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Purification of two distinct proteins of approximate M_r 80000 from human epithelial cells and identification as proper substrates for protein kinase C

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A M_r-80000 acidic phosphoprotein ('80K protein') is a specific substrate for protein kinase C. We attempted to purify the 80K protein from a human squamous-cell carcinoma cell line, Ca9-22, by the sequential use of heat treatment, $(NH_4)_2SO_4$ precipitation, Mono Q column chromatography, proRPC column chromatography and gel filtration. The 80K protein was assayed by phosphorylation *in vitro* by using partially purified human type III protein kinase C, and was fractionated into two distinct molecular species with slightly different M_r values, designated 80K-L and 80K-H proteins. Phosphorylation occurred mainly at serine residues of these proteins. Two-dimensional phosphopeptide maps after trypsin digestion and kinetic profiles of phosphorylation were different from each other. Ca^{2+}- and phospholipid-dependency of the phosphorylation *in vitro* confirmed that both 80K-L and 80K-H proteins are true substrates for three subtypes of protein kinase C. The 80K-L protein was a preferential substrate for type III protein kinase C, and the 80K-H protein was phosphorylated more effectively by type I and type II protein kinase C. The possible roles of these two distinct 80K proteins in signal transduction are discussed.

INTRODUCTION

Protein phosphorylation is postulated to play an important role in the initial steps of signal transduction induced by the binding of growth factor to receptor (for review see Rozengurt [1]). The Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C) is thought to be a prominent kinase in signal transduction, because platelet-derived growth factor (PDGF), fibroblast growth factor and epidermal growth factor (EGF) enhance phosphatidylinositol (PtdIns) turnover, activating protein kinase C [2–4]. Other signals, including expression of the proto-oncogenes c-*myc* and c-*fos*, are mediated via protein kinase C [5]. Although the entire cascade of protein phosphorylation has not been determined, the M_r-80000 acidic phosphoprotein ('80K protein') is thought to be an integral target protein [2–8].

Phosphorylation of the 80K protein is enhanced in intact cells by the addition of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) in some fibroblast and cancer cell lines [2–6,8]. Purified protein kinase C, moreover, can phosphorylate the 80K protein *in vitro* in synaptosome membrane fractions [7]. The 80K protein was detected at M_r 70000–90000 in previous studies [2–10]. They seem to be the same proteins, because they share some common characteristics: they have similar M_r and pI values, and they are substrates for protein kinase C. In the present paper, we use '80K protein' as the abbreviation for the most prominent cellular substrates for protein kinase C, the M_r-70000–90000 proteins. Furthermore, there exists a heterogeneity in 80K protein, which was detected in Swiss 3T3 cells [11] and rat [9,12] and bovine [13] brain. Up to now, 80K protein has been purified from rat [11,12] and bovine [14] brain. We also attempted to purify the 80K protein substrate for protein kinase C from a human squamous-cell carcinoma cell line, Ca9–22. In Ca9-22 cells, EGF receptors are overproduced, and not only TPA but also EGF can enhance the 80K protein phosphorylation [4], as observed in A431 cells [3]. A series of chromatographic procedures resulted in the two separate fractions of 80K protein, designated as 80K-H and 80K-L proteins. Phosphopeptide mapping and phosphorylation patterns by three subtypes of protein kinase C indicate that these are two distinct molecular species with an M_r of approx. 80000. Recently, we isolated cDNAs encoding human 80K-H protein [15]. Stumpo *et al.* [16] also isolated cDNAs encoding bovine 80K protein (termed MARCKS). Our preliminary results of peptide sequencing of human 80K-L protein indicated that the 80K-L protein in human was the MARCKS protein in bovine. These results strongly suggested that two distinct molecular species existed as protein kinase C substrates, with an M_r of approx. 80000. The possible roles of these two distinct 80K proteins in signal transduction are discussed.

MATERIALS AND METHODS

Purification of protein kinase C

Protein kinase C was partially purified from human cancer cells, UCVA-1 and A549 cells [17], or from rat brain by the published method [17,18]. Briefly, cells or tissues were homogenized in buffer A [20 mM-Tris/HCl (pH 7.5), 0.25 M-sucrose, 10 mM-EGTA, 2 mM-EDTA and leupeptin (20 μg/ml)]. The homogenate was clarified by centrifugation at 100000 rev./min (356160 *g*) for 15 min with a Beckman TLA100.2 rotor. The supernatant was applied to a Mono Q column (HR5/5; Pharmacia, Uppsala, Sweden), equilibrated with buffer B [20 mM-Tris/HCl (pH 7.5), 0.5 mM-EGTA, 0.5 mM-EDTA, 10 mM-2-mercaptoethanol]. Protein kinase C was eluted from the Mono Q column with a linear gradient of NaCl. The fractions containing protein kinase C activity were then applied to a hydroxyapatite column (type S; Koken, Tokyo, Japan), equilibrated with buffer C [20 mM-potassium phosphate buffer (pH 7.5), 0.5 mM-EGTA, 0.5 mM-EDTA, 10% (v/v) glycerol, 10 mM-2-mercaptoethanol]. The protein kinase C was eluted with a linear gradient (20–280 mM) of potassium phosphate in buffer C, and activity-peak fractions were collected and stored as small batches at −70 °C in buffer C containing 20% glycerol and 0.05% Triton

Abbreviations used: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; TPCK, *N*-tosyl-L-phenylalanylchloromethane.

