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Purification of a 20 kDa phosphoprotein from epithelial cells and identification as a myosin light chain

Phosphorylation induced by enteropathogenic *Escherichia coli* and phorbol ester

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Previous studies on the mechanism of enteropathogenic *Escherichia coli* (EPEC) infection have revealed an increase in the phosphorylation state of a number of proteins in human laryngeal HEp-2 cells. The most prominent was an acidic phosphoprotein(s) of M_r 20–21 kDa. The present study reports: (a) a simple method for purification of phosphorylated 20 kDa protein: (b) identification of the 20 kDa phosphoprotein as myosin light chain; and (c) that the phorbol ester, TPA, also increased the phosphorylation of the 20 kDa myosin light chain. In contrast to the effects of EPEC, TPA stimulation resulted in the dissociation of myosin from the cytoskeleton to the cytosol.

Myosin light chain; Protein phosphorylation; Protein kinase C; Enteropathogenic *Escherichia coli;* Caco-2 cell; HEp-2 cell

1. INTRODUCTION

Isolates, taken from cases of severe infantile diarrhea, of non-toxin secreting enteropathogenic *E. coli* (EPEC) [1] have a pronounced effect on the phosphorylation levels of proteins in human laryngeal (HEp-2) epithelial cells, used as a model for studying the pathogenic mechanism of the disease [2]. We have observed similar effects on the levels of phosphorylation with okadaic acid (Manjarrez et al., in preparation). This toxin is the causative factor in 'diarrhetic shellfish poisoning' [3]. Using Caco-2 cells (derived from human colon), Burnham and Fondacaro [4] have shown that both the 20 and 21 kDa light chains of myosin are phosphorylated after treatment with secretagogues that induce increased membrane chloride permeability. We have demonstrated an increase in the phosphorylation of the 20–21 kDa proteins after EPEC infection [2] and now present evidence for their identification as myosin light chain.

Non-muscle myosins have been identified in many cell types. Most consist of two heavy chains and two pairs of light chains [5]. A 21 dDa light chain, thought to be an isoform of the 20 kDa regulatory form has also been identified in pancreatic acini and stimulation of acinar secretion has been shown to be accompanied by an increase in phosphorylation of both these forms to roughly equal extents [6]. In non-muscle cells the 20 kDa myosin light chain (MLC) is phosphorylated by a specific calcium-activated/calmodulin-dependent enzyme, MLC kinase [7]. This enzyme activity has been identified in enterocyte brush-border preparations [8] which are the most extensively non-muscle cell in terms of cytoskeletal structure and the role of MLC phosphorylation in cell function.

In this paper we describe a simple purification procedure for the phosphorylated 20 kDa protein from Hep-2 and Caco-2 epithelial cells. These latter cells are of particular importance since they are able to express spontaneous differentiation features which are characteristic of typical brush-border microvilli [9]. Also reported is the characterisation and subsequent identification of the 20 kDa phosphoprotein as MLC with the use of antibodies. Identification of the EPEC-induced phosphoproteins may be of particular importance in understanding the mechanism of pathogenicity of EPECC-induced diarrhetic disease. Their respective roles and those of the associated kinases and phosphatates are important in the function of the intestinal epithelium with respect to shape, change and secretion.

Abbreviations: DOC, deoxycholate; EPEC, enteropathogenic *Escherichia coli*; MHC, myosin heavy chain; MLC, myosin light chain; PKC, protein kinase C; TPA, tetradecanoylphorbol-13-acetate.

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2. MATERIALS AND METHODS

2.1. *Materials*

[^{32}P]Orthophosphate (carrier free; 1 mCi/ml) was obtained from Amersham International, UK. Purified antibodies which specifically bind to residues 6–23 of the phosphorylated form of regulatory light chain of smooth muscle myosin and antimyosin heavy chain (smooth muscle) antiserum were kindly supplied by Dr J. Bennett, Department of Anatomy and Cell Biology, St Mary's Hospital Medical School and Dr A.I. Magee, National Institute for Medical Research, Mill Hill, London, respectively. Antimyosin (bovine smooth and skeletal muscle) antiserum was obtained from Sigma. All other reagents were analytical grade, from BDH or Sigma.

