32. Furthermore, they also show that aldolase phosphorylation by PKC\(_\mu\) immunoprecipitates from the soluble cell fraction could be readily discerned. They explain these results by suggesting that p32 regulates PKC activity in a compartment-specific fashion and that binding of p32 to the kinase catalytic domain might only restrict, by steric hindrance, the substrate access to PKC\(_\mu\). It is important to take into account that the p32 portion obtained from the construct employed by us corresponds to the mature form of p32 (predominantly found inside cells), which lacks a 73-residue signal sequence at the NH\(_2\) terminus, necessary to target the protein to mitochondria (22). Thus, the discrepancy with our data can be explained by the fact that Storz et al. (25) in their experiments used a construct that included all of the open reading frame of p32. On the other hand, it has been reported recently (36) that 14-3-3 proteins stimulate classical and \(\zeta\) PKC isoforms up to 2.5-fold in an apparently nonspecific manner solely because of the acidic nature of these proteins, as other acidic proteins such as lactoglobulin and apotransferrin stimulated PKC\(_\zeta\) in a similar fashion. Because p32 is a highly acidic protein having a pI of 4.15, it would be predicted that it may activate PKC activity in the same way, but it remains to be clarified as to which mechanism stimulates PKC activity. In this respect, it is noteworthy that despite the differences in affinity displayed by p32 in binding PKC isoforms, its effects on PKC activities are stimulatory for all isoforms.

Immunofluorescence experiments with cultured rat hepatocytes in our current work demonstrated that p32 co-distributes with PKC\(\theta\) and PKC\(\delta\) in vivo. Our data indicate that whereas p32 and PKC\(\theta\) are constitutively associated, PKC\(\delta\) activation is required for its association with p32 at the perinuclear region in intact cells. It is interesting to note how the same protein, p32, is able to behave as a scaffold (for PKC\(\theta\)) or as a receptor for activated C-kinase (RACK) (for PKC\(\delta\)) depending upon which PKC isoform associates with it. In addition, a salient finding obtained here is that p32 is transiently translocated to the cell nucleus in response to TPA treatment, co-localizing there with PKC\(\delta\), and detected as a nucleoplasmic speckled pattern, typical of nucleoplasmic small nuclear ribonucleoprotein particles. This location is therefore consistent with the functional proposed roles for p32 in transcriptional regulation of viral gene expression (24) and as a regulatory factor of RNA splicing by inhibiting ASF/SF2 RNA binding and phosphorylation (23). There has been some controversy regarding the subcellular localization of p32. Although some authors have reported p32 to be present predominantly in the mitochondria (22, 25), others have found it at the cytoplasm (21), cell surface (19), and nucleus (20, 21). Recently, using immunogold electron microscopy to evaluate the p32(gC1q-BP) distribution in immunogold-labeled cells, Soltys et al. (37) have shown specific labeling of mitochondria, condensing vacuoles, endoplasmic reticulum, nuclei, and cell surface in several cultured cell lines and in rat tissues (including rat liver) under normal physiological conditions. Furthermore, the distribution of p32 appears to be altered during adenovirus infection, with p32 migrating to the nucleus together with a viral core protein (21), suggesting that p32 plays a bridging role in nucleus-mitochondrion interactions. Therefore, it seems that p32 does not have a unique location within a cell, and our immunofluorescence data support this notion. With respect to the p32 carbohydrate content, it is important to mention that although up until the mid 1980s it was widely believed that glycosylation was restricted largely to proteins localized on the cell surface, the widespread existence of complex glycoconjugated proteins within the nucleus and cytoplasm is now known (38).

Another remarkable issue is that p32 shares with another PKC-binding protein, calreticulin (18), the property to bind C1q complement factor. Several binding molecules and potential receptors for C1q have been described: a cell surface receptor, calreticulin; a 60-kDa homologue, termed cC1q-R, that binds to the collagenous region of C1q; and a 32-kDa glycoprotein with affinity for the globular heads, or gC1q-R (39). Obrehehiwet et al. have found that p32 and cC1q-R often coelute during purification and are able to associate with each other forming a complex on the cell surface (39). The functional significance of the interaction between these two proteins and PKC remains to be established.

Finally, it is also astonishing that the solved crystal structure of the p32(gC1q-BP) mature core determined at 2.25 Å (35) revealed a doughnut-shaped protein composed of three monomers, in close resemblance with the propeller-like three-dimensional structure of the receptors for activated protein kinase C, RACK1 and \(\beta\)-COP, that in turn belong to the WD-40 family of regulatory proteins (15, 18). Important questions remain concerning the biological significance of this similarity as well as the molecular mechanisms involved in the functional coupling between p32 and protein kinase C.
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