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Phorbol acetate enhances the phosphorylation of cytokeratins 8 and 18 in human colonic epithelial cells

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The phosphorylation of epithelial-specific cytokeratin (CK) 8 and 18 was studied in the human colonic cell line HT29. Metabolic labelling of cells with orthophosphate resulted in phosphorylation of cytokeratins 8/18 on serine residues. When phorbol acetate was added to labelled cells, a 2.2-fold increase in CK8/18 phosphate labelling was noted, whereas increasing intracellular cAMP levels using forskolin or 8-Br-cAMP showed no significant change in CK phosphorylation. CKs8/18 were also phosphorylated by added PKC in the presence of [γ-^{32}P]ATP. Tryptic peptide map analysis of the phosphorylated CK8 species showed that treatment of cells with 8-Br-cAMP or phorbol acetate generated a phosphopeptide not seen in control cells. In contrast, tryptic peptide maps of phosphorylated CK18 showed no discernable differences. Our results support a role for PKC in the phosphorylation of epithelial cytokeratins, with some phosphorylation sites being modulated by cAMP dependent protein kinase.

Cytokeratin phosphorylation; Protein kinase C; Phorbol acetate

1. INTRODUCTION

Intermediate filaments (IF) are a heterogeneous group of proteins that form an important part of the cytoskeleton and nuclear envelope of most eukaryotic cells ([1–3] for reviews). The 5 major types of IF show organelle (e.g. type V lamins) or tissue specificity (e.g. types I and II cytokeratins) depending on their constituent structural domains [1,2]. Cytokeratins, which encompass approximately 30 different proteins, are expressed primarily in epithelial cells and are considered a marker for epithelial cell differentiation [4,5]. They form obligate non-covalent heteropolymers by the interaction of an acidic type I keratin with a neutral/basic type II keratin. As a group, cytokeratins undergo post-translational modification including O-linked glycosylation [6,7] and phosphorylation on serine residues [8–11].

Although phosphorylation of several of the cytokeratin chains has been reported [8–11], the kinases that are involved in their phosphorylation remain sketchy. Some in vitro evidence suggests that cAMP dependent protein kinase can phosphorylate hoof cytokeratins [10], human cytokeratin 13 [9], and human keratins 1, 10 and 11 [8]. This is supported by the presence of multiple potential cAMP dependent protein kinase sites in several of the cytokeratins that have been studied [8]. However, the effect of agents that increase intracellular cAMP levels on CK phosphorylation has been variable. For example, dibutyryl cAMP and isoproterenol increased the phosphorylation of CK13 2- and 5-fold respectively, but no effect was noted for CK5 [9]. It was therefore suggested that other kinases may also play a role in CK phosphorylation although the nature of these kinases has not been studied.

Protein kinase C (PKC) is a serine kinase with at least 7 subspecies that regulate multiple cellular events ([12–14] for reviews). The role of PKC in cytokeratin phosphorylation has not been studied although PKC has been shown to play a role in the phosphorylation of other intermediate filament proteins such as lamin B [15]. In addition, PKC associates with several cytoskeletal components [16,17] and may consequently phosphorylate cytoskeletal proteins. In this report, we used agents that modulate the activity of cAMP dependent protein kinase and PKC to ask if these 2 kinases play a role in cytokeratin 8 and 18 phosphorylation.

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2. MATERIALS AND METHODS

2.1. *Materials*

Phorbol 12-myristate 13-acetate (PMA), 4 α-phorbol, ionomycin, forskolin, 8-bromo-cAMP, staurosporine, and anti-epithelial cytokeratin (anti-cytokeratin 8 and 18) antibody termed CK5 were obtained from Sigma. Other materials used were: BAPTA/AM and Pansorbin (Calbiochem); carrier-free [^{32}P]phosphoric acid and [γ-^{32}P]ATP (3000 Ci/mmol) (New England Nuclear); cAMP ^{125}I detection kit (Amersham); PVDF membranes (Immobolin P, Millipore). PKC-ϵ was a generous gift from Dr Berta Strulovici (Syntex Research, Palo Alto, CA). HT29 cells (human colon adenocarcinoma cell line) were grown in RPMI 1640 media supplemented with 10% fetal calf serum, penicillin (100 μg/ml) and streptomycin (100 μg/ml).