2.2. *Bacterial strains and culture*

Single colonies of EPEC strain E2348-69 (0127), isolated from a case of severe infantile diarrhea [10] and laboratory *E. coli* strain HB101 (included as a negative control) [1] were inoculated into 10 ml aliquots of Luria broth and incubated overnight at 37°C without agitation to the mid-exponential phase before use.

2.3. *Cell culture and protein phosphorylation in intact cells*

(HEp-2) cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100% (v/v) foetal calf serum (FCS) in Nunc cell factories (culture area 6000 cm^2. Caco-2 cells were grown in 8 cm diameter Petri dishes in DMEM/20% (v/v) FCS and were allowed to fully differentiate [9]. Cells were maintained at 37°C in a humidified atmosphere of air enriched with 5% CO_2.

Prior to phosphorylation experiments, cells were maintained for 20 h in DMEM containing 0.5% (v/v) FCS to obtain quiescent cells, followed by 4 h in phosphate-free DMEM/0.5% (v/v) FCS and 50–100 μCi of carrier-free $^{32}P_i$ (to label the intracellular ATP pool). HEp-2 and Caco-2 cells were infected with a bacterial suspension (10^8 CFU in 0.3 ml) for 2 h at 37°C. HEp-2 cells were also stimulated with tetradecanoylphorbol-13-acetate (TPA: 200 nM) for 2 h. To terminate the phosphorylation reaction, cells were rapidly washed with ice-cold phosphate-buffered saline, removed from the dish by scraping with a rubber policeman and transferred into Eppendorf tubes containing 50 mM NaF, 1 mM PMSF. The cells were frozen, and complete lysis was ensured by sonication on ice for 5–10 s. The cytosolic (supernatant) fraction was separated from the membrane (pellet) fraction by centrifugation for 15 min at 4C in a microcentrifuge.

2.4. *Polyacrylamide gel electrophoresis and immunoblotting*

SDS-PAGE was adapted from Laemmli [11] using 10 and 12.5% gels, stained either with Coomassie brilliant blue or silver adapted from Blum et al. [12]. Two-dimenstional gel electrophoresis was performed according to O'Farrell [13]. Western blotting was performed according to [14].

2.5. *Purification of the 20 kDa phosphoprotein*

The supernatant (2 ml) from HEp-2 or Caco-2 cells (following TPA or EPEC treatment) was applied in 0.5 ml aliquots to size-exclusion column (TSK G2000 SW; 7.5 mm × 30 cm), equilibrated with buffer A (25 mM phosphate buffer pH 5.5), containing 10% (v/v) propan-1-ol (flow rate 0.5 ml/min). 1 ml fractions were collected and the presence of the 20 kDa protein detected as described below. Fractions containing the 20 kDa protein were pooled and frozen at −20°C. After thawing, the fractions were centrifuged (microfuge, 15 min) and the supernatant applied to a Mono S cation-exchange column (Pharmacia; 5 × 50 mm) equilibrated in buffer A on B Pharmacia FPLC system. The column was extensively washed with buffer A and bound proteins were eluted by application of a linear NaCl gradient, 0–1.0 M over 20 min at 1 ml/min. Fractions (1.0 ml) containing the 20 kDa phosphoprotein were pooled, desalted using a Sephadex column (Pharmacia PD-10) and concentrated by Centricon 10 microconcentrators (Amicon).

Following each stage of the purification, the 20 kDa phosphoprotein was detected by running aliquots on SDS polyacrylamide gels with subsequent autoradiography on Kodak X OMAT S at −70°C. To assess recoveries, the 10 kDa phosphoprotein (located by autoradiography of wet unfixed gels) was cut from the gel and radioactivity counted using Cerenkov counting. The remainder of the gel lane was cut into slices of equivalent size as that containing the 20 kDa protein, and counted. Summation of the counts from all slices gave a value for total radioactivity from which recovery of the 20 kDa protein was calculated. In order to overcome apparent losses through radioactive decay, samples from pools before and after each purification stage were run on the same polyacrylamide gel.

2.6. *Amino acid analysis and protein estimation*

Hydrolysis was carried out at 100°C for 24 h with 6 M HCl/2 mM phenol in the vapour phase under nitrogen. Amino acids were detected with an Applied Biosystems 420A derivatiser fitted with an on-line 130A phenylthiocarbamyl amino acid analyser, using reagents supplied by the manufacturer.