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X-100. No noticeable loss of kinase activity was detected, at least up to 3 months. Three types of protein kinase C (I, II and III) were purified from a rat brain as described above, and further purified by using anti-protein kinase C subtype-specific monoclonal antibody (MBL, Nagoya, Japan) [19] bound to Protein A–Sepharose CL-4B beads (Pharmacia). Each subtype of protein kinase C immobilized on beads was directly used for phosphorylation assay. There were no contaminating substrates in these purified protein kinase C preparations [4].

Phosphorylation of the 80K protein and histone IIIS with protein kinase C

The standard reaction mixture for 80K protein phosphorylation (in a final volume of 25 μl) contained 20 mM-Tris/HCl (pH 7.5), 10 mM-$MgCl_2$, 25 μg of phosphatidylserine (PtdSer)/ml, 4 μg of TPA/ml, 0.2 mM-$CaCl_2$, 2 μM-[γ-^{32}P]ATP (50 Ci/mmol; Amersham International, Amersham, Bucks., U.K.), purified protein kinase C and a sample of column fraction. The reaction was initiated by addition of ATP, and incubation was continued for 15 min at 30 °C. The reaction was terminated by the addition of 10 μl of 4-fold-concentrated Laemmli's sample buffer [20], followed by heating in boiling water for 5 min. The phosphorylated proteins were resolved by SDS-PAGE (7.5%-acrylamide gels) and detected by autoradiography. The 80K protein band was cut from the gel and the incorporated [^{32}P]phosphate was quantified by Čerenkov counting. The reaction mixture for histone-IIIS phosphorylation (final volume 100 μl) was the same as for the 80K-protein phosphorylation, except that 40 μg of histone IIIS was added instead of 80K protein. The reaction was initiated by addition of 9 μM-[γ-^{32}P]ATP (1.1 Ci/mmol) and terminated by applying 75 μl on to a phosphocellulose P81 filter (3 cm × 3 cm; Whatman, Maidstone, Kent, U.K.). The filters were then washed with four changes of water, air-dried, and the radioactivity was counted as above. The protein kinase inhibitors H-7 [21] and staurosporine [22] were obtained from Seikagaku Kogyo (Tokyo, Japan) and Kyowa Hakko (Tokyo, Japan) respectively.

Purification procedure for the 80K protein

Ca9-22 cells [(5–10) × 10^9 cells], derived from a human tongue squamous carcinoma [23], were homogenized in buffer A with a Teflon homogenizer. The homogenate was clarified by centrifugation at 100000 rev./min (356160 *g*) for 15 min with a TLA100.2 rotor. The supernatant (S100 fraction) was kept frozen at −70 °C until use. The S100 fraction was heated for 10 min in boiling water, quickly cooled and incubated for 30 min in ice/water, and then centrifuged at 14500 rev./min (17000 *g*) for 40 min with a TOMY No. 4 rotor. The supernatant was first precipitated by the addition of solid $(NH_4)_2SO_4$ to 30% saturation at 4 °C, and centrifuged at 14500 rev./min for 40 min. This supernatant was then precipitated by the addition of solid $(NH_4)_2SO_4$ to 65% saturation, and centrifuged at 14500 rev./min for 40 min. The precipitate was suspended in a minimal volume of dialysis buffer [20 mM-Tris/HCl (pH 7.5), 0.5 mM-EGTA, 0.5 mM-EDTA, 10 mM-2-mercaptoethanol, 0.1 mM-phenylmethanesulphonyl fluoride], and dialysed against two changes of dialysis buffer at 4 °C by using Spectra/Por 7 (cut-off M_r 50000; Spectrum, Los Angeles, CA, U.S.A.) for 18 h. The dialysis residue was applied to a Mono Q column (Pharmacia), equilibrated with buffer B, and the proteins were eluted with a linear gradient of 0.2–0.6 M-NaCl (in buffer B) at a flow rate of 1 ml/min (Fig. 1). The fractions (0.5 ml each) containing the 80K protein were pooled and applied to a proRPC column (HR5/10; Pharmacia) equilibrated with 0.1% trifluoroacetic acid in water. The proteins were eluted with a linear gradient of 20–50% acetonitrile. Gel filtration on a TSKG3000SW gel column (LKB, Bromma, Sweden) was performed with 0.1% trifluoroacetic acid in water.

Cell culture

Ca9-22 cells were grown in Dulbecco's modified Eagle's medium containing 8% (v/v) fetal-calf serum (Boehringer, Mannheim, Germany), in either 15 cm-diam. dishes or a multi-tray culture system [24].

Two-dimensional PAGE and phosphopeptide mapping

Two-dimensional PAGE, phosphopeptide mapping using *N*-tosyl-L-phenylalanylchloromethane (TPCK)-treated trypsin and phosphoamino acid analysis were performed as described in refs. [4,25–27].

