PKCε-Related Kinase Associates with and Phosphorylates Cytokeratin 8 and 18

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Abstract. A 40-kD protein kinase C (PKC)ε related activity was found to associate with human epithelial specific cytokeratin (CK) polypeptides 8 and 18. The kinase activity coimmunoprecipitated with CK8 and 18 and phosphorylated immunoprecipitates of the CK. Immunoblot analysis of CK8/18 immunoprecipitates using an anti-PKCε specific antibody showed that the 40-kD species, and not native PKCε (90 kD) associated with the cytokeratins. Reconstitution experiments demonstrated that purified CK8 or CK18 associated with a 40-kD tryptic fragment of purified PKCε, or with a similar species obtained from cells that express the fragment constitutively but do not express CK8/18. A peptide pseudosubstrate specific for PKCε inhibited phosphorylation of CK8/18 in intact cells or in a kinase assay with CK8/18 immunoprecipitates. Tryptic peptide map analysis of the cytokeratins that were phosphorylated by purified rat brain PKCε or as immunoprecipitates by the associated kinase showed similar phosphopeptides. Furthermore, PKCε immunoreactive species and CK8/18 colocalized using immunofluorescent double staining. We propose that a kinase related to the catalytic fragment of PKCε physically associates with and phosphorylates cytokeratins 8 and 18.

CYTOKERATINS (CK)[1] are a group of intermediate filament (IF) proteins which are expressed primarily in epithelial tissues (Lazarides, 1982; Steinert and Roop, 1988; Franke et al., 1981; Osborn and Weber, 1986). The 30 or so polypeptides which make up the family of cytokeratin proteins are divided into acidic (type I) and basic/neutral (type II) keratins. In epithelial cells, CK are found as mosaic noncovalent polymers with an assembly consisting of at least one type I and one type II CK (Steinert and Roop, 1988). For example, "simple" single layer epithelial cells such as intestinal epithelia express CK8 (type II) and CK18 (type I), whereas esophageal epithelial cells express CK4 (type II) and CK13 (type I) predominantly. Cytokeratins are not only important as tissue-specific markers, they also form important markers of cell differentiation.

IF proteins, including cytokeratins, undergo several post-translational modifications such as NH_2-terminal acetylation (Steinert and Idler, 1975), glycosylation (King and Hounsell, 1989; Roberts and Brunt, 1986), and serine/threonine phosphorylation (Steinert, 1988; Gilmartin et al., 1984; Yeagle et al., 1990; Baribault et al., 1989). The functional role of IF protein phosphorylation is not well understood but phosphorylation of vimentin, desmin, neurofilaments, and nuclear lamins appears to be important in regulating the assembly of these filaments during mitosis (Inagaki et al., 1987, 1988; Gonda et al., 1990). The kinases that are involved in CK phosphorylation have not been identified although evidence exists that cAMP-dependent protein kinase can phosphorylate several cytokeratins (Steinert, 1988; Gilmartin et al., 1984). In addition, protein kinase C (PKC) appears to play a role in CK8/18 phosphorylation (Chou and Omary, 1991).

PKC is a serine/threonine protein kinase with important functional roles in the cellular responses of many tissues (for reviews see Nishizuka 1988, 1989; Parker et al., 1989; Bell and Burns, 1991). At least eight subspecies of PKC have been identified (α, βI, βII, γ, δ, ε, ζ, and η) with differences in structure, substrate, and calcium dependence of the PKC subspecies described (Nishizuka, 1988; Osada et al., 1990; Bacher et al., 1991). PKCε (90 kD), is a calcium-independent member of the PKC family with a broad tissue distribution as determined by Northern blot analysis of rat tissue mRNA (Ono et al., 1988; Schaap et al., 1989), or by Western blot analysis using specific PKCε antisera (Strulovici et al., 1991).

Limited in vitro proteolytic digestion of PKC with trypsin or calpain results in the generation of a stable fragment of 40–50 kD, (Kishimoto et al., 1989; Schaap et al., 1990;

1. *Abbreviations used in this paper*: CK, cytokeratins; IEF, isoelectric focusing; IF, intermediate filament; PKC, protein kinase C; SCLC, small cell lung carcinoma.

Takai et al., 1977), which displays catalytic activity in the absence of PKC allosteric activators. Different substrate specificity is exhibited by the catalytic fragment of PKCε as compared to the intact enzyme (Schaap et al., 1990). It is unclear if the described PKC fragments play any physiological role, however, several recent investigations suggested that such fragments may be generated with cellular activation. For example, a catalytic 40-kD PKCε immunoreactive fragment has been noted in the human small cell lung carcinoma (SCLC) cell line NCI-N417. The level of this 40-kD species increased after treating cells with phorbol myristate acetate (TPA) or gastrin-releasing peptide (Baxter et al., 1992). The 40-kD species noted in the NCI-N417 cells may represent a transcript variant (Schaap et al., 1989, 1990), an alternate gene product (Ono et al., 1988) or a proteolytically activated fragment of the parent PKCε. The latter possibility is likely to occur in several systems as was shown with f-Met-Leu-Phe or TPA-stimulated neutrophils which generated a 50-kD PKC species within minutes (Pontremoli et al., 1990). In addition, stimulation of rat adipocytes with insulin resulted in a decrease in cytosolic 80-kD PKC with concomitant increase in a 50-kD protein recognized by an anti-PKC antibody (Ishizuka et al., 1990).

Treatment of intact epithelial cells with TPA resulted in an increase in the level of CK8/18 phosphorylation supporting a role for PKC in the phosphorylation of epithelial cytokeratins (Chou and Omary, 1991). It also appeared that CK phosphorylation may be calcium independent since agents which alter available intracellular calcium concentrations did not alter the level of CK phosphorylation (Chou and Omary, 1991). Hence, if a PKC subspecies is indeed involved in cytokeratin phosphorylation, a potential candidate would be PKCε which in most systems that have been tested appears to be a calcium-independent kinase.

The observation that PKC may play a role in CK phosphorylation led us to our present study. Here we show that cytokeratin polypeptides 8 and 18 associate with a 40-kD PKCε-related kinase, possibly a PKCε catalytic fragment. Further, CK 8 and 18 appear to serve as substrates for PKCε-related species.

Materials and Methods

Cells and Reagents

The human cell lines HT29 (colonic epithelial) and NCI-N417 (small cell lung carcinoma) were grown in RPMI 1640 medium supplemented with 10% FCS, penicillin (100 U/ml) and streptomycin (100 μg/ml). The NCI-N417 cell line was established by Dr. Adi Gazdar (National Cancer Institute, Bethesda, MD) and was a gift from Dr. Michael Bishop (University of California, San Francisco, CA). Fresh human placenta was obtained from the Labor and Delivery wing at Stanford University (Stanford, CA) under a protocol that was approved by the Human Subjects Committee. Rabbit anti-PKCε antibody was generated after immunizing with a peptide corresponding to residues 721-737 of PKCε, followed by affinity purification using the immunizing peptide coupled to epoxy-activated Sepharose 6B (Strulovici et al., 1991). mAbs used were: anti-human CK8/18 (L2A1) (Chou and Omary, 1991); anti-human CK8/18 antibody termed CK5 and anti-actin immunoprecipitating antibody (Sigma Chemical Co., St. Louis, MO); anti-adenocarcinoma/epithelial specific antigen (I4D4) and anti-transferrin receptor (B3/25) (Omary et al., 1991). Other reagents used were: histone IIA and IIIS, casein, chicken muscle actin (Sigma Chemical Co.); carrier-free [^{32}P]orthophosphoric acid and [γ-^{32}P]ATP (3,000 Ci/mmol) (New England Nuclear, Boston, MA); enolase, bovine CK8 and 18 (Boehringer Mannheim Biochemicals, Indianapolis, IN); FITC-rabbit anti-mouse F(ab')$_2$ antibody (Zymed Laboratories, San Francisco, CA); Texas-red donkey anti-rabbit Ig antibody (Amersham Corp., Arlington Heights, IL). Rat brain PKCε was purified from COS-1 cells that transiently express the enzyme (Knopf et al., 1986).