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2.2. *Immunoprecipitation*

Cytokeratin 8/18 immunoprecipitation was carried out using a mouse IgG1 monoclonal antibody (MAb) termed L2A1 obtained in our laboratory. The conclusion that MAb L2A1 recognizes CK8/18 is supported by extensive tissue distribution studies, microsequencing of the species corresponding to putative CK18 with 100% sequence homology to characterized human CK18 [18], and immunodepletion experiments using the anti-epithelial cytokeratin specific antibody CK5 [19] (data not shown). Orthophosphate ($^{32}PO_4$) labelling of intact cells was done as described before [20]. After labelling, cells were solubilized using 1% Nonidet P-40 in phosphate buffered saline (pH 7.2) containing: aprotinin (25 μg/ml), leupeptin (10 μM), pepstatin (10 μM) and phenylmethylsulfonyl fluoride (0.1 mM). After removal of nuclei, 100 μl of lysate (from 1–2 × 10^6 cells) were incubated with 5 μg of purified L2A1 antibody or 1 μl of ascites or 20 μl of purified L2A1 antibody coupled with sepharose. Immune complexes were collected using 100–150 μl of 2% formalin fixed *staphylococcus aureus* Pansorbin. Phosphorylated amino acids were analyzed by 2 dimensional electrophoresis after extraction of the phosphate labelled cytokeratin protein band from SDS-polyacrylamide gels and hydrolysis in-vacuo with constant boiling HCl [21].

2.3. *Cytokeratin phosphorylation*

For the experiments described in Table I and Fig. 2, HT29 cells (2 × 10^6) were labelled with 250 μCi [^{32}P]orthophosphate in 1 ml of phosphate-free media for 2 h. PMA and ionomycin (20–30 min before), and 8-Br-cAMP (10 min before) were added before the completion of labelling; staurosporine and BAPTA/AM were added 30 min before the start of labelling. Forskolin was added at the indicated time points before the completion of labelling. After labelling, CK immunoprecipitates were prepared followed by analysis using SDS-polyacrylamide gel electrophoresis (PAGE, 8% gel). To test if CK can