RESULTS

Purification of the 80K protein

To purify the 80K protein from Ca9-22 cell homogenates, we employed heat treatment (100 °C for 10 min), $(NH_4)_2SO_4$ fractionation (30–65% saturation) and size-exclusion dialysis using Spectra/Por 7 tubing, which removes proteins with M_r less than 50000. The dialysis residue was then applied to a Mono Q column (Fig. 1). Each fraction was assayed for the presence of 80K protein by using phosphorylation *in vitro* by type III protein kinase C partially purified from human cancer cells, because that protein kinase C is dominant in Ca9-22 cells, and then phosphoproteins were separated on SDS-PAGE (Fig. 1*b*). As shown in Fig. 1(*a*), the 80K protein was eluted as a broad peak with a small shoulder. The broad peak fractions (12–16) were combined and analysed on a proRPC column (Figs. 2*a* and 2*b*). The 80K protein was eluted as a single peak. Similarly, the small shoulder fractions (17 and 18 in Fig. 1*a*) were combined and applied to a proRPC reverse-phase column. As shown in Figs. 2(*c*) and 2(*d*), the 80K protein was clearly separated into two distinct peaks. These peak fractions showed slightly different M_r values on SDS-PAGE, as clearly shown below in Fig. 3, and therefore were designated the 80K-L (the first peak, containing fractions 12–14 in Fig. 2*c*) and the 80K-H (the second peak, containing fractions 18 and 19 in Fig. 2*c*). The 80K-H protein from the second peak fractions was found to be homogeneous when analysed on SDS-PAGE followed by silver staining (results not shown). The 80K-L protein from the first peak fractions was purified by further use of a TSKG3000SW gel-filtration column (results not shown), and also found to be homogeneous when judged by SDS-PAGE followed by silver staining (results not shown). By this purification procedure, 3 μg of 80K-L and 60 μg of 80K-H proteins were obtained from 256 mg of proteins in the S100 fraction.

Analysis of the phosphorylated 80K protein

The purified 80K-L and 80K-H proteins were individually phosphorylated *in vitro* by using purified type III protein kinase C, and the reaction products were analysed by two-dimensional PAGE (Fig. 3). The 80K-H protein migrated as a condensed spot (Fig. 3*b*), whereas the 80K-L protein migrated diffusely, as though still heterogeneous (Fig. 3*a*). A mixture of the 80K-H and 80K-L proteins clearly showed a composite profile (Fig. 3*c*). When a cytosol fraction from Ca9-22 cells was incubated with type III protein kinase C, two phosphorylated spots corresponding to the 80K-H and 80K-L species were detected (Fig. 3*d*). Phosphoamino acid analysis revealed that phosphorylation of the 80K protein occurs exclusively on serine residues in both molecular species (results not shown).

The 80K-H and 80K-L proteins which were phosphorylated *in vitro* by type III protein kinase C were completely digested with