In Vivo and In Vitro Labeling

HT29 cells were labeled with orthophosphate as described (Omary and Trowbridge, 1980). In vitro kinase assays were carried out on washed immunoprecipitates using 25 μl of 10 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM DTT, 50 mM Pipes (kinase buffer) containing 2.5–10 μCi of [γ-^{32}P]ATP (2 μM ATP). After a 15 min reaction time (equilibrium conditions) at 22°C, the kinase reaction was stopped by adding an equal volume of sample buffer containing 4% SDS and 20% glycerol.

Immunoprecipitation and Cytokeratin Isolation

Cells (1×10^7) were solubilized for 30–60 min (4°C) in 1 ml of 1% Nonidet P-40 (NP-40) in PBS (pH 7.2) containing: aprotinin (25 μg/ml), leupeptin (10 μM), pepstatin (10 μM), and PMSF (0.1 mM). After removal of nuclei, 1/10 volume of 10% deoxycholate, 10% NP-40, 1% SDS in PBS were added to the lysate followed by the addition of 100 μl of 10% (vol/vol) formalin-fixed *Staphylococcus aureus* Pansorbin® per ml of lysate. After 20 min at 4°C, the bacteria were removed by centrifugation followed by the addition of 10-30 μl of 1:10 dilution of mAb ascites per 100-200 μl of lysate. Immune complexes were collected using 150 μl of 2% Pansorbin® followed by three washes in 0.5% deoxycholate, 0.5% NP-40, and 0.05% SDS. For in vitro kinase assays, immunoprecipitates underwent one additional wash in the kinase buffer.

To evaluate CK isolation under several extraction conditions, cytokeratins were extracted using our standard solubilization conditions and compared with a described CK isolation using Triton X-100 (TX-100) followed by high salt extraction (Achtstaetter et al., 1986). HT29 cells were grown in serum free medium for 12 h followed by solubilization with 1% NP-40 in PBS for 45 min, or TX-100 in PBS for 45 or 2 min. After centrifugation (12,000 *g* for 2 min), supernatants of the lysates were saved for immunoblotting and a sample buffer containing 2% SDS and 10% glycerol was added to the pellets for solubilization. The pellet from the 2 min TX-100 treatment was further extracted using a high salt solution (1.5 M KCl, 5 mM EDTA, 0.5% TX-100) for 30 min followed by pelleting (12,000 *g* for 10 min) then solubilization of the pellet in 2% SDS, 10% glycerol sample buffer. Protein concentration was determined as described (Bradford, 1976). A discontinuous buffer system was used for SDS-polyacrylamide gel analysis (Laemmli, 1970). Autoradiography for ^{32}P was performed using intensifying screens (Laskey and Mills, 1977).

Immunofluorescence

Immunofluorescence staining was performed using confluent HT29 cells grown on culture chamber slides, (Nunc, Roskilde, Denmark) in serum-containing medium then switched to serum-free medium for 12 h. Cells were fixed by dipping in −20°C acetone followed by washing in PBS. First antibody incubations (L2A1 mAb, and rabbit anti-PKCε) were done in PBS containing 1% BSA, 0.02% NaN_3, and 10% human serum for 30 min (22°C) with shaking. After washing, fluorescent second antibodies were added at dilutions recommended by the manufacturer (30 min at 22°C) followed by washing. Fluorescence microscopy was carried out with a microscope (Nikon Inc., Garden City, NJ) using a 40× objective. Photos were taken using Kodak Ektachrome 400 film (Eastman Kodak Co., Rochester, NY).

Western Blotting and Two-Dimensional Analysis

Isoelectric focusing (IEF) was performed using a pH 3–10 gradient as described (O'Farrell, 1975). For Western blot analysis, immunoprecipitates were transferred from SDS gels to PVDF or nitrocellulose membranes using the manufacturer recommendations in a buffer containing 192 mM glycine, 25 mM Tris, and 20% methanol (Towbin et al., 1979). Transfer was carried out as described (Strulovici et al., 1989) or by using Trans-Blot transfer cell (semi-dry; Bio-Rad Laboratories, Cambridge, MA) for 30 min at 15 V. After transfer, membranes were incubated with the appropriate antibody for 2 h (22°C) followed by washing. Specific bands were visualized by incubating with ^{125}I-protein A or ^{125}I-sheep anti-mouse IgG followed by washing and autoradiography (Strulovici et al., 1989). Analysis of tryptic peptides of the cytokeratins was carried out as described before (Omary and Trowbridge, 1980), except that carboxymethylation was done before SDS-PAGE and the protein bands were extracted by electroelution. Phosphory-

lated amino acids were analyzed by two-dimensional electrophoresis after extraction of the phosphate-labeled cytokeratin protein band from the SDS-polyacrylamide gels, and hydrolysis in vacuo with constant boiling HCl (Cooper et al., 1983).

Results

CK8/18 and PKCε Immunoreactive Species Colocalize Using Double Immunofluorescent Staining

Recent evidence from our laboratory suggested that intact cell phosphorylation of CK8/18 may involve a calcium-independent PKC (Chou and Omary, 1991). We used the human colonic epithelial cell line HT29 which expresses PKCε, a calcium-independent PKC isoform, and compared its subcellular localization with that of cytokeratin polypeptides 8 and 18. Rabbit anti-PKCε antibody and mAb L2A1 which recognized CK8/18 were used to stain their respective antigens by immunofluorescence. A similar pattern of fluorescent staining was noted for CK8/18 (Fig. 1 *C*) or PKCε (Fig. 1 *D*). Faint background immunofluorescence staining was noted in the absence of the primary antibody (Fig. 1, *A* and *B*). Both anti-PKCε and anti-CK8/18 antibodies showed staining to be primarily localized to the periphery of the cell with some filamentous cytoplasmic staining.

Cytokeratins 8 and 18 Associate with a Serine Kinase Activity

The colocalization of CK8/18 and PKCε in HT29 cells prompted us to ask whether any kinase activity associated with CK8/18 immunoprecipitates. Epithelial-specific cytokeratins 8 and 18 were studied using mAb L2A1 which immunoprecipitated CK8/18 as a noncovalent heteropolymer. As shown in Fig. 2, CK8/18 immunoprecipitates, obtained either from HT29 cells or from fresh homogenates of human placenta, were phosphorylated after [γ-^{32}P]ATP was added. The specificity of association of CK8/18 with the kinase activity was confirmed by using immunoprecipitates obtained with mAb I4D4 (which recognizes an adenocarcinoma/epithelial specific antigen on HT29 cells) and mAb B3/25 (which recognizes the transferrin receptor on placental tissue) (Fig. 2). The cytokeratin associated kinase activity also phosphorylated exogenous substrates such as myelin basic protein, casein alpha chain, histones IIA and IIIS, and enolase (not shown).