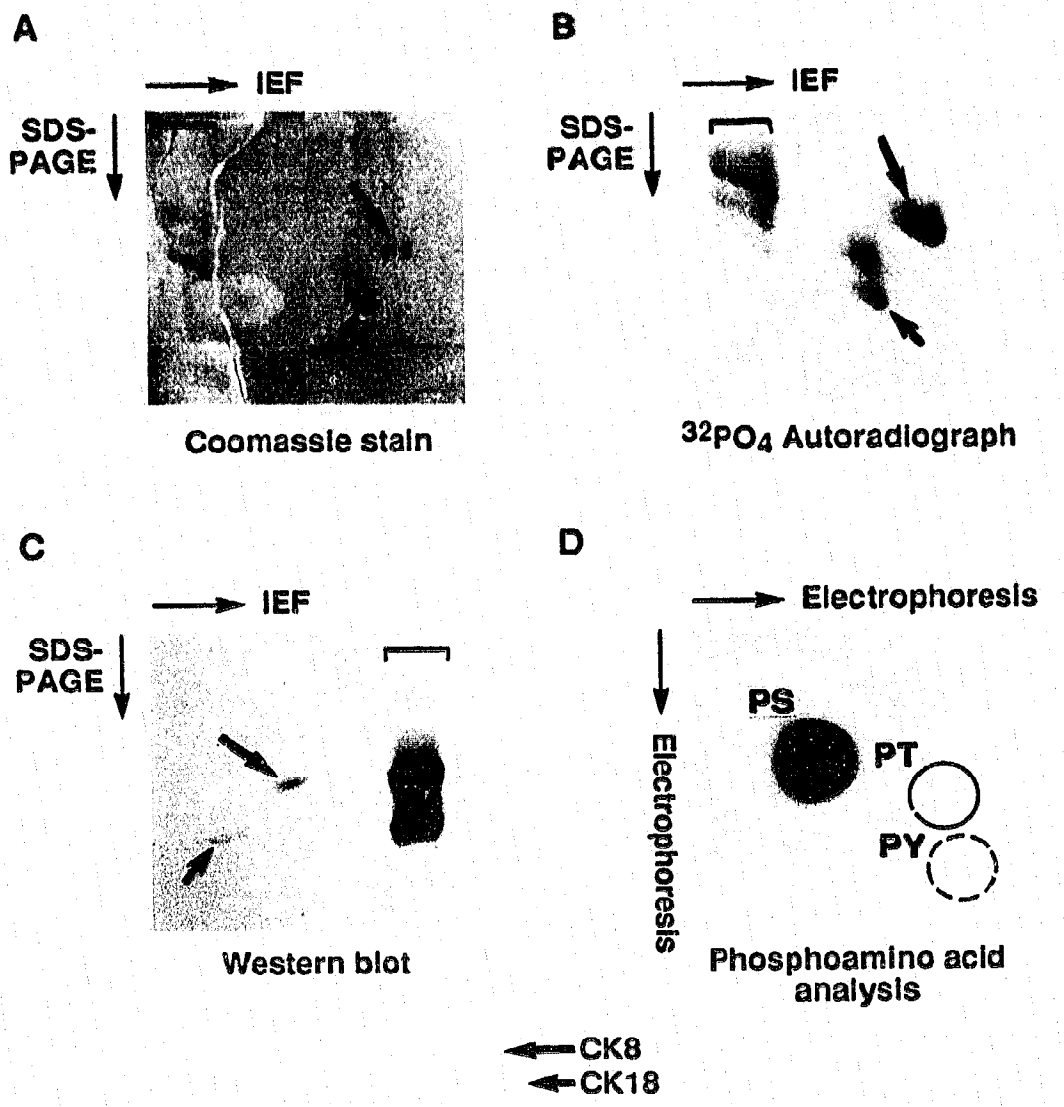

Fig. 1. Cytokeratins 8 and 18 are recognized by MAb L2A1 and are phosphorylated on serine residues. Immunoprecipitates of CK8 and 18 were obtained from [^{32}P]orthophosphate labelled HT29 cells after metabolic labelling for 2 h in phosphate-free media. Panels A–C show 2D analysis with horizontal arrows indicating isoelectric focusing (IEF) and vertical arrows indicating SDS electrophoresis (basic peptides are to the left). Panel B shows an autoradiograph of the dried Coomassie stained gel shown in panel A. Panel C represents a separate CK immunoprecipitate analyzed by a 2D gel followed by Western blotting. Panels A–C also include immunoprecipitates that were analyzed only in the second dimension (horizontal bracket) to allow easier identification of the 2D spots. Panel D shows results of phosphoamino acid analysis of a pooled mixture of CK 8 and 18. PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine.

act as a substrate for PCK-ϵ, duplicate immunoprecipitates of cytokeratin were obtained from non-labelled cells, boiled, then 10 μCi of [γ-^{32}P]ATP in 10 mM $MnCl_2$, 1 mM dithiothreitol and 50 mM PIPES were added in the presence or absence of 100 ng of PKC-ϵ. After 15 min, 2 × non-reducing sample buffer was added followed by analysis on an 8% SDS-polyacrylamide gel.

2.4. *2D gels, Western blotting and tryptic peptide mapping*

Isoelectric focusing (IEF) was performed using a pH 3–10 gradient as in [22]. For Western blot analysis, immunoprecipitates were transferred from SDS gels to PVDF membranes (0.45 μm pore size) using the manufacturer's recommendations in a buffer containing 192 mM glycine, 25 mM Tris, and 20% methanol [23]. Transfer was carried out using Bio-Rad Trans-Blot transfer cell (semi-dry) for 30 min at 15 volts. After transfer, the PVDF membrane strips were incubated with appropriate MAb supernatant at 1:1 dilution for 2 h (22°C) followed by washing. Specific bands were visualized by incubating with goat anti-mouse IgG alkaline phosphatase conjugate and developing using the manufacturer's recommendations (Bio-Rad). Analysis of tryptic peptides of the cytokeratins was carried out exactly as in [20] exept that carboxymethylation was done before SDS polyacrylamide gel electrophoresis.