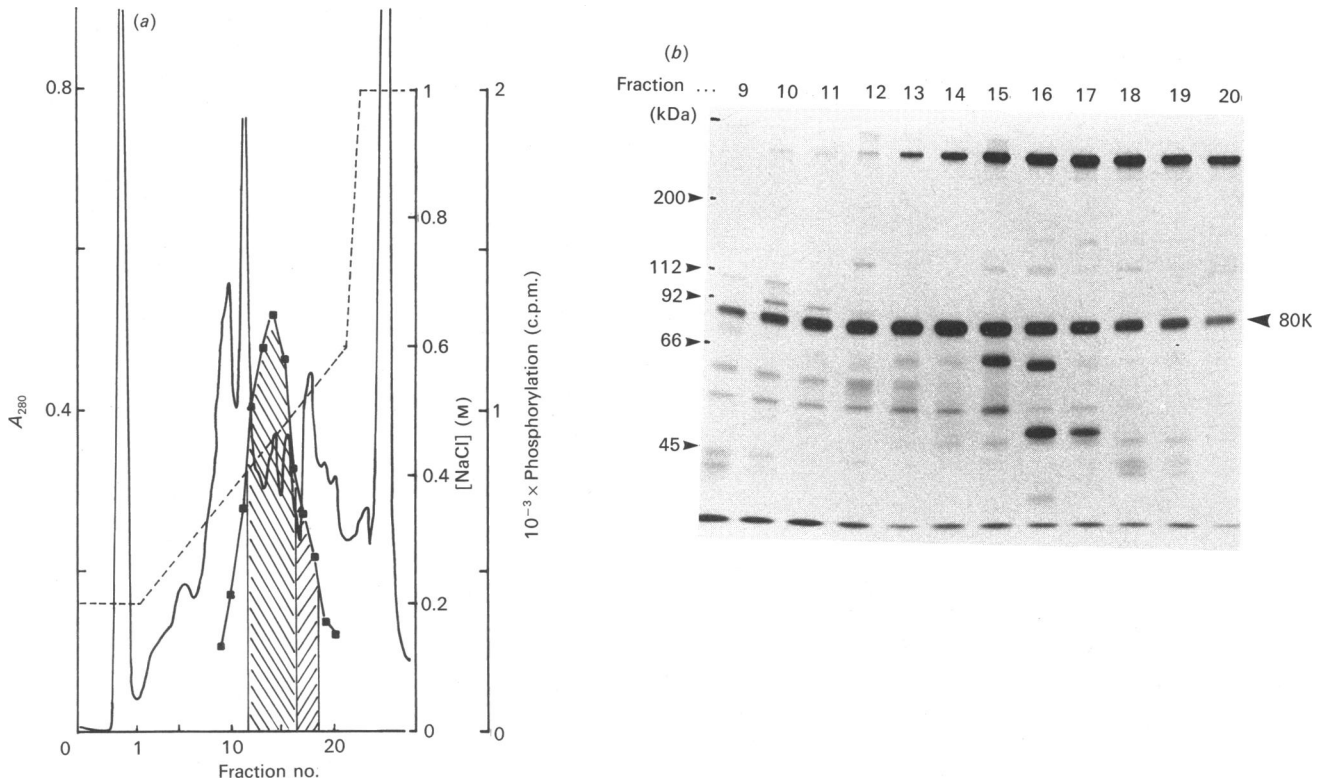

Fig. 1. Mono Q column chromatography

The dialysis residue from Spectra-Por 7 was applied to a Mono Q column and eluted with a linear gradient of 0.2–0.6 M-NaCl at a flow rate of 1 ml/min (*a*); 0.5 ml fractions were collected. Proteins were phosphorylated by type III protein kinase C *in vitro* and assayed by SDS-PAGE (7.5% gel) (*b*). ■, Phosphorylation of 80K protein; ----, NaCl gradient; ——, A_{280}.

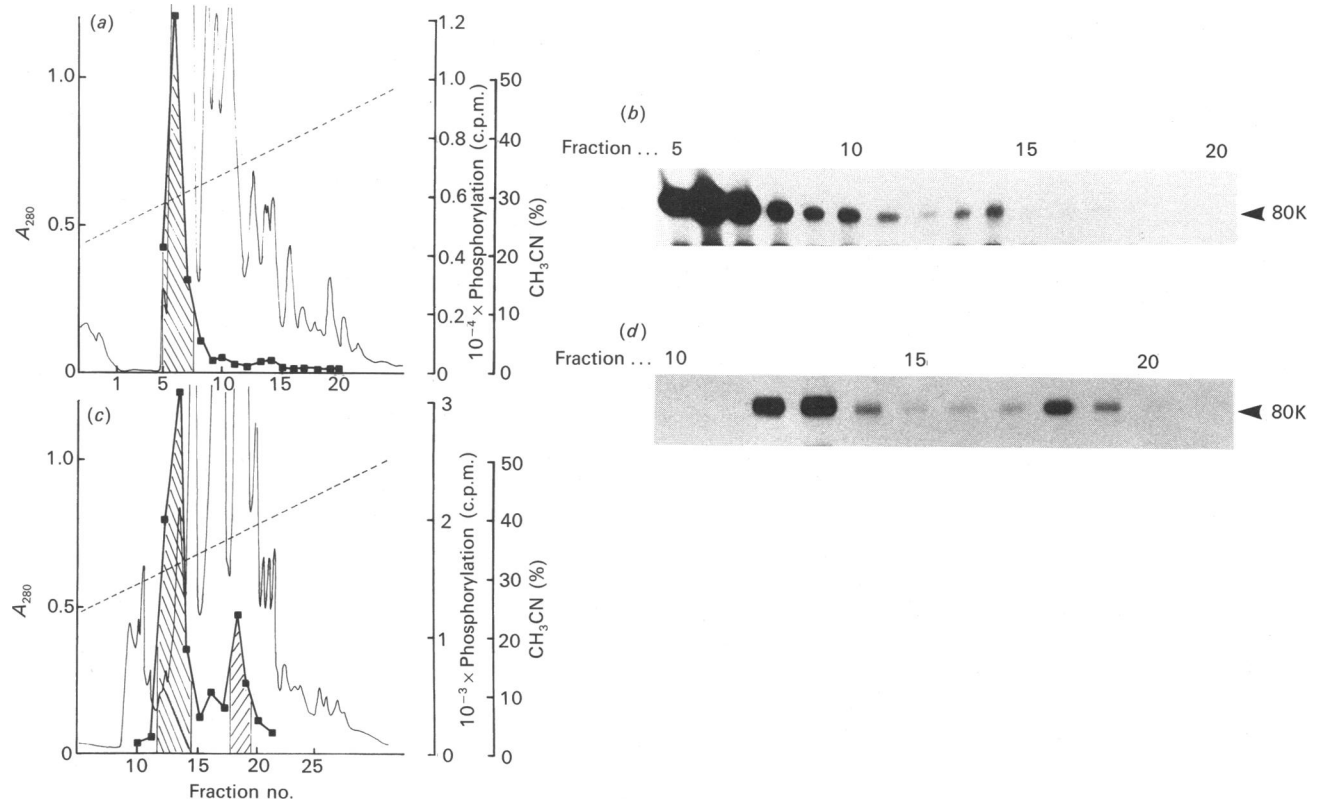

Fig. 2. ProRPC column chromatography

The fractions containing 80K proteins in Fig. 1 were applied to a proRPC column and eluted with a linear gradient of 20–50% acetonitrile (CH_3CN) at a flow rate of 0.25 ml/min; 0.5 ml fractions were collected and assayed by SDS-PAGE. (*a*) and (*b*) Fractions 12–16 from Fig. 1; (*c*) and (*d*), fractions 17 and 18 from Fig. 1. ■, Phosphorylation of 80K protein; ----, CH_3CN gradient; ——, A_{280}.