Phosphorylation of CK8/18 shown in Fig. 2 was further resolved using two-dimensional analysis. As shown in Fig. 3 *A*, mAb L2A1 immunoprecipitated three major bands as determined by Coomassie blue staining. The upper and lower bands corresponded to CK8 and 18, respectively. The middle band (not reproducibly seen) was not observed when immunoprecipitates were phosphorylated in vitro but was phosphorylated when cells were labeled in vivo followed by immunoprecipitation (Fig. 3 *A*). This middle band likely represents a proteolytic fragment of CK8 (based on two-dimensional tryptic peptide mapping, data not shown) and was not studied further. The pI of CK 8 and 18 shown in Fig. 3 *A* was 6–6.2 and 5.6–5.8, respectively, which is similar to that reported before (Moll et al., 1982). Multiple charged isomers are noted for in vitro phosphorylated CK8/18 (Fig. 3) whereas one to two major phosphorylated species are seen for in vivo–labeled cytokeratins (Fig. 3 and Gilmartin et al., 1984). The least charged isomer of in vitro and in vivo phosphorylated CK8 and CK18 (i.e., most basic isomer) corresponds to the second major Coomassie-stained spot (i.e., the more acidic spot seen in Fig. 3). Therefore, a substantial portion of CK8 and CK18 is not phosphorylated in vivo (Fig. 3 and Gilmartin et al., 1984) or in vitro (Fig. 3).

Examination of the in vitro phosphorylated CK8 and 18 showed serine to be the only phosphorylated amino acid (Fig. 3 *B*). Hence, the kinase activity observed in CK8/18 immunoprecipitates phosphorylated serine residues similar to what was observed for cytokeratin phosphorylation in intact cells (Steinert 1988; Chou and Omary, 1991).

Comparison of CK8/18 Tryptic Phosphopeptides Labeled Using Purified PKCε or by the Keratin Associated Kinase

To assess the possibility that CK8/18 phosphorylation is the result of a physical association with PCKε, CK8, and 18 tryptic phosphopeptide maps from CK8/18 immunoprecipitates were compared with those obtained after the addition of exogenous PKCε to the cell-free kinase assay. To eliminate the contribution of the associated kinase(s), CK8 and 18 immunoprecipitates were subjected to boiling before exogenous PKCε was added to the reaction mixture. The two-dimensional tryptic phosphopeptide analysis of CK8 isolated from immunoprecipitates clearly shows that, of the eight sites phosphorylated by the kinase associated with the immunoprecipitates, five were also phosphorylated by the exogenously added purified brain PKCε (Fig.4 *c*, *arrows*). A similar result was obtained for CK18 after CK8/18 immunoprecipitates were subjected to the cell-free kinase assay in the absence (Fig. 4 *d*) or presence (Fig. 4 *e*) of exogenous PKCε.

Although the pattern shown in Fig. 4 is similar when comparing CK8/18 tryptic phosphopeptides from immune complex associated kinase activity with exogenously added PKCε, several quantitative and qualitative differences are evident. These differences may be explained by the boiling treatment used to eliminate any associated kinase activity, which may alter the availability of potential phosphorylation sites. Some differences may also be explained by the association of kinases other than PKCε.

Demonstration of Physical Association between CK8/18 and a 40-kD PKCε Immunoreactive Protein

Several observations suggested to us that PKCε is a likely candidate for the CK-associated kinase. Namely, the above described CK8/18 serine associated kinase activity, the colocalization of PKCε and CK8/18 by immunofluorescence, and the similarity of CK8/18 tryptic phosphopeptide maps in the presence or absence of exogenous PKCε. To test the hypothesis that CK8 and 18 are physically associated with endogenous PKCε, CK8/18 immunoprecipitates from HT29 detergent cell lysates were immunoblotted with anti-PKCε antibody. Western blot analysis of PKCε in HT29 cells revealed two PKCε-immunoreactive proteins of 90 and 40 kD (Fig. 5, lane *a*). When CK8/18 immunoprecipitates were blotted with anti-PKCε antibody, only the 40-kD protein was noted (Fig. 5, lane *b*). After immunodepletion of CK8/18, the remaining HT29 cell lysate lacked any residual 40-kD PKCε immunoreactive protein (Fig. 5, lane *c*) but did contain some

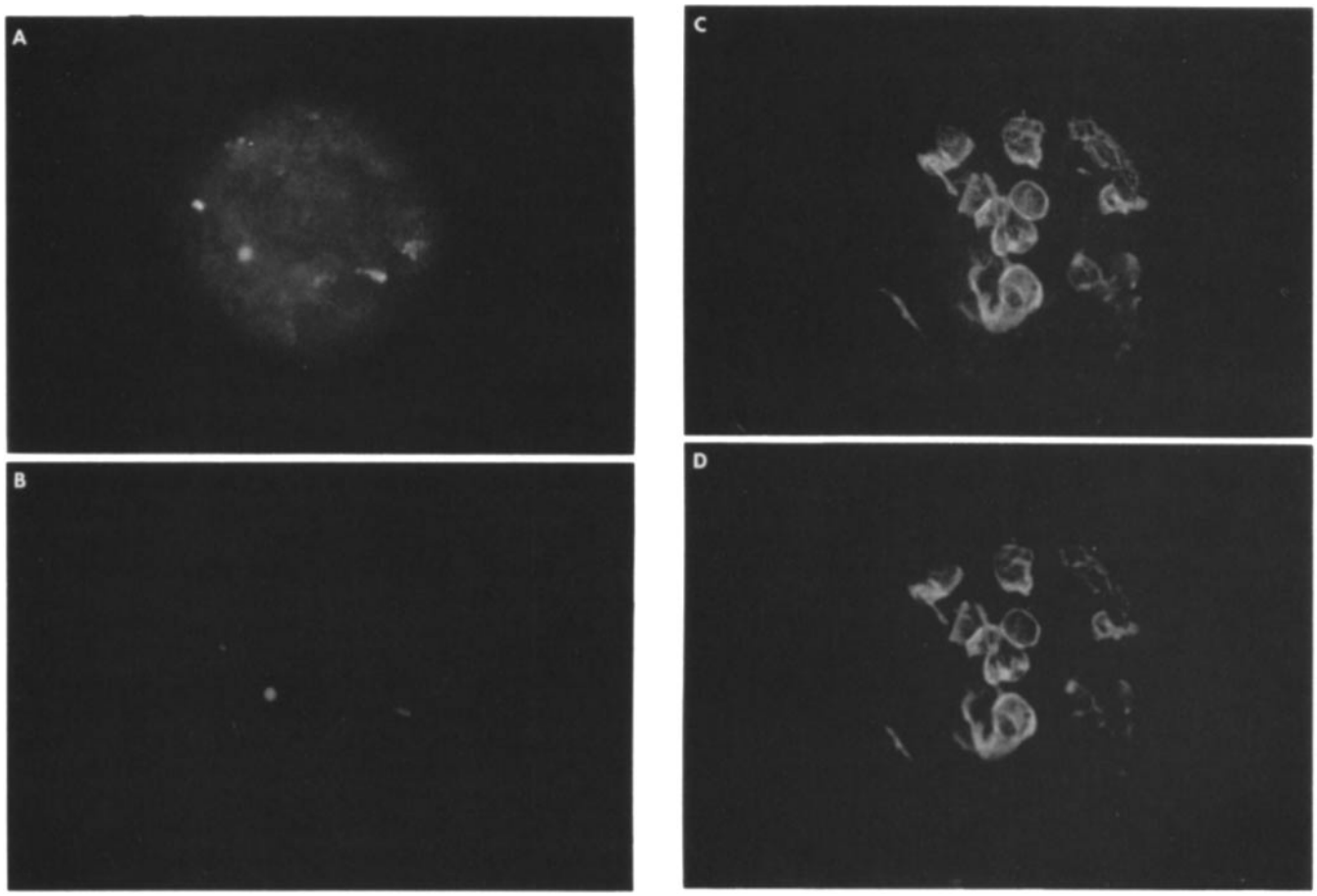

Figure 1. Double immunofluorescence staining of HT29 cells. Cells were fixed with acetone then stained with antibodies as described in Materials and Methods. For background staining, normal mouse ascites (*A*) and prebleed rabbit antibody (*B*) were used. The specific staining was obtained using mAb L2A1 (anti-CK8/18) (*C*) and rabbit anti-PKCε (*D*). Fluorescent antibodies used were FITC-rabbit anti-mouse $F(ab')_2$ and Texas red donkey anti-rabbit Ig antibodies. Green fluorescein staining (*A* and *C*) was used to visualize CK8/18 (*C*) and Texas red staining (*B* and *D*) was used to visualize PKCε immunoreactive proteins (*D*).

of the 90-kD PKCε species (which migrated slightly faster than 90 kD likely secondary to proteolysis). This indicated that most if not all of the 40-kD PKCε immunoreactive protein associated with CK8/18. The intense 80kD band Fig. 5, lanes *a*, *c*, and *f*) represents a non-specific band that is seen when cells are grown in serum containing medium for <2 d (Fig. 5, lane *g*) and is absent upon serum starvation (Fig. 5, lane *h*). Its nonspecific nature was shown previously (Strulovici et al., 1991).