3. RESULTS

MAb L2A1 immunoprecipitated 2 major bands corresponding to CK8 and CK18 as seen on Coomassie blue staining in Fig. 1A. The 3rd prominent middle band may correspond to CK7 but was not investigated further. The p*I* of CK8 and 18 shown in Fig. 1 was 6.0–6.2 and 5.6–5.8 respectively and was similar to that reported by others [4]. Both CK8 and 18 were phosphorylated (Fig. 1B), and the peptide chains recognized by the MAb by Western blotting (Fig. 1C), corresponded to the phosphorylated species (autoradiograph of Fig. 1C, not shown). Analysis of the phosphorylated residues on CK8 and 18 revealed that phosphorylation occurred mainly on serine residues with less than 5% of the radioactivity associated with threonine residues (Fig. 1D).

We then asked if protein kinase C or cAMP dependent protein kinase plays a role in the phosphorylation of CK8/18 by using agents that modulate the function of these kinases. Increasing intracellular cAMP levels using forskolin or 8-Br-cAMP resulted in increasing intracellular cAMP levels by 30–200 fold (Fig. 2A). These elevations in cAMP levels did not result in any significant change in the level of phosphorylation of either CK8 or 18 (Fig. 2A and Table I). In contrast, the addition of phorbol acetate to cells labelled with [^{32}P]orthophosphate increased the level of CK8/18 phosphorylation by 2-fold (mean 2.2, n = 7) (Fig. 2B). Staurosporine, a PKC inhibitor [24], caused a 60% decrease in CK phosphorylation; whereas 4α-phorbol, ionomycin and the cell permeable calcium ion chelator BAPTA/AM [25] had no significant effect on CK phosphorylation (Table I).

The increase in CK phosphorylation in the presence of phorbol acetate is consistent with PKC acting as the kinase. The lack of effect of calcium mobilization by ionomycin or chelation by BAPTA/AM suggested that