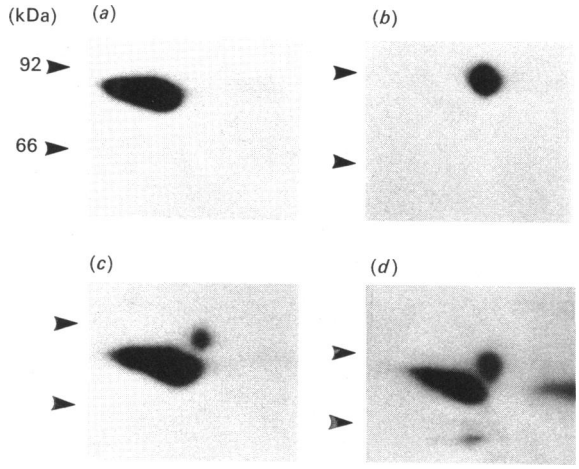

Fig. 3. Two-dimensional PAGE analysis of the purified 80K proteins

Samples were phosphorylated *in vitro* by protein kinase C and separated by two-dimensional PAGE as described in the Materials and methods section. (*a*) 80K-L protein; (*b*) 80K-H protein; (*c*) mixture of the 80K-H and 80K-L proteins after phosphorylation; (*d*) cytosol of Ca9-22 cells.

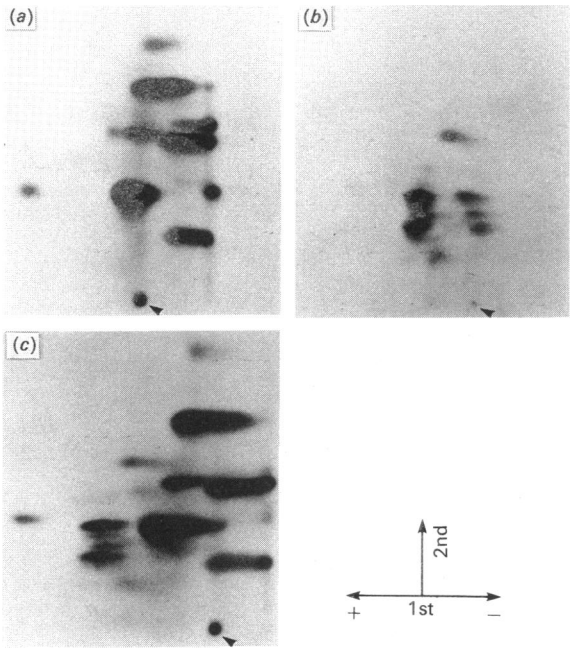

Fig. 4. Two-dimensional phosphopeptide maps of the 80K protein

The purified 80K-H and 80K-L proteins were phosphorylated *in vitro* by protein kinase C. The phosphorylated '80K proteins' were purified by SDS-PAGE, subjected to digestion with TPCK-treated trypsin (50 μg) at 37 °C for 24 h, and separated by thin-layer electrophoresis (1st) and chromatography (2nd). (*a*) 80K-L protein (817 c.p.m. loaded); (*b*) 80K-H protein (193 c.p.m.); (*c*) mixture of both 80K-H and 80K-L proteins (1256 c.p.m.). The exposure time with an intensifying screen was 4 days. The arrowheads indicate the origin.

TPCK-treated trypsin and analysed on two-dimensional t.l.c. plates. Figs. 4(*a*) and 4(*b*) show clear difference in the phosphopeptide maps of the two molecular species. There is no overlap of individual phosphopeptide components when mixed together (Fig. 4*c*).

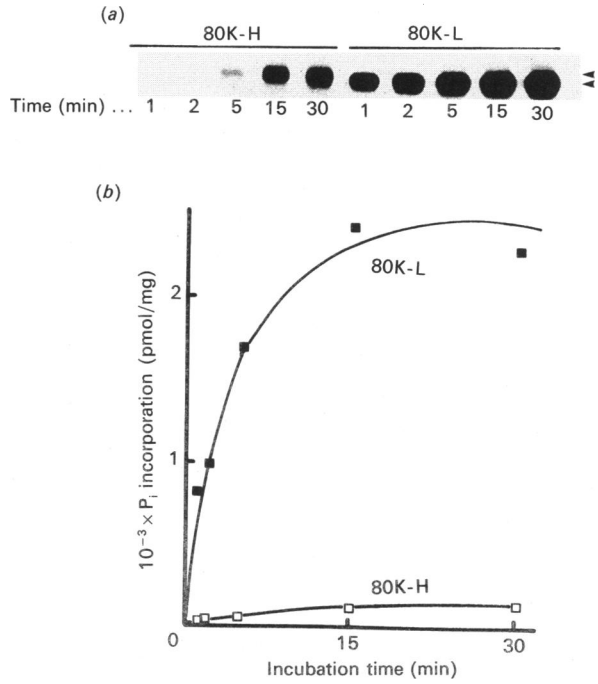

Fig. 5. Time course of 80K protein phosphorylation

The purified 80K-H (50 ng) and 80K-L (10 ng) proteins were phosphorylated *in vitro* by protein kinase C. Samples were removed from the reaction mixture and added to Laemmli sample buffer to terminate the reaction at the indicated times. Phosphoproteins were separated by SDS-PAGE (*a*), and the bands corresponding to the 80K proteins were excised and their radioactivity was measured by Čerenkov counting (*b*). ■, 80K-L protein; □, 80K-H protein.