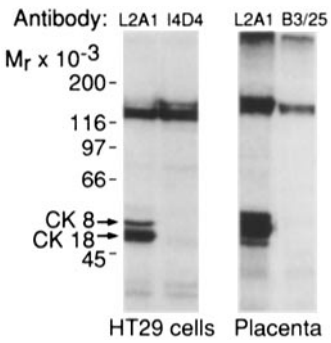

Figure 2. CK8/18 immunoprecipitates have an in vitro kinase-associated activity. Kinase assays were performed using immunoprecipitates obtained from detergent extracts of HT29 cells or homogenized human placenta as described in Materials and Methods. The figure shows an autoradiograph (2-h exposure) of a kinase assay performed on material immunoprecipitated with the indicated antibodies. mAbs used were: L2A1 (*anti-CK8/18*); I4D4 (*anti-adenocarcinoma/epithelial specific antigen*); B3/25 (*anti-transferrin receptor*).

The rabbit anti-PKCε antibody did not recognize CK8/18 (not shown) and mAb L2A1 did not cross react with the 40-kD protein (Fig. 5, lane *e*). After correcting for the amount of lysate used in Fig. 5 (lane *a*) with that used for CK8/18 immunoprecipitation, it was estimated that >90% of the 40-kD protein present in the detergent lysate associated with CK8/18 immunoprecipitates.

We have recently reported the constitutive presence of a PKCε-related 40-kD kinase in the SCLC cell line NCI-N417 (Baxter et al., 1992). Comparison of the SDS-PAGE migration properties of the SCLC 40-kD kinase and the 40-kD PKCε-immunoreactive protein in HT29 cells is shown in Fig. 5 (lanes *a*, *b*, and *d*). A close inspection of the 40-kD protein obtained from HT29 cells showed that it consisted of a doublet (Fig. 5, lane *a* and *b*), whereas the 40-kD species obtained from the NCI-N417 cells migrated as a single band (Fig. 5, lane *d*). The mix of HT29 and NCI-N417 detergent cell lysates still showed the doublet (Fig. 5, lane *f*). It is unclear whether the doublet corresponded to two different proteins or different phosphorylation levels of the same protein. The PKCε immunoreactivity and similarity in SDS-PAGE migration properties suggested to us that the 40-kD protein

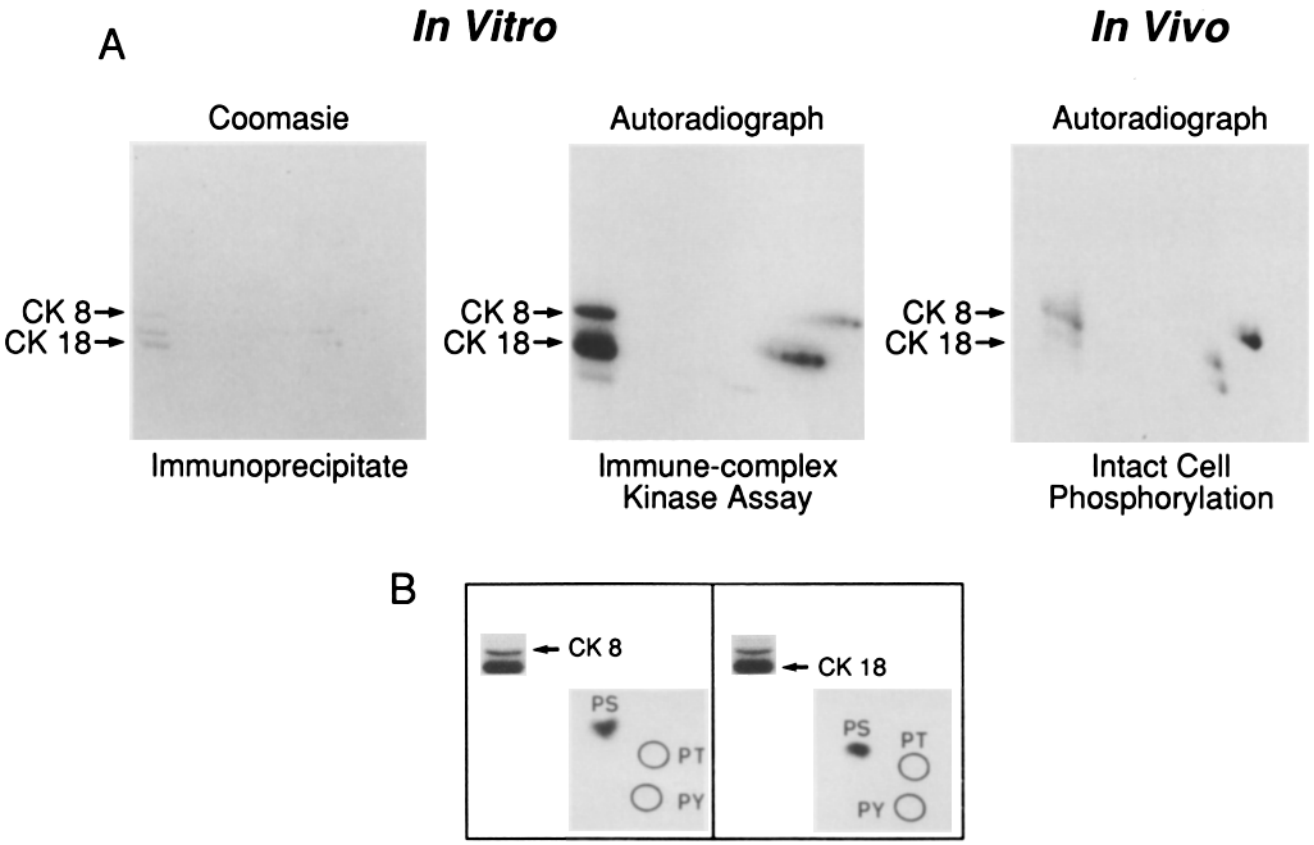

Figure 3. 2D gel analysis of CK8/18 immunoprecipitates labeled in vitro or in vivo. (*A*) Immunoprecipitates of CK8/18 were obtained from detergent solubilized HT29 cells using mAb L2A1 followed by analysis using isoelectric focusing (*horizontal axis*) and SDS-PAGE (*vertical axis*). The left of each panel shows an immunoprecipitate (identical to that used for two-dimensional gel analysis) that was analyzed only in one dimension using SDS-PAGE. For in vitro labeling, a kinase assay was performed using [γ-^{32}P]ATP and CK8/18 immunoprecipitates. The autoradiogram was obtained from the Coomassie-stained gel shown to the left after gel drying. For in vivo labeling, HT29 cells were labeled using [^{32}P]orthophosphate followed by immunoprecipitation and two-dimensional gel analysis as described in Materials and Methods. Basic peptides are to the right. (*B*) CK8/18 immunoprecipitates were incubated with [γ-^{32}P]ATP in kinase buffer. Phosphorylated species were analyzed using SDS-PAGE followed by electroelution of the indicated band and phosphoamino acid analysis using two-dimensional electrophoresis as described in Materials and Methods. (*PS*) phosphoserine; (*PT*) phosphothreonine; (*PY*) phosphotyrosine.