Protein content was estimated by the method according to Lowry [15] or by quantitave amino acid analysis.

2.7. *Immunoprecipitation*

After protein phosphorylation experiments, cells were rapidly washed in cold PBS, and were removed from the dish in PBS containing 50 mM NaF and 1 mM PMSF. Cells were frozen and complete lysis was ensured by sonication on ice for 5–10 s. (i) Immunoprecipitation was achieved, using antibodies to denatured phosphorylated myosin regulatory light chains and protein-A-Sepharose beads (Pharmacia), from cell lysates after denaturing in SDS-PAGE sample buffer (100°C, 5 min). (ii) To immunoprecipitate native cytosolic proteins the supernatant fraction was diluted with 2 vols of cold RIP buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 50 mM Tris-HCl, pH 8.0), protease inhibitors were added (0.2 mM leupeptin, 20 μg/ml pepstain A, 0.3 mg/l aprotinin and 10 mM benzamidine) and incubated with either antimyosin or antimyosin heavy chain serum (1 : 10 dilution) on ice for 1 h. To collect the antibody–antigen immunocomplexes, 30 μl of 1 : 1 suspension of protein-A-sepharose beads (Pharmacia) was added and incubated for 1 h at 4°C with rocking. The resulting complexes were collected by brief centrifugation, the pellet was washed three times with ice cold RIP buffer and once with 50 mM Tris-HCl, pH 6.8. (iii) To immunoprecipitate cytoskeleton associated proteins, cell pellets were resuspended in cold lysis buffer (1.0% NP-40, 40 mM sodium pyrophosphate, pH 7.4, 0.4 M NaCl, 10 mM EDTA, 0.1 M NaF, 1 mM benzamidine). After standing on ice for 30 min, the lysed cells were briefly sonicated and were centrifuged in a bench microcentrifuge for 20 min. The supernatant was incubated with the antisera as described in (ii). Immunoprecipitated proteins were analysed by either one or two-dimensional PAGE.

3. RESULTS

3.1. *Phosphorylation of the 20–21 kDa protein in HEp-2 and Caco-2 cells after treatment with TPA or following infection with EPEC isolates*

Incubation of HEp-2 monolayers for 2 h with either TPA or EPEC strain E2348-69 caused increased phosphorylation of both 20 and 21 kDa proteins (Fig. 1). Treatment of Caco-2 cells for 2 h with EPEC strain E2348-69 also resulted in a significant enhancement of the phosphorylation state of these proteins. The phosphorylation patterns resulting from EPEC infection in the two cell lines were similar.

3.2 *Purification and amino acid analysis of the 20 kDa phosphoprotein*

The elution profile of ^{32}P-radiolabelled cytosolic

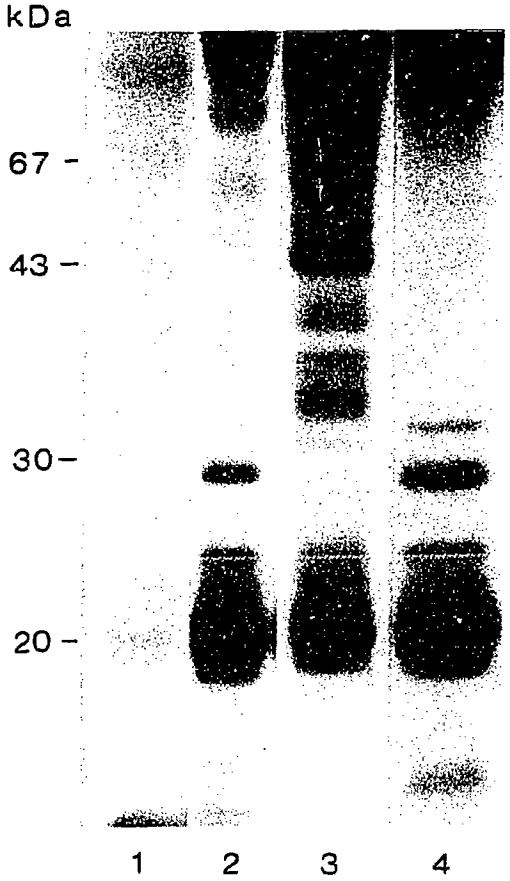

Fig. 1. Effect of EPEC and TPA on HEp-2 and Caco-2 cell protein phosphorylation. Autoradiograph of SDS-PAGE analysis of proteins phosphorylated after incubation for 2 h of: lane 1, (control) untreated HEp-2 cells; lane 2, HEp-2 cells after treatment with EPEC strain E2348-69, lane 3, HEp-2 cells after treatment with TPA; lane 4, Caco-2 cells after treatment with EPEC strain E2348-69. The level of phosphorylation in unstimulated Caco-2 cells was similar to that of untreated HEp-2 cells (data not shown).