Table 1. Ca^{2+}-, phospholipid- and TPA-dependency of 80K protein phosphorylation

The purified 80K-H (75 ng) and 80K-L (10 ng) proteins or histone IIIS (12 μg) were incubated with protein kinase C in the presence or absence of 2 mM-EGTA, 25 μg of PtdSer/ml, 4 μg of TPA/ml and 0.2 mM-$CaCl_2$. The incorporated radioactivity was quantified by Čerenkov counting. Results are averages of three experiments: S.E.M. is within 20%.

Addition				Protein kinase C substrate...	Phosphorylation (pmol/min per mg)		
TPA	PtdSer	Ca^{2+}	EGTA		Histone IIIS	80K-L	80K-H
			+		1.73	12.6	15.2
		+			2.02	7.40	6.58
	+		+		2.06	17.8	5.78
+			+		33.9	41.1	7.82
	+	+			60.6	163	5.87
+	+		+		41.1	168	7.38
+		+			34.6	72.7	8.80
+	+	+			73.3	130	12.5

Kinetic and enzymological characterization of the 80K protein phosphorylation

Fig. 5 shows the time course of the 80K protein phosphorylation *in vitro* by type III protein kinase C. Phosphorylation of both molecular species reached a plateau within 15 min, but the maximum phosphorylation level of the 80K-L protein was 10 times that of the 80K-H protein. Table 1 shows the Ca^{2+}-,

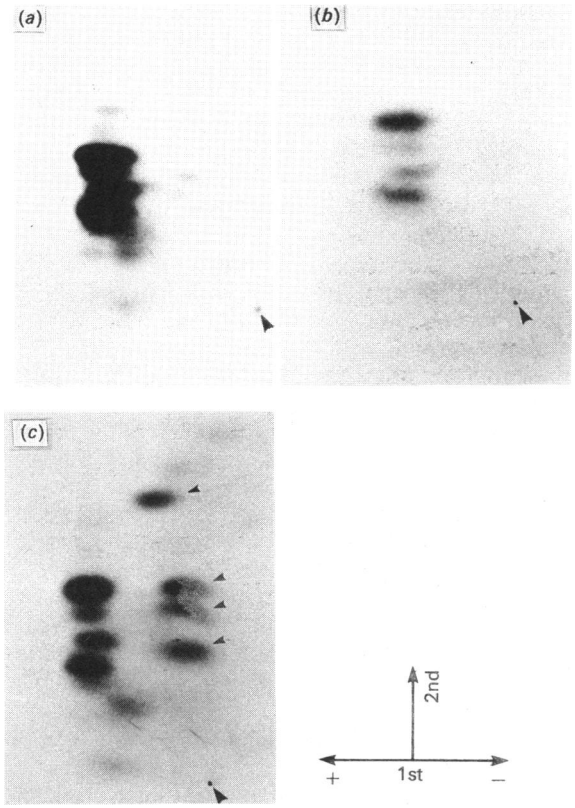

Fig. 6. Two-dimensional phosphopeptide maps of the 80K-H protein phosphorylated under different conditions

The purified 80K-H protein was incubated with protein kinase C in the presence or absence of various effectors. The phosphorylated 80K-H proteins were purified by SDS-PAGE, subjected to digestion with TPCK-treated trypsin (50 μg) at 37 °C for 24 h, and separated by thin-layer electrophoresis (1st) and chromatography (2nd). Samples were made in the presence of EGTA (*a*; 769 c.p.m.), Ca^{2+} (*b*; 64 c.p.m.) and Ca^{2+}, PtdSer and TPA (*c*; 579 c.p.m.). The exposure time with an intensifying screen was 2 days. The large arrowheads indicate the origin.

PtdSer- and TPA-dependency of the phosphorylation reaction. The 80K-L protein exhibited a profile of effector dependence similar to that of histone IIIS, whereas the 80K-H protein showed a unique profile. The 80K-H protein was phosphorylated to a high degree in the presence of EGTA and in the absence of Ca^{2+}, PtdSer and TPA. Phosphopeptide maps of the 80K-H protein indicate that phosphorylation in the presence of EGTA occurred mainly on four tryptic peptides (Fig. 6*a*), and this phosphorylation was greatly decreased in the presence of Ca^{2+} (Fig. 6*b*). Additionally, four tryptic peptides were phosphorylated in a Ca^{2+}-, PtdSer- and TPA-dependent manner (Fig. 6*c*; small arrowheads). These peculiar phosphorylation patterns were obtained by using a type III protein kinase C partially purified from human cancer cells. To elucidate further the specificity of 80K-H and 80K-L proteins, we performed the phosphorylation assay *in vitro* by using three subtypes (I, II and III) of protein kinase C partially purified from rat brain. As shown in Table 2, 80K-H protein was phosphorylated by rat type I and type II protein kinase C in a Ca^{2+}- and phospholipid-dependent manner. The 80K-H protein phosphorylation by a rat type III protein kinase C occurred in the presence of EGTA, as observed for human type III protein kinase C, although slight Ca^{2+}-dependency was also observed. The 80K-L protein was phosphorylated by three subtypes of protein kinase C in a Ca^{2+}- and phospholipid-dependent manner. However, when type III protein kinase C was affinity-purified by using anti-type III monoclonal antibody, Ca^{2+}- and phospholipid-dependent phosphorylation of the 80K-H protein was observed (Table 3). Phosphopeptide mapping analysis indicated that phosphorylation by type I and type II protein kinase C occurred only in the same tryptic peptides as those that were phosphorylated by type III protein kinase C in a

Table 2. Phosphorylation *in vitro* of 80K-L and 80K-H proteins by partially purified subtypes of protein kinase C

Protein kinase C was purified from a rat brain and separated into types I, II and III on a hydroxyapatite column. The purified 80K-L (10 ng) or 80K-H (50 ng) protein was incubated with each type of protein kinase C in the presence of EGTA or Ca^{2+}/PtdSer/TPA. Results are averages of two experiments: S.E.M. is within 10%.