in HT29 cells and the SCLC 40-kD kinase were related. Since NCI-N417 cells lack any detectable CK8/18, as assessed by immunoblotting with mAb L2A1 (not shown), there was no association noted between the 40-kD protein and CK8/18 immunoprecipitates obtained from NCI-N417 cells (Fig. 5, lane *e*), thus further demonstrating the specificity of association between CK8/18 and the 40-kD PKCε-immunoreactive protein. A 60-kD PKCε immunoreactive species is occasionally seen in HT29 cells (Fig. 5, lanes *g* and *h*) and N417 cells (see Fig. 7, lane *4*) and likely corresponds to a degradation product from the 90-kD PKCε.

Cosolubilization of the 40-kD PKCε Kinase with the Cytoskeleton Fraction

Given that cytokeratins are insoluble in neutral aqueous buffers (Lazarides et al., 1982), we tested the efficiency of solubilization of CK8/18 under various conditions and the association of PKCε or the 40-kD PKCε kinase with the cytokeratins. Solubilization with 1% NP-40 or TX-100 for 45 min results in 50% or more recovery of the cellular keratin (Fig. 6 *A*). A brief solubilization (2 min with TX-100) does not result in any keratin recovery (Fig. 6 *A*, lane *5*) whereas high salt extraction yielded a pellet that contained essentially all of cellular CK8/18. As shown in Fig. 6 *A*, CK8/18 were identified by immunoblotting using mAb L2A1 that we previously characterized (Chou and Omary, 1991) (Fig. 6 *A*, lanes *a*) or using a commercially available anti-CK8/18 mAb that was characterized previously (Tolle et al., 1985).

Solubilization of cells for 45 min with 1% NP-40 or TX-100 resulted in generating the 90- and 40-kD PKCε species in the soluble fraction (Fig. 6 *B*, lanes *1* and *3*). Only the 90-kD PKCε is seen after a brief 2-min solubilization (lane *5*). In contrast the 40-kD PKCε species fractionated in all cases with the "insoluble" keratin-containing fraction (Fig. 6 *B*, lanes *2*, *4*, and *6*) and with prolonged solubilization (i.e., 30–45 min) which resulted in 50% or more efficiency in CK recovery (Fig. 6 *B*, lanes *1* and *3*).

The major proteins in the high salt extracted pellet (Fig. 6, *A* and *B*, lane *6*) are keratins as compared with the heterogeneous mixture of cellular proteins loaded in lanes *1* to *5* (Fig. 6) (determined by Coomassie staining, not shown). This explains the relatively intense 40-kD band seen in Fig. 6 *B*, lane *6* as compared with the remaining lanes in Fig. 6 *B*.

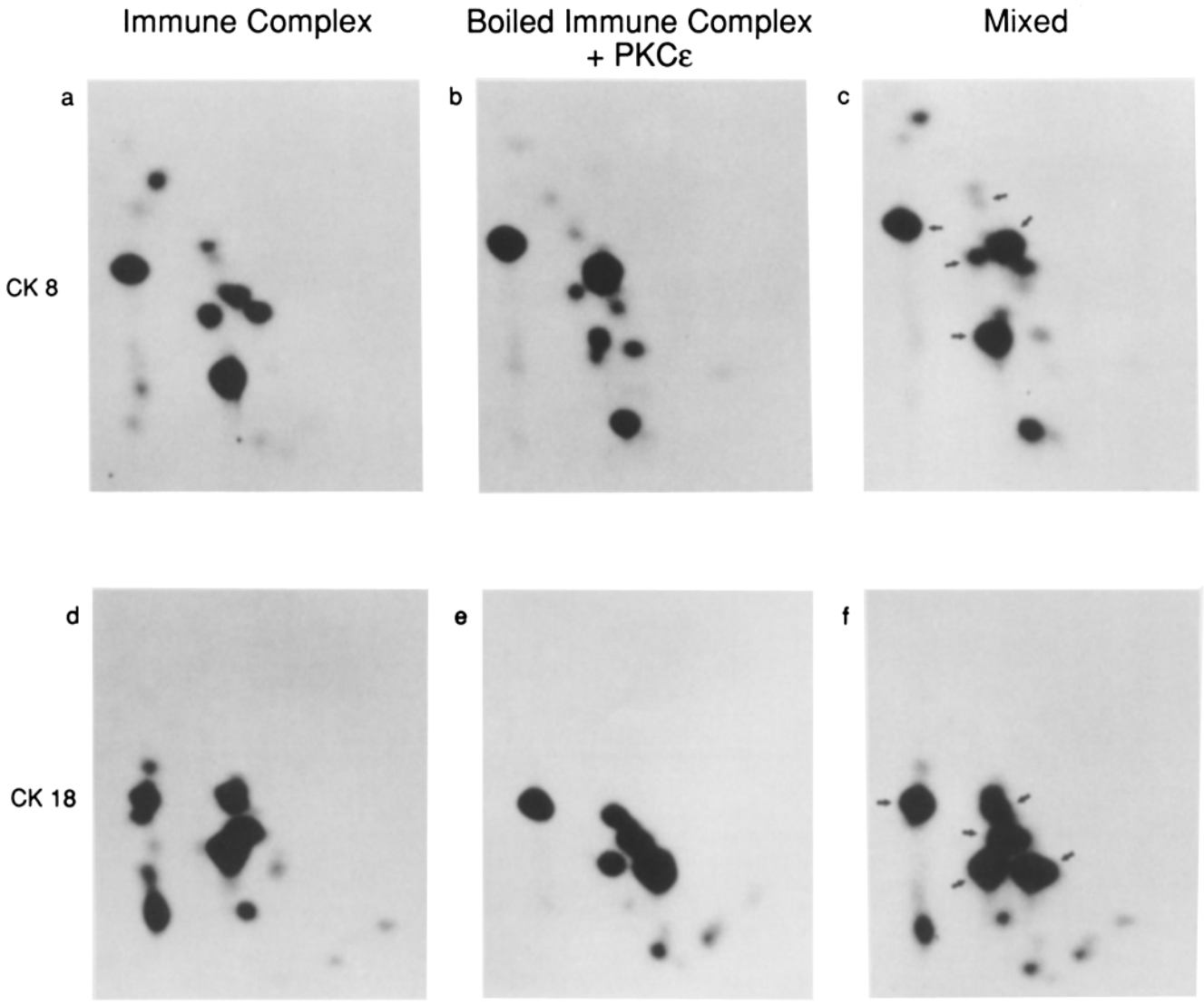

Figure 4. Tryptic phosphopeptide analysis of CK8 and 18. Immunoprecipitates of CK8/18 were first prepared followed by phosphorylation directly using an immune complex kinase assay or phosphorylation by adding purified rat brain PKCε to preboiled immunoprecipitates. Individual polypeptides corresponding to CK8 or CK18 were electroeluted from SDS–polyacrylamide gels and processed for peptide mapping as described in Materials and Methods. Approximately 2,000 cpm of phosphorylated immune complex or preboiled immune complex plus rat brain PKCε or a mix of each of the labeled cytokeratin was loaded onto the cellulose plate followed by electrophoresis in the horizontal axis and chromatography in the vertical axis. Arrows indicate shared peptides.