proteins separated on a size-exclusion column from TPA-stimulated HEp-2 cells is shown in Fig. 2A. Analysis of the fractions by SDS-PAGE and subsequent autoradiography (Fig. 2B) showed that the 20 kDa protein eluted with an apparent molecular weight of 45 000. Most of the protein in fractions containing the 20 kDa phosphoprotein (pooled from multiple TSK G2000 SW separations) was observed to precipitate after freezing and thawing; 95% of the 20 kDa phosphoprotein (estimated from SDS-PAGE and Cerenkov counting) was recovered in the supernatant of this preparation after microfuging, with 80% of the total protein (estimated by Lowry determination) contained in the pellet. As a final purification, the supernatant was loaded on a Mono S cation-exchange column. The 20 kDa protein eluted from the column as two discrete peaks; 65% of the recovered 20 kDa protein did not bind to mono S and was contained in the flowthrough, with the remainder eluting as a broad peak at approximately 0.4 M NaCl (Fig. 3). Analysis of the latter peak

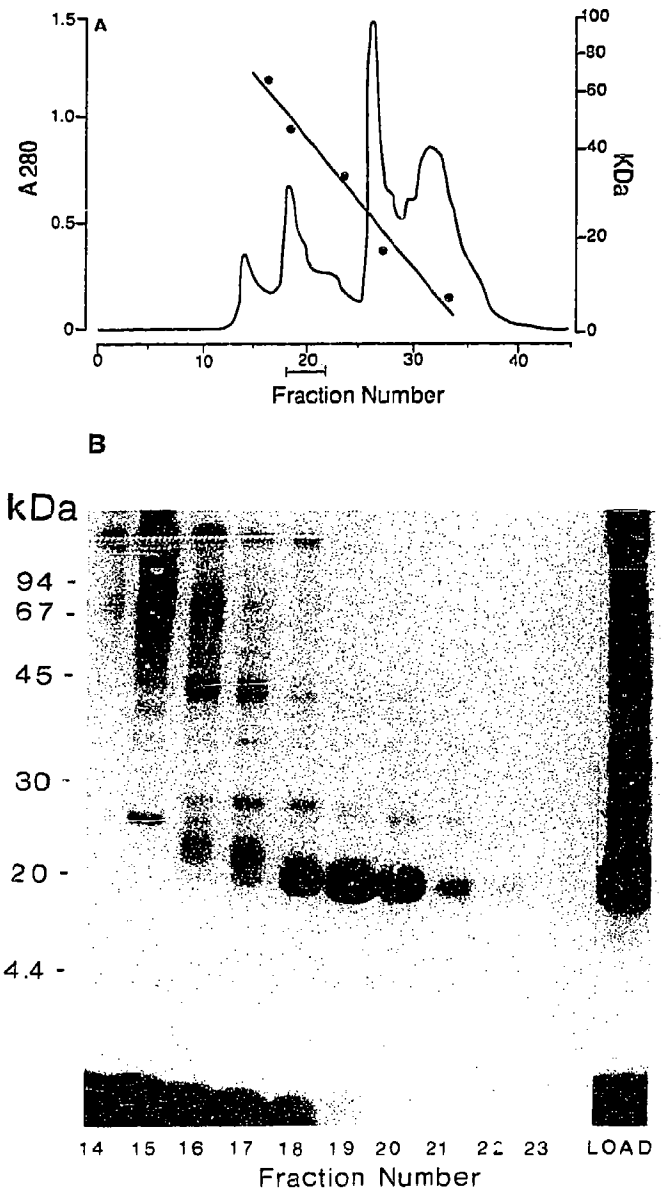

Fig. 2. (A) Size-exclusion chromatography of HEp-2 cytosolic proteins, following TPA stimulation, on TSK G2000 SW column, eluted with 0.25 mM phosphate buffer, pH 5.5, monitored at 280 nm (A_{280}) and collected in 0.5 ml fractions. The column was calibrated using molecular weight marker proteins (●); bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), myoglobin (17.3 kDa) and cytochrome *c* (12.3 kDa). The elution position of the 20 and 21 kDa proteins is marked by the horizontal bar. (B) Autoradiograph of SDS-PAGE analysis of successive fractions (14–23) from the size-exclusion column. The positions of molecular weight proteins; phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and lactalbumin (14.4 kDa), are indicated (–) from top to bottom, respectively. The lane marked 'load' is of the supernatant applied to the column.