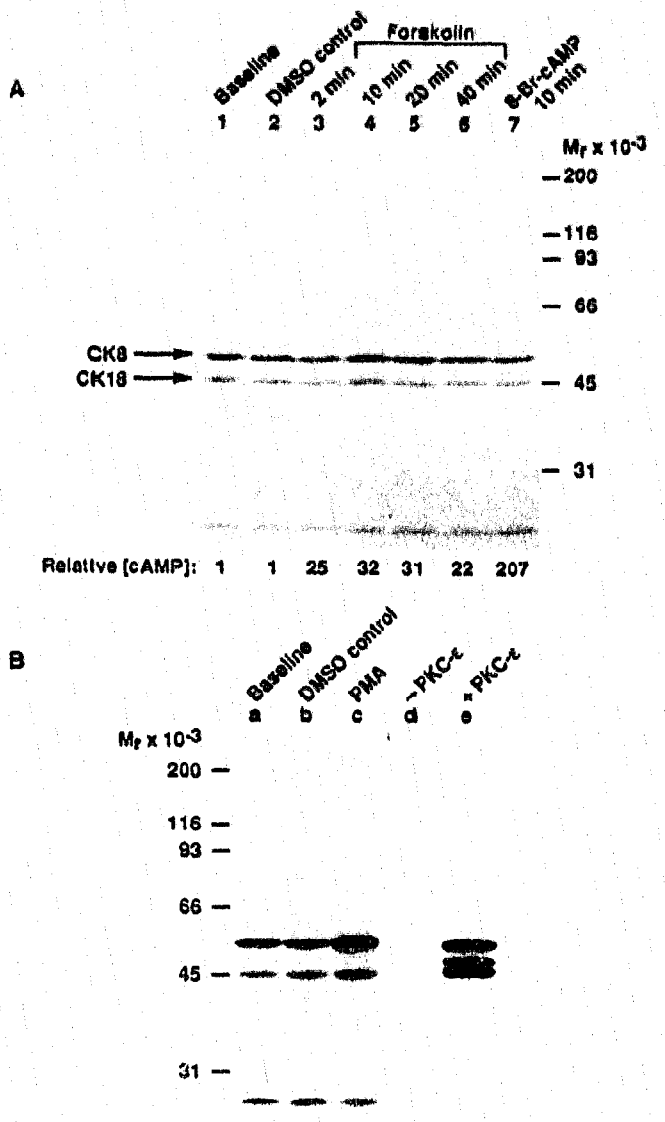

Fig. 2. Phosphorylation of cytokeratins 8/18 in the presence of forskolin, 8-Br-cAMP, PMA and by PKC-ϵ. The figure shows autoradiograms of: (A) Cells in duplicates were either labelled with orthophosphate or incubated under the same conditions as labelled cells. Forskolin (in DMSO, 10 μl/ml added) or 8-Br-cAMP (in H_2O) were added for the indicated timepoints to duplicate dishes, followed by immunoprecipitation and SDS/PAGE (labelled cells) or measurement of cAMP levels (unlabelled duplicate). Lane 2 shows immunoprecipitates from control cells, to which 10 μl of DMSO was added. The measured cAMP corresponding to lane 1 was 75 fmol per 1 × 10^6 HT29 cells. (B) Cytokeratin phosphorylation with or without added PMA (200 ng/ml). Also shown are unlabeled cytokeratin immunoprecipitates in the presence of [γ-^{32}P]ATP and added PKC-ϵ (lane e) or without PKC-ϵ (lane d).

the involved kinase may be calcium independent. We therefore tested if CK8/18 can serve as an in vitro substrate for PKC-ϵ, a calcium independent isoform of PKC [12,13]. As shown in Fig. 2B, PKC-ϵ phosphorylated both CK 8 and 18 which were isolated from HT29 cells using the L2A1-sepharose coupled MAb.

Table I

Relative level of cytokeratin 8 and 18 phosphorylation in HT29 cells

Agent added	CK8 (n)[a]	CK18 (n)
None (baseline)	1.00	1.00
Forskolin (20 min) 10 μM	1.01 (3)	1.01 (3)
8-Br-cAMP (30 min) 1 mM	0.98 (3)	1.03 (3)
4α-phorbol, 200 ng/ml	1.02 (2)	1.01 (2)
PMA, 200 ng/ml[b]	2.15 ± 0.6 (7)	2.10 ± 0.6 (7)
Staurosporine, 10^{-8} M	0.43 (2)	0.35 (2)
Ionomycin, 20–500 nM	1.1 ± 0.1 (5)	1.1 ± 0.1 (5)
BAPTA/AM, 10 μM	1.00 (2)	1.04 (2)

Numbers in table correspond to the mean level of $^{32}PO_4$ incorporated into CK8 or 18, obtained either by cpm counting or densitometric scanning of the individual bands. Numbers were normalized to baseline phosphorylation where no agent is added.

[a] *n* indicates number of experiments

[b] represents optimum concentration in range of 20–500 ng/ml tested

We also examined the tryptic peptide profile of CK 8 and 18 to determine if any specific peptides could be identified that become phosphorylated when cells are induced with phorbol acetate or 8-Br-cAMP. As shown in Fig. 3, PMA and 8-Br-cAMP resulted in the phosphorylation of a peptide in CK8 that was not phosphorylated in control CK8 (faint peptide indicated by large arrow). In contrast, CK18 showed a similar phosphopeptide pattern under all these conditions. A faint peptide seen in PMA-treated CK18 peptide map (small arrow) was also seen on longer exposure in control and 8-Br-cAMP CK18 maps and hence was not considered significant (not shown).