Protein kinase C	Addition	Phosphorylation (pmol/min per mg) 80K-L	80K-H
Type I	EGTA	16.0	0
	Ca^{2+}/PtdSer/TPA	293	56.8
Type II	EGTA	20.0	0
	Ca^{2+}/PtdSer/TPA	473	52.3
Type III	EGTA	6.00	27.2
	Ca^{2+}/PtdSer/TPA	223	42.4

Table 3. Phosphorylation *in vitro* of 80K-H protein by immunoaffinity-purified subtypes of protein kinase C

Rat brain protein kinase C was further purified with anti-subtype-specific monoclonal antibodies bound to Protein A–Sepharose CL-4B beads. Results are averages of two experiments: S.E.M. is within 20%.

Protein kinase C	Addition	80K-H phosphorylation (pmol/min per mg)
II	EGTA	0.53
	Ca^{2+}/PtdSer/TPA	14.0
III	EGTA	0.40
	Ca^{2+}/PtdSer/TPA	1.87

Table 4. Effect of protein kinase inhibitors on 80K-H phosphorylation

80K-H protein (10 ng) or histone IIIS was incubated with human type III protein kinase C in the absence or presence of H-7 (50 μM) or staurosporine (10 nM). Values in parentheses are percentages of controls. Results are averages of two experiments: S.E.M. is within 20%.

Addition	Inhibitor	Phosphorylation (pmol/min per mg) 80K-H	Histone IIIS
EGTA	(−)	5.48 (100)	4.32 (100)
	H-7	3.00 (54.7)	2.55 (59.1)
	Staurosporine	4.12 (75.3)	3.22 (74.6)
Ca^{2+}/PtdSer/TPA	(−)	11.3 (100)	24.5 (100)
	H-7	4.50 (39.8)	10.7 (43.5)
	Staurosporine	2.62 (23.2)	5.53 (22.5)

Ca^{2+}-dependent manner (those indicated by small arrowheads in Fig. 6c) (results not shown). Furthermore, we analysed the effect of protein kinase C inhibitors on Ca^{2+}-dependent or -independent phosphorylation (Table 4). Both H-7 and staurosporine inhibited not only Ca^{2+}-dependent but also Ca^{2+}-independent phosphorylation. These results indicate that the 80K-H protein is phosphorylated by each of the three types of protein kinase C, but is apparently the preferential substrate for type I and II protein kinase C.

DISCUSSION

Protein kinase C is thought to play an important role in signal transduction for cell growth [28]. Although several substrates for protein kinase C have been identified, and cDNAs of some of them have been isolated [29,30], the essential substrate(s) for signal transduction has not yet been determined. The 80K protein was thought to be one of the important substrates for the signal transduction of cell growth and neurotransmission [8,9,12]. PDGF and bombesin enhance PtdIns turnover and activate protein kinase C, which stimulates phosphorylation of the 80K protein in mouse 3T3 cells [2]. EGF enhances 80K protein phosphorylation in EGF-receptor-hyperproducing tumour cells such as A431 [3] and Ca9-22 [4] cells, but not in normal cells [2]. Thus growth factors activate not only receptor tyrosine kinase but also protein kinase C, and some of the signals for cell growth are believed to be transmitted via protein kinase C-mediated 80K protein phosphorylation. TPA, a phorbol ester tumour promoter, also stimulates phosphorylation of the 80K protein in a number of cells [2–6,8,31]. Several studies reported the purification of the 80K protein as a substrate of protein kinase C from bovine [14] and rat [11,12] brain. Since no studies have been reported for human material, we attempted purification and characterization of the human 80K protein from a large number of cultured epithelial cells.

Previously, heterogeneity of the 80K protein was reported in Swiss 3T3 cells [11], rat [9,12] and bovine [13] brain. These heterogeneous 80K proteins reported were all heat-stable and acidic. Kligman & Patel observed two (lower and higher) molecular forms of the 80K protein in rat brain [9,12] and purified the higher-M_r form as the major component [12]. These authors thought that the higher- and lower-M_r 80K protein were the same protein, because of the identity of its one-dimensional peptide mapping profile after protease V8 digestion [9]. In the present study, we also observed the presence of distinct molecular species for human 80K protein in Ca9-22 epithelial cells, and these were designated as 80K-H and 80K-L proteins. Our 80K-L and 80K-H proteins had several characteristics similar to the lower- and higher-M_r 80K proteins of Kligman & Patel [9,12]: quantities, heat-stability, migration pattern on two-dimensional PAGE and elution profile from Mono Q column chromatography (Figs. 1 and 3). Furthermore, one-dimensional phosphopeptide mapping using protease V8 indicated that 80K-L and 80K-H proteins showed very similar profiles (results not shown) to those for the 80K proteins of Kligman & Patel [9,12]. However, two-dimensional phosphopeptide mapping using TPCK-treated trypsin revealed that the 80K-L and 80K-H proteins are absolutely different (Fig. 4). Studies using antiserum raised against 80K protein indicated the existence of two distinct molecular species of 80K protein [32].