by SDS-PAGE showed a single silver staining band with an apparent molecular weight of 20 000 (see Fig. 4A, lane 4), which was also detected by autoradiography (Fig. 4B, lane 4). This phosphorylated prepara-

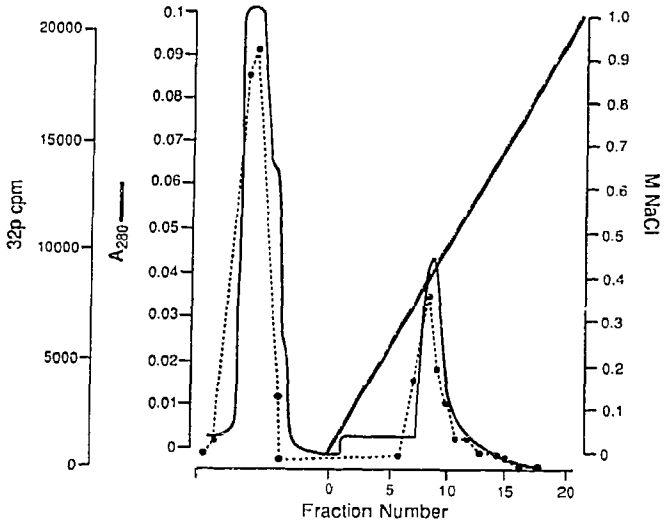

Fig. 3. Elution of the 20 kDa phosphoprotein by Mono S cation-exchange chromatography. Supernatant, resulting from centrifugation after freezing and thawing of size-exclusion chromatography fractions containing the 20 and 21 kDa phosphoproteins, was loaded on to a Mono S column, and bound protein (A_{280}) eluted with a linear concentration gradient of NaCl. Two peaks of radioactivity both containing the 20 kDa protein were identified and pooled separately: one was contained in the flow-through of the column (also containing the 21 kDa phosphoprotein), and the second eluted at approximately 0.4 M NaCl on the gradient.

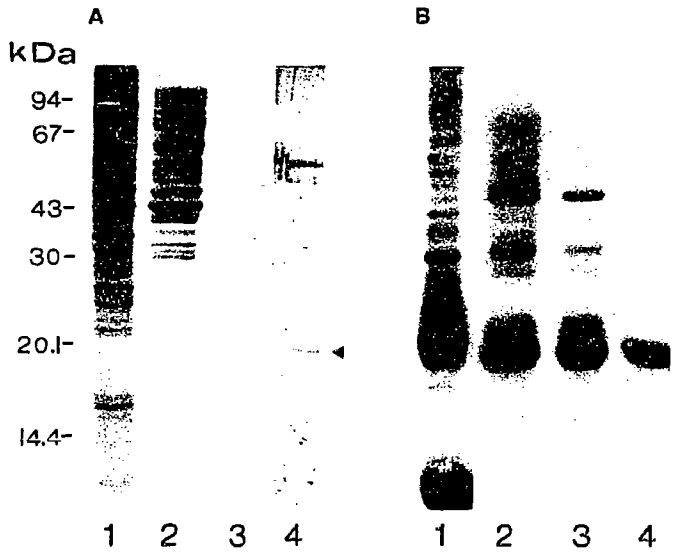

Fig. 4. Polyacrylamide gel electrophoresis of the 20 kDa phosphoprotein at each stage of purification. (A) SDS polyacrylamide gel analysis of HEp-2 cytosolic proteins, lane 1 (Coomassie stained); lane 2, 20 and 21 kDa phosphoprotein pool after size-exclusion chromatography (Coomassie stained); lane 3, supernatant resulting from freezing/thawing this pool (Coomassie stained); lane 4, peak eluting from Mono S cation-exchange column containing 20 kDa phosphoprotein (silver stain, highlighted by the arrow). Note the staining on lane 4 at approximately 60 kDa is artifactual (this may be keratin) and is routinely seen on silver stained 'blank lanes'. (B) The corresponding autoradiograph of the gel lanes shown in A.