4. DISCUSSION

Although no differences were seen in the phos-

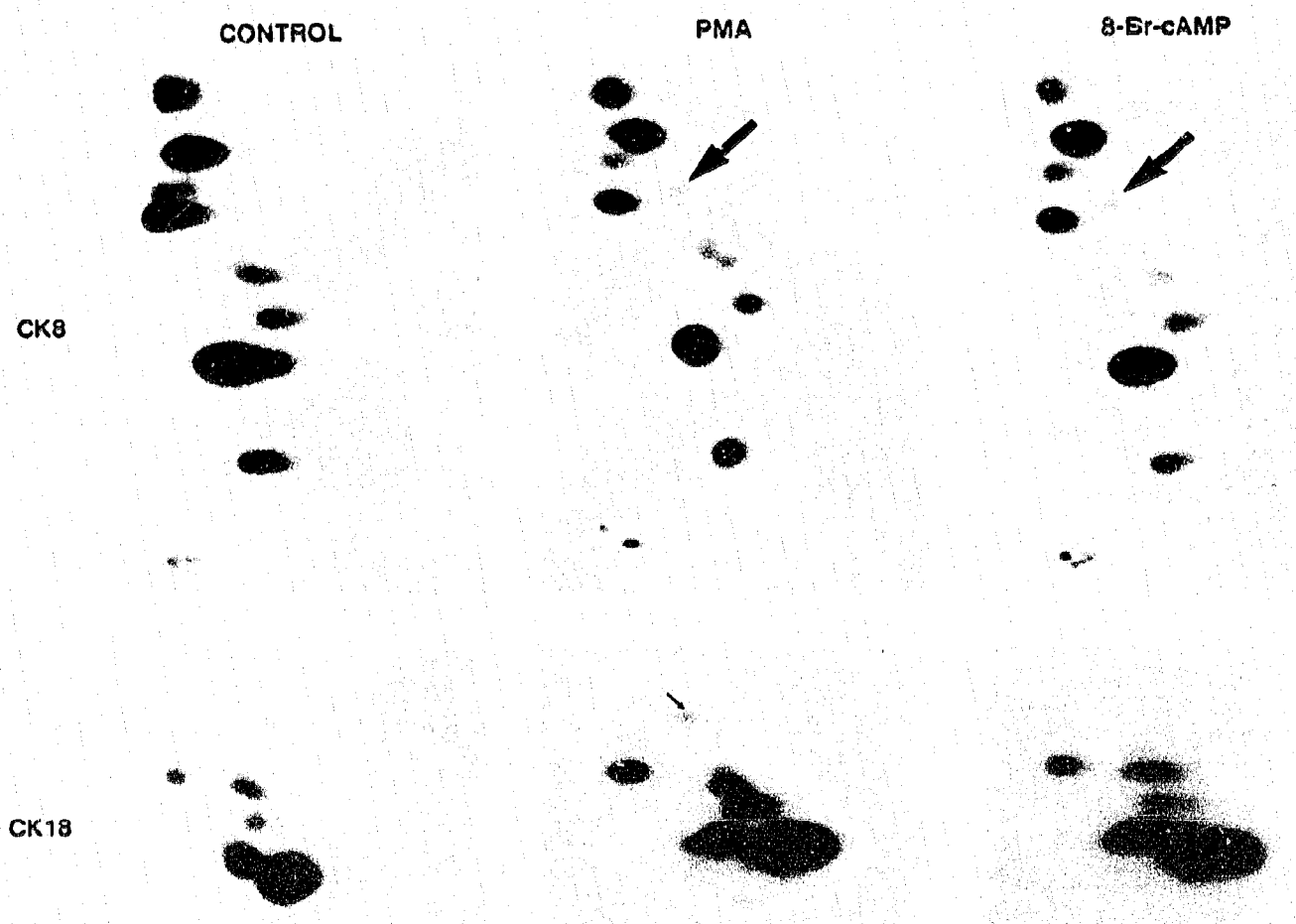

Fig. 3. Tryptic phosphopeptide analysis of phosphate labelled CK8 and 18 in the presence of PMA and 8-Br-cAMP. Individual polypeptides corresponding to CK8 or CK18 were electroeluted from SDS-polyacrylamide gels and processed for peptide mapping as described in Section 2. Approximately 1200 cpm of each trypsin-digested polypeptide was loaded onto the cellulose plates followed by electrophoresis in the horizontal axis and chromatography in the vertical axis. Exposure of the autoradiograph was for 3–6 days.

phorylation of CK8/18 after an increase in intracellular cAMP levels, a more subtle difference was observed for CK8 after detailed analysis using tryptic peptide mapping. Earlier studies showed that increasing intracellular cAMP levels manifested variable differences in the level of cytokeratin phosphorylation, depending on the cytokeratin that was studied [9]. However, these studies did not examine individual tryptic peptides. Interestingly, the same peptide appeared after increasing cAMP levels as after stimulation with PMA. It remains to be determined if the same serine or threonine residue is being phosphorylated in the presence of the different stimulating agents, and whether the phosphorylation involves a single or a cascade of kinases. A role for cAMP dependent protein kinase in the phosphorylation of CK8/18 is not surprising given that CK8 contains a total of 59 serine residues with 6 sequence motifs (basic-X-ser) representing potential cAMP protein kinase phosphorylation sites [26], wheras CK18 contains 37 serine residues with basic-X-ser motifs [18].