We recently isolated and sequenced a cDNA clone encoding the 80K-H protein and deduced the amino acid sequence [15]. We also isolated a cDNA clone encoding the human 80K-L protein and found the sequence almost identical with that of the bovine 80K protein (MARCKS protein) ([16]; K. Sakai, M. Hirai, S. Minoshima, J. Kudoh, R. Fukuyama & N. Shimizu, unpublished work). The MARCKS protein consists of 335 amino acids (M_r 31949), is highly enriched in alanine, and contains an *N*-terminal myristoylation consensus sequence [16]. The 80K-H protein consists of 527 amino acids (M_r 59300), is highly enriched in glutamic acid and contains no myristoylation consensus sequence [15]. Furthermore, Northern-blot analysis using the specific cDNAs revealed mRNA of 2.6/4.4 kb and 2.3/3.5 kb for 80K-L and 80K-H proteins respectively ([15]; K. Sakai, M. Hirai, S. Minoshima, J. Kudoh, R. Fukuyama & N Shimizu, unpublished work). These results strongly support the idea that 80K-H protein is different from 80K-L protein and that 80K-L protein is a human counterpart of the bovine MARCKS protein.

The phosphopeptide map of the 80K-L protein (Fig. 4) was very similar to that of the phosphorylated 80K protein for ^{32}P-labelled Ca9-22 cells [4]. The 80K-L protein was present in the cytosol fraction of Ca9-22 cells, as was found for the mouse 80K protein [2]. The higher-M_r form of rat and bovine 80K proteins is phosphorylated on serine residues, with a stoichiometry of 2 mol of phosphate/mol of protein [12]. The phosphorylation *in vitro* of the 80K-L protein by type III protein kinase C was 10 times that of the 80K-H protein and exhibited the typical dependency on effectors of protein kinase C (Fig. 5 and Table 1). The sequence data indicated that the MARCKS protein contains four protein kinase C phosphorylation sites, which are located in a 25-residue basic domain, whereas the same number of phosphorylation sites is scattered in the less basic region of the 80K-H protein [15,16]. This structural difference may be related to the substrate specificity. Thus the 80K-L protein appears to be a better substrate than the 80K-H protein for type III protein kinase C, which is more abundant in epithelial cells.

Surprisingly, the 80K-H protein was much more efficiently phosphorylated by type III protein kinase C in the absence of Ca^{2+} (Table 1). A similar observation was previously reported for a rat '70K protein', which is immunologically related to 80K protein [10]. In the present study, however, at least two groups of phosphorylation sites, one Ca^{2+}-dependent and the other Ca^{2+}-independent, were detected by phosphopeptide mapping (Fig. 6). More importantly, the 80K-H protein was phosphorylated in a Ca^{2+}-dependent manner when type I or type II protein kinase C was used (Table 2). Furthermore, the 80K-H protein was phosphorylated in a Ca^{2+}-dependent manner when the affinity-purified type III protein kinase was used (Table 3). The inhibitors of protein kinase C inhibited Ca^{2+}-independent phosphorylation of 80K-H protein (Table 4). Thus Ca^{2+}-independent phosphorylation observed for partially purified type III protein kinase C may be due to a contamination with either an unknown type of protein kinase or an activator for protein kinase [33], but the data do not allow us to distinguish between them. These results again support the idea that the 80K-H protein is a true substrate for three subtypes of protein kinase C.

The 80K-H and 80K-L proteins are distinct substrates for protein kinase C, and therefore may have separate functions in signal transduction in cellular events. We have recently provided cell-genetic evidence that the intracellular translocation of type III protein kinase C and the kinase C-mediated phosphorylation of the 80K protein are involved in the mechanisms of mitogenic signal transfer in mouse fibroblast cells [34]. The mouse 80K protein was found to be equivalent to human 80K-L protein, which is also translocated upon mitogenic stimulation ([4]; Y. Shimizu & N. Shimizu, unpublished work). In this regard, it is reasonable to speculate that the human 80K-L protein may also be involved in delivering a growth-related signal via type III protein kinase C, which is predominant in fibroblasts and epithelial cells. To clarify further the relation between 80K-L and 80K-H proteins, we recently prepared the antiserum specific for human 80K-H protein by using the synthetic peptide corre-

sponding to an *N*-terminal region of the 80K-H protein ([15]; M. Hirai, R. Fukuyama, S. Minoshima, K. Sakai & N. Shimizu, unpublished work). To our surprise, preliminary results indicated that this antiserum stains heavily the peripheral and central nerve axons. The sequence database revealed that the 80K-H protein has similarity to the neurofilament triplet L protein [15]. The 80K-H protein was found in human epithelial cells and was shown to be a true substrate for protein kinase C, based on several biochemical criteria. However, enhancement of the 80K-H protein phosphorylation by EGF or TPA in intact cells has not been observed. The 80K-H protein phosphorylation may be extremely rapid and transient or non-functional in epithelial cells. Nevertheless, the preliminary indication of the possible relation to neurofilament components tempts us to postulate that the 80K-H protein may play a role in neuronal signal transmission via type I and/or type II protein kinase C, which are predominant in brain.

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