CK8 and 18 Associate with the Catalytic Fragment of Brain PKCε and with the SCLC 40-kD Kinase In Vitro

We tested whether the association of the 40-kD PKCε immunoreactive protein from HT29 cells with CK8/18 was a property of a PKCε catalytic fragment regardless of its tissue of origin. To address this we designed a reconstitution experiment using either the brain PKCε catalytic domain generated by limited proteolysis or the PKCε 40-kD fragment from NCI-N417 cells which lack CK8 and 18, and purified bovine cytokeratins 8 and 18 that lacked any residual bound PKCε (as determined by immunoblotting, data not shown). Previous studies showed that trypsin treatment of PKCε generated a catalytic fragment of 50–60 kD depending on the trypsin concentration used (Schaap et al., 1990). Treatment of purified rat brain PKCε with trypsin generated two major immunoreactive species (Fig. 7, lanes *1* and *2*), that displayed kinase activity as shown previously (Baxter et al., 1992). Incubation of the trypsin generated catalytic fragment of PKCε with CK18 followed by cytokeratin immunoprecipitation and Western blotting with the anti-PKCε antibody resulted in the identification of the PKCε catalytic fragment which physically associated with CK18 immunoprecipitates (Fig. 7, lane *3*). There was no association of the 40-kD species with actin (Fig. 7, lane *7*) nor with an irrelevant control antibody (Fig. 7, lane *6*). Identical results were obtained after incubation of the purified CK18 with a homogenate of NCI-N417 cells (used as a source of the 40-kD PKCε catalytic fragment) (Fig. 7, lane *5*). When CK8 was incubated with either the tryptic catalytic fragment of brain PKCε or with the NCI-N417 cell homogenate, the results were similar to those obtained with CK18 (data not shown).

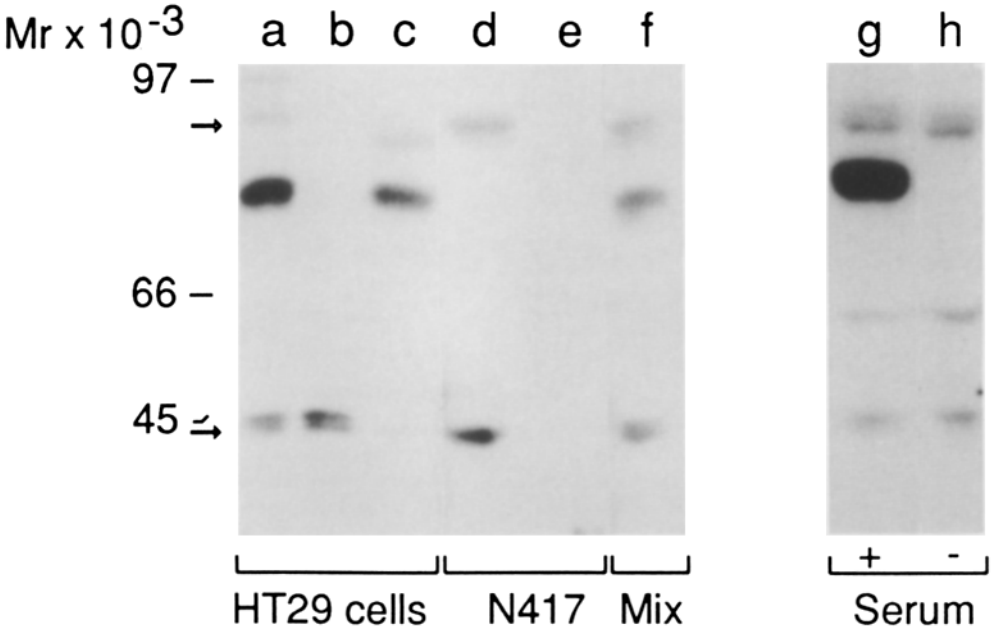

Figure 5. CK8/18 associated kinase is a PKCε immunoreactive protein. Equal amounts of protein (4 mg/ml) from detergent lysates of HT29 or NCI-N417 cells were used to immunoprecipitate CK8/18 using mAb L2A1. Immunoprecipitates (lanes *b* and *e*), detergent lysates (lanes *a*, *d*, and *f–h*; 150 μg from each cell line or 75 μg from each for the mix) or the remaining of the lysate after removal of the immune complexes (lane *b*) were analyzed on SDS–polyacrylamide gels followed by Western blot analysis using rabbit anti-PKCε antibody, as described in Materials and Methods. Arrows correspond to the 90-kD PKCε and the 40-kD immunoreactive species. Lanes *g* and *h* show an identical blot using 150 μg of a detergent lysate of HT29 cells grown without serum for 12 h or with serum.

Phosphorylation of CK8/18 Is Blocked by Pseudo-ε Peptide in Intact Cells, Immunoprecipitates, and in a Reconstituted System

The physical association of the 40-kD kinase with CK8/18 immunoprecipitates and their phosphorylation in the absence of added PKC allosteric activators, suggested that a constitutively active PKCε-related kinase might play a role in CK8/18 phosphorylation in situ. To further test this notion, we used a synthetic peptide corresponding to the pseudosubstrate domain of PKCε (ERMRPRKRQAVRRRV) (House and Kemp, 1987), which inhibits PKCε (not shown) and the SCLC 40-kD kinase (Baxter et al., 1992). Introduction of pseudo-ε into intact HT29 cells by scrape loading resulted in a decrease in CK phosphorylation (Fig. 8 *A*). Similarly, this synthetic peptide blocked CK8/18 phosphorylation in immunoprecipitates (Fig. 8 *B*), or when purified CK18 (Fig. 8 *C*) or CK8 (not shown) were incubated with purified rat brain PKCε.

Discussion

The salient finding of the present investigation is that a 40-kD PKCε–related kinase is associated with CK8 and 18 in HT29 colonic epithelial cells. Biochemical and immunological data are presented demonstrating that CK8 and 18 physically associate with a catalytic fragment of PKCε constitutively present in these cells. The association was assessed in CK8/18 immunoprecipitates by Western blotting with an anti-PKCε antiserum and by the kinase activity that was shown to be associated with the cytokeratin immunoprecipitates. Moreover, we were able to mimic such an association in a reconstituted system, using purified components such as CK8 or CK18 and the proteolytic fragment of brain PKCε containing the catalytic domain. In addition, double immunofluorescent staining shows that a PKCε immunoreactive species and CK8/18 colocalize in HT29 cells.

We have recently demonstrated the constitutive presence of a 40-kD PKCε–related kinase in several SCLC cell lines (Baxter et al., 1992). Partial purification followed by assessment of the kinase activity indicated that the 40-kD kinase from NCI-N417 cells, an established SCLC cell line, is independent of PKC activators, suggesting it might be a constitutively active catalytic fragment of PKCε. Moreover, peptide mapping with V8 protease from *S. aureus* and the inhibitory action of pseudo ε and staurosporine further indicated its similarity with the catalytic domain of brain PKCε (Baxter et al., 1992).

Here we show that a 40-kD PKCε immunoreactive protein is constitutively present, along with the 90-kD PKCε, in the epithelial colonic cancer cell line HT29. Several criteria suggest that the 40-kD protein present in HT29 cells is similar to or identical with the 40-kD kinase identified before in NCI-N417 cells. First, it is recognized by a PKCε antibody made against the carboxy-terminal region, i.e., the kinase-containing domain of PKCε. Second, they co-migrate in SDS–polyacrylamide gels when mixed. Third, both cell types contain a 90- and a 40-kD PKCε immunoreactive species, which are related if not identical as was shown by peptide mapping (Baxter et al., 1992). Here we add a new criterion, namely, physical association with CK8/18. We show that, regardless of the source of the PKCε catalytic fragment, i.e., brain PKCε tryptic fragment, NCI-N417 cell homogenate or HT29 cells, they all have the ability to physically associate with these intermediate filament proteins.