Previous studies had suggested that CK phosphorylation involved other kinases in addition to cAMP dependent protein kinase [8,9], although the nature of these kinases has not been investigated. Our data support a role for PKC in cytokeratin phosphorylation. This is based on enhancement of the phosphorylation level in the presence of PMA and inhibition of phosphorylation in the presence of staurosporine. Furthermore, CKs8/18 are good phosphorylation substrates when mixed with PKC-ϵ. Although other studies are required, it appears that within the PKC family, PKC-ϵ is a possible candidate as a protein kinase involved in cytokeratin phosphorylation. This hypothesis is based on the lack of effect of the intracellular calcium modulators ionomycin and BAPTA/AM on cytokeratin phosphorylation. To this end, HT29 cells contain significant levels of immunoreactive PKC-ϵ as determined by Western blotting using an anti-PKC-ϵ specific antibody (Berta Strulovici, personal communication).

Other indirect evidence also supports a role for PKC in the phosphorylation of cytoskeletal component proteins. For example, activation of PKC by bryostatin resulted in its translocation to the nuclear envelope and phosphorylation of the nuclear envelope intermediate filament protein lamin B [15]. In addition, the calcium dependent PKC-α appears to associate with the cytoskeletal focal contact proteins vinculin and talin, which in turn mediate the attachment of microfilaments with the plasma membrane [17].

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REFERENCES

[1] Lazarides, E. (1982) Annu. Rev. Biochem. 51, 219–250.
[2] Steinert, P.M. and Roop, D.R. (1988) Annu. Rev. Biochem. 57, 593–625.
[3] Osborn, M. and Weber, K. (1986) Trends Biochem. Sci. 11, 469–472.
[4] Moll, R., Franke, W.W., Schiller, D.L., Geiger, B. and Krepler, R. (1982) Cell 31, 11–24.
[5] Franke, W.W., Schiller, D.L., Moll, R., Winter, S., Schmid, E., Engelbrecht, I., Denk, H., Krepler, R. and Platzer, B. (1981) J. Mol. Biol. 153, 933–959.
[6] Roberts, G.P. and Brunt, J. (1986) Biochim. Biophys. Acta 883, 413–419.
[7] King, L.A. and Hounsell, E.F. (1989) J. Biol. Chem. 264, 14022–14028.
[8] Steinert, P.M. (1988) J. Biol. Chem. 263, 13333–13339.
[9] Gilmartin, M.E., Mitchell, J., Vidrich, A. and Freedberg, I.M. (1984) J. Cell Biol. 98, 1144–1149.
[10] Yeagle, P.L., Frye, J. and Eckert, B.S. (1990) Biochemistry 29, 1508–1514.
[11] Baribault, H., Blouin, R., Bourgon, L. and Marceau, N. (1989) J. Cell Biol. 109, 1665–1676.
[12] Nishizuka, Y. (1988) Nature 334, 661–665.
[13] Parker, P.J., Kour, G., Marais, R.M., Mitchell, F., Pears, C., Schaap, D., Stabel, S. and Webster, C. (1989) Mol. Cell. Endocrinol. 65, 1–11.
[14] Berry, N. and Nishizuka, Y. (1990) Eur. J. Biochem. 189, 205–214.
[15] Fields, A.P., Pettit, G.R. and May, W.S. (1988) J. Biol. Chem. 263, 8253–8260.
[16] Jaken, S., Leach, K. and Klauck, T. (1989) J. Cell Biol. 109, 697–704.
[17] Papadopoulos, V. and Hall, P.F. (1989) J. Cell Biol. 108, 553–567.
[18] Romano, V., Hatzfeld, M., Magin, T.M., Zimbelmann, R., Frank, W.W., Maier, G. and Ponstingl, H. (1986) Differentiation 30, 244–253.
[19] Tölle, H-G, Weber, K. and Osborn, M. (1985) Eur. J. Cell Biol. 38, 234–244.
[20] Omary, M.B. and Trowbridge, I.S. (1980) J. Biol. Chem. 255, 1662–1669.
[21] Cooper, J.A., Sefton, B.M. and Hunter, T. (1983) Methods Enzymol. 99, 387–402.
[22] Knowles, R.W. (1987) in: Histocompatibility Testing (Dopont B. ed.) Springer, New York, pp. 1–45.
[23] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[24] Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986) Biochem. Biophys. Res. Commun. 135, 397–402.
[25] Tsien, R.Y. (1981) Nature 290, 527–528.
[26] Krauss, S. and Franke, W.W. (1990) Gene 86, 241–249.