tion was used for amino acid analysis. The 21 kDa phosphoprotein co-purified with the 20 kDa protein until the Mono S purification step. The 20 kDa protein bound and could be eluted from Mono S by a salt gradient, whereas the 21 kDa form was not retained by the column. A very similar elution pattern at each stage of the purification procedure was obtained for phosphorylated 20 and 21 kDa proteins from Caco-2 cells after EPEC infection. However, the recovery of the 20 kDa phosphoprotein was low (20% compared to TPA stimulated HEp-2 cells).

Both the 20 and 21 kDa phosphoproteins were poorly stained by Coomassie blue, probably because of their unusually acidic pI. They could be detected with the carbocyanine dye 'Stains-All' under conditions optimized for phosphoproteins (data not shown). Fig. 4 shows a 12.5% SDS polyacrylamide gel and corresponding autoradiograph of the 20 kDa protein preparation from HEp-2 cells at various stages of purification. The recovery of the 20 kDa phosphoprotein is shown in Table I.

Amino acid analysis of the purified 20 kDa phosphoprotein is shown in Table II. The values are in good agreement with those for an average of two mammalian smooth muscle light chains and a human non-sarcomeric 20 kDa regulatory light chain, except for Glu/Gln, Ile and Phe. From unsuccessful attempts at direct protein sequencing, we have concluded that the 20 kDa protein is N-terminally blocked.

3.3. *Immunoprecipitation*

Quiescent cells, labelled with [^{32}P]orthophosphate, were treated with either TPA or EPEC and immunoprecipitation was performed with three different antibodies. Following lysis in SDS-PAGE sample buffer, antibodies against phosphorylated myosin light chain showed cross-reactivity with the 20 kDa phosphoprotein on Western blot analysis of SDS-PAGE (data not shown) and by immunoprecipitation with subsequent 2D PAGE analysis (Fig. 5). Following lysis in buffer containing protease and phosphatase inhibitors but excluding detergents, the cytosolic (supernatant) fraction was immunoprecipitated using antibodies raised against myosin complex (anti-M) or antibodies raised against the myosin heavy chain (anti-MHC); Fig. 6A shows subsequent SDS-PAGE (Coomassie blue staining and autoradiography). Bands corresponding to myosin heavy chain (MHC), myosin light chain (MLC) and actin (A) were observed only after TPA treatment (lane b). Their corresponding autoradiography shows that both MHC and MLC were phosphorylated (lane b). The co-precipitation of actin was explained by persistence of actomyosin complexes under the mild lysis conditions. After EPEC treatment myosin complex is still largely associated with the cytoskeleton and it remains in the membrane fraction (pellet). In contrast, incubation with anti-MHC antibodies (Fig. 6B) resulted in the appearance of bands corresponding to ^{32}P-la-

belled MHC, unlabelled MLC and actin (lane b and b'). However the ^{32}P-phosphorylated 20–21 kDa did not immunoprecipitate with these antibodies (lane b'), suggesting that phosphorylated MLC is dissociated from the myosin complex when cells are treated with TPA. None of these proteins were immunoprecipitated if the antiserum was replaced by normal rabbit serum (data not shown). Phosphorylated MHC and MLC were detected by immunoprecipitation when non-ionic detergents were used to lyse the cells and solubilize proteins in EPEC-treated cells (data not shown).