The Western blotting and double immunofluorescent data does not distinguish between preferential binding of PKCε to CK8 or CK18. However, incubation of purified CK8 or CK18 with the catalytic fragment of purified brain PKCε showed similar association with both cytokeratins. An association of

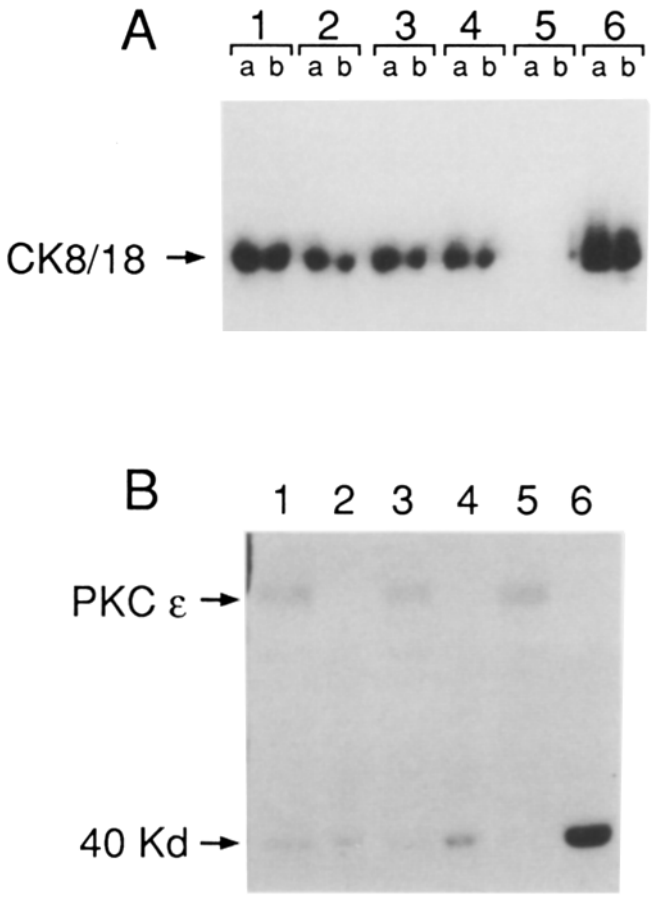

Figure 6. Efficiency of CK8/18 solubilization: cosegregation of the 40-kD PKCε kinase with the CK fraction. Immunoblots are shown of nonionic detergent solubilized and remaining nonsolubilized material from HT29 cells using anti-CK8/18 mAb's (*A*) L2A1 (lanes *a*) and CK5 (lanes *b*) and rabbit anti-PKCε antibody (*B*). Solubilization conditions were as described in Materials and Methods. For each lane 20 μg of protein were loaded. Lane *1*, supernatant of 1% NP40 soluble fraction (45 min); lane *2*, insoluble fraction after 45 min solubilization with 1% NP-40; lane *3*, supernatant of 1% TX-100 soluble fraction (45 min); lane *4*, insoluble fraction of lane *3*; lane *5*, supernatant of 1% TX-100 solubilized fraction (2 min); lane *6*, remaining material after high salt extraction of the insoluble fraction of lane *5*. Of note, the high salt extract contained minimal cytokeratin as determined after dialysis to remove the salt then blotting (not shown).

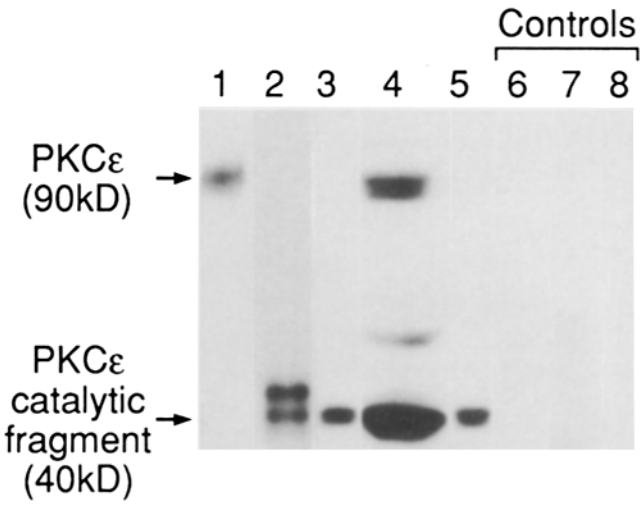

Figure 7. Tryptic fragment of purified brain PKCε or PKCε-related kinase from NCI-N417 cells associate with purified cytokeratin 18. The figure shows results of Western blot analysis using rabbit anti-PKCε antibody. Purified brain PKCε (500 ng) was incubated with trypsin (100 μg/ml) for 10 min, 22°C in the presence of BSA (400 μg/ml) in 50 μl. Protease inhibitors were added (0.1 mM PMSF 2 μl, 10 mM leupeptin 1 μl, 10 mM pepstatin 1 μl, 5 mg/ml soybean trypsin inhibitor 10 μl) followed by adding 10 μg of actin or purified bovine CK18 in 150 μl of 1% NP-40. After a 1-h incubation, immunoprecipitates were prepared using mAb to CK8/18 (lane *3*), actin (lane *7*), and an irrelevant antigen (immunoglobulin isotype matched control) (lane *6*). For coincubation with NCI-N417 NP-40 lysate, 200 μl of lysate (100 μg protein) were incubated with 10 μg of purified bovine CK18 for 1 h followed by immunoprecipitation using mAb to CK8/18 (lane *5*) or isotype matched antibody (lane *8*). Lane *1* shows 10 ng of intact PKCε, lane *2* shows 100 ng of trypsin digested PKCε and lane *4* shows 150 μg of NCI-N417 lysate.

PKCε with CK8 and CK18 would require binding to regions of structural or conformational similarity. Comparison of the amino acid sequence of CK8 (Krauss and Franke, 1990) with CK18 (Kulesh and Oshima, 1989) showed 31% overall homology. Most of the homology occurs in the rod region of the cytokeratin (Steinert and Roop, 1988) which consequently represents one potential site of CK8/18-PKCε 40-kD species association.

Although HT29 cells express both the 90- and 40-kD PKCε immunoreactive proteins, only the 40-kD protein remains associated with CK8/18 after immunoprecipitation. Furthermore, although only the PKCε (90 kD) species is seen in the soluble fraction (Fig. 6 *B*, lane *5*) which does not contain any cytokeratin (Fig. 6 *A*, lane *5*), only the 40-kD species is found in the cytokeratin containing particulate fraction (Fig. 6 *B*, lane *6*) and in detergent insoluble fractions (Fig. 6 *B*, lanes *2* and *4*). Similarly, although the NCI-N417 cell homogenate contains both the 90-kD PKCε and the 40-kD fragment, only the 40-kD protein from NCI-N417 cell homogenates associates with purified CK8 or CK18 after coincubation. Moreover, purified CK8 and 18 physically associate with the brain catalytic fragment of PKCε in a reconstituted system. Further studies will be needed to determine the sequence of events in the association of PKCε immunoreactive species with CK8/18. For example, it is unclear whether PKCε binds CK8/18 initially then becomes converted to a smaller catalytic fragment or whether the 40-kD fragment which is present constitutively in these cells (Fig. 5 and 6) associates directly with CK8/18.