4. DISCUSSION

Adherence of EPEC strain E2348-69 to HEp-2 and differentiated Caco-2 cells induced pronounced phosphorylation of several groups of proteins (Fig. 1). Of these the 20–21 kDa phosphoproteins have been shown to appear after treatment of HEp-2 cells with a variety of agents including protein kinase activators (TPA, epidermal growth factor, phosphatidic acid, phospholipase C) and angiotensin II [2], (and Manjarrez et al., in preparation). Based on molecular weight, pI and reactivity with antibodies, the 20 kDa phosphoproteins have now been identified as the phosphorylated forms of MLC. In support of this finding, okadaic acid (the inhibitor of protein phosphatases 1 and 2A) which produces an increase in the phosphorylation states of MLC in non-muscle cells [16], also caused a net increase in phosphorylation of the 20–21 kDa proteins in both HEp-2 and Caco-2 cells (Manjarrez et al., in preparation). Since the anti-M antibodies recognise both MHC and MLC, immunoprecipitation of the phosphorylated 20 kDa MLC from cytosolic extracts of TPA stimulated cells does not indicate whether this protein is part of an intact myosin complex. This is clarified by using anti-MHC antibodies when both MHC and unphosphorylated MLC were immunoprecipitated but the phosphorylated 20 kDa protein was not (Fig. 6B, lane b and b'). We conclude that phosphorylated 20 kDa MLC is no longer part of intact myosin following TPA stimulation. Presumably, TPA causes a net release of myosin and/or actomyosin from the cytoskeleton with dissociation of the heavy and light chains, involving phosphorylation

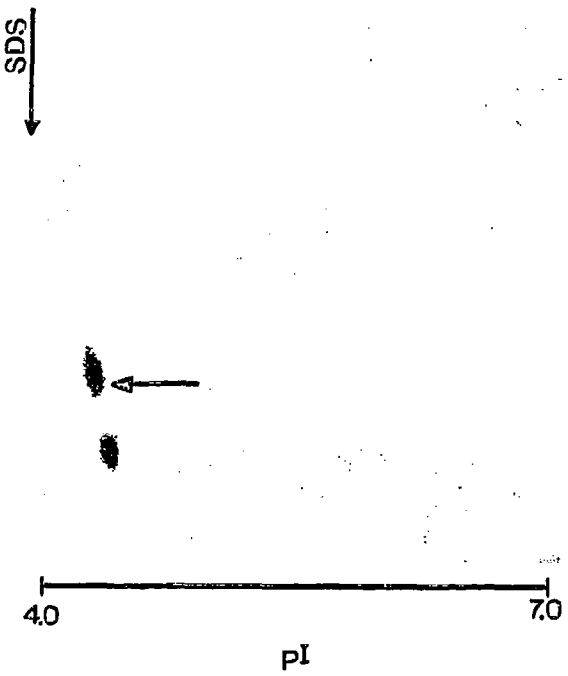

Fig. 5. 2-D PAGE analysis of phosphoproteins from Caco-2 after incubation for 2 h with EPEC strain E2348-69 and immunoprecipitation with antibodies specific for the phosphoform of the regulatory light chain. The only silver stainable protein present (data not shown) was that corresponding to the area blackened on the autoradiograph. The position of the 20 kDa protein is shown arrowed together with the pI range. The lower M_r radioactive spot is probably due to partially proteolyzed phosphorylted myosin light chain.

by protein kinase C. As a consequence, this phosphoprotein, now identified as a phosphorylated MLC could be purified by the method described above.

It has been shown that the regulatory MLC of some invertebrate myosins can be selectively dissociated from myosin by a combination of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and EDTA [7]. The 20 kDa phosphoproteins were immunoprecipitated after EPEC infection of HEp-2 cells only from solubilised cytoskeletal proteins. This would indicate that phosphorylation of MLC, in this case, does not correlate with release of myosin or myosin light chain from the cytoskeleton. Therefore, this may indicate that phosphorylation

Table I

Purification table of the 20 kDa phosphoprotein

Purification step	Pool volume (ml)	Protein (mg)	20 kDa (cpm × 10^{-3})	cpm/mg × 10^{-3}
Cell sonication/centrifugation	2.0	45.0	118.7	2.6
Size exclusion chromatography	8.0	6.0	94.1	15.7
Freezing/thawing centrifugation	7.5	0.78	70.6	90.5
Cation-exhange chromatography	10.0	0.02	24.7	1235.0

Recovery of the 20 kDa phosphoprotein from HEp-2 monolayers (from 20 × 8 cm diameter Petri dishes) after EPEC infection. The recovery was scaled up using Nunc cell factories.