If the 40-kD species does indeed represent a fragment of PKCε, the question arises as to its functional significance. Several potential physiological roles for the proteolysis of PKC have been proposed (Kishimoto et al., 1989; Kikkawa et al., 1989; Pontremoli et al., 1990). These include either the generation of a catalytically independent, constitutively active form of PKC or the generation of one or more fragments as an initial step towards complete degradation. Our data in the NCI-N417 (Baxter et al., 1992) and HT29 cells (present study) show that the 40-kD kinase is constitutively present, thus suggesting it may play a physiologically relevant role. Its physical association with the IF CK8 and 18 and their phosphorylation in situ points to such a possibility. Although the role of phosphorylation in intermediate filament

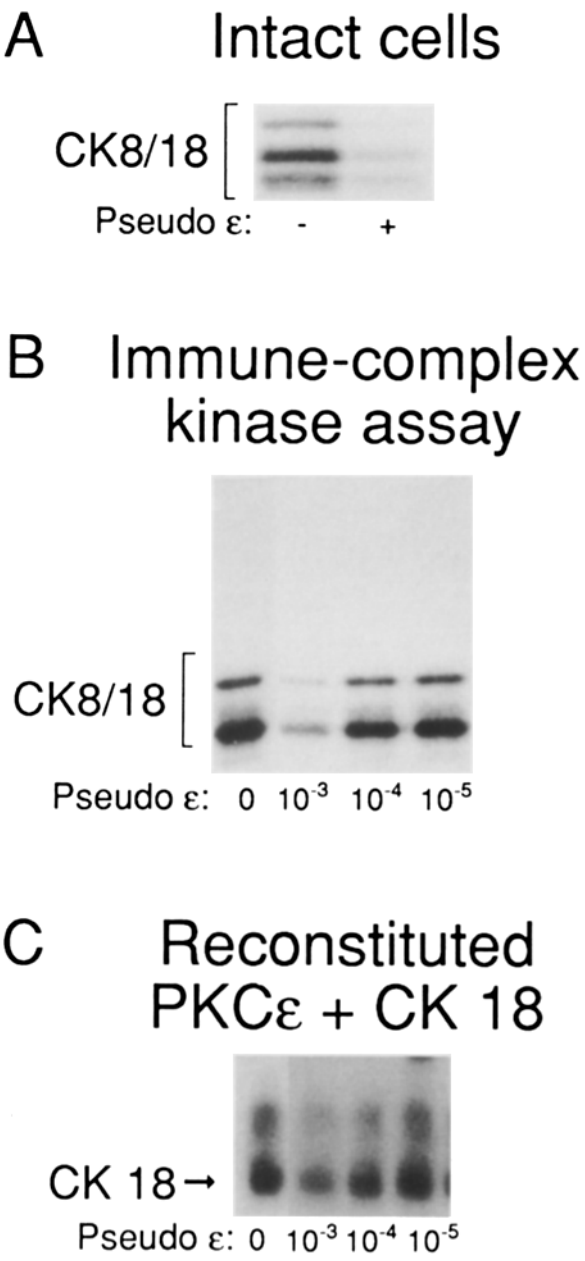

Figure 8. Effect of PKCε pseudosubstrate on CK8/18 phosphorylation. (*A*) HT29 cells (4×10^6) were scraped then incubated in 1 ml of phosphate-free medium with 10^{-4} M PKCε pseudosubstrate peptide for 1 h, followed by the addition of 250 μCi of [^{32}P]orthophosphate. After 90 min of labeling, cells were solubilized followed by immunoprecipitation of CK8/18 and analysis by SDS-PAGE and autoradiography. (*B*) Immunoprecipitates of CK8/18 were obtained from HT29 cells then used in a kinase assay in the presence of the indicated amounts of PKCε pseudosubstrate. (*C*) Purified rat brain PKCε (100 ng) was mixed with 1 μg of bovine CK18 in the presence of the indicated amounts of PKCε pseudosubstrate peptide.

function is not fully understood, phosphorylation does appear to play a role in the assembly and disassembly of vimentin and desmin (Inagaki et al., 1987, 1988). Alternatively, binding of the fragment to the cytokeratin may protect the catalytic domain from further degradation and provide a kinase that has different substrate and regulatory requirements.

Immunofluorescent staining of HT29 cells using both anti-CK8/18 and anti-PKCε antibodies showed a similar staining profile with immunofluorescence seen in both the cell surface and cytoplasm. Since the anti-PKCε antibody is directed to the carboxy terminus, it should recognize intact as well as fragments of PKCε containing only the catalytic domain. Leach et al. showed that staining of phorbol ester–treated NIH-3T3 cells with antibodies recognizing the catalytic domain of PKC-α resulted in cell surface and cytoplasmic staining (Leach et al., 1989). In contrast, only cytoplasmic staining was noted when anti-regulatory domain antibodies were used. Although an anti-PKCε antibody that is specific for the regulatory domain is lacking at present, our Western blot data would predict a different staining pattern with such an antibody, since only the 40-kD kinase and not intact PKCε associated with CK from HT29 cell homogenates or in a reconstituted system.

Several reports presented evidence for the association of kinases with cytoskeletal elements. For example, immunofluorescent techniques were used to show that PKC-α associated with the focal contact proteins vinculin and talin (Jaken et al., 1989). No association was seen with actin or with the intermediate filament protein examined in that study, namely vimentin. The intermediate filament proteins, lamins, were also shown to be associated with a Ca^{2+}- and cAMP-independent kinase activity although the identity of the kinase was not determined (Dessev et al., 1988). In addition, cytoskeletal preparations from adrenal cells were shown to be associated with PKC (Papadopoulos and Hall, 1989) and with cAMP-dependent protein kinase (Osawa and Hall, 1985). Furthermore, the protooncogene product pp39^{mos} was recently shown to associate with and phosphorylate tubulin (Zhou et al., 1991).

The phosphorylation data presented herein suggest that CK8 and 18 are substrates for a PKCε-related kinase. We previously showed that CK8/18 phosphorylation was calcium independent as determined by the lack of effect of ionomycin and the calcium chelator BAPTA/AM (Chou and Omary, 1991). Therefore, other calcium-independent PKC subspecies, such as PKCζ which is present in HT29 cells (not shown), may also play a role in cytokeratin phosphorylation. It remains to be determined if PKCε or the 40-kD PKCε–related kinase or both act as the CK8/18 kinases. Evidence supporting a role for the 40-kD PKCε catalytic fragment is the phosphorylation of CK8/18 in intact cells in the absence of a PKC activator, the inhibition of CK8/18 phosphorylation in intact cells and immunoprecipitates by the PKCε pseudosubstrate peptide and its physical association with CK8/18. Furthermore, comparison of the tryptic phosphopeptide pattern of CK8/18 obtained from in vivo labeled cells and from in vitro labeled immune complexes showed overlap of some of the peptides (not shown). However, the effect of PKCε pseudosubstrate on CK8/18 phosphorylation may partially affect other PKC subspecies since pseudosubstrate peptides are not entirely selective in their inhibition of different PKC isoforms (not shown and Schaap et al., 1989).

Our data suggest that the association between the 40-kD PKCε catalytic fragment and CK8/18 may not obey the rules of a "classical" enzyme-substrate interaction: (*a*) it exists in

cells that were not pre-activated (Fig. 2); (*b*) it can be reproduced by the simple coincubation of the purified components (Fig. 7), in the absence of any cofactors; (*c*) the complex cosegregates under several harsh conditions, including high salt and detergent extraction (Fig. 2 and 6); (*d*) the association does not change in the presence or absence of phosphorylating conditions (not shown). In conclusion, we have shown that the PKCε catalytic fragment physically associates with and phosphorylates CK8/18 HT29 cells. The nature of this association and its physiological significance remain to be determined.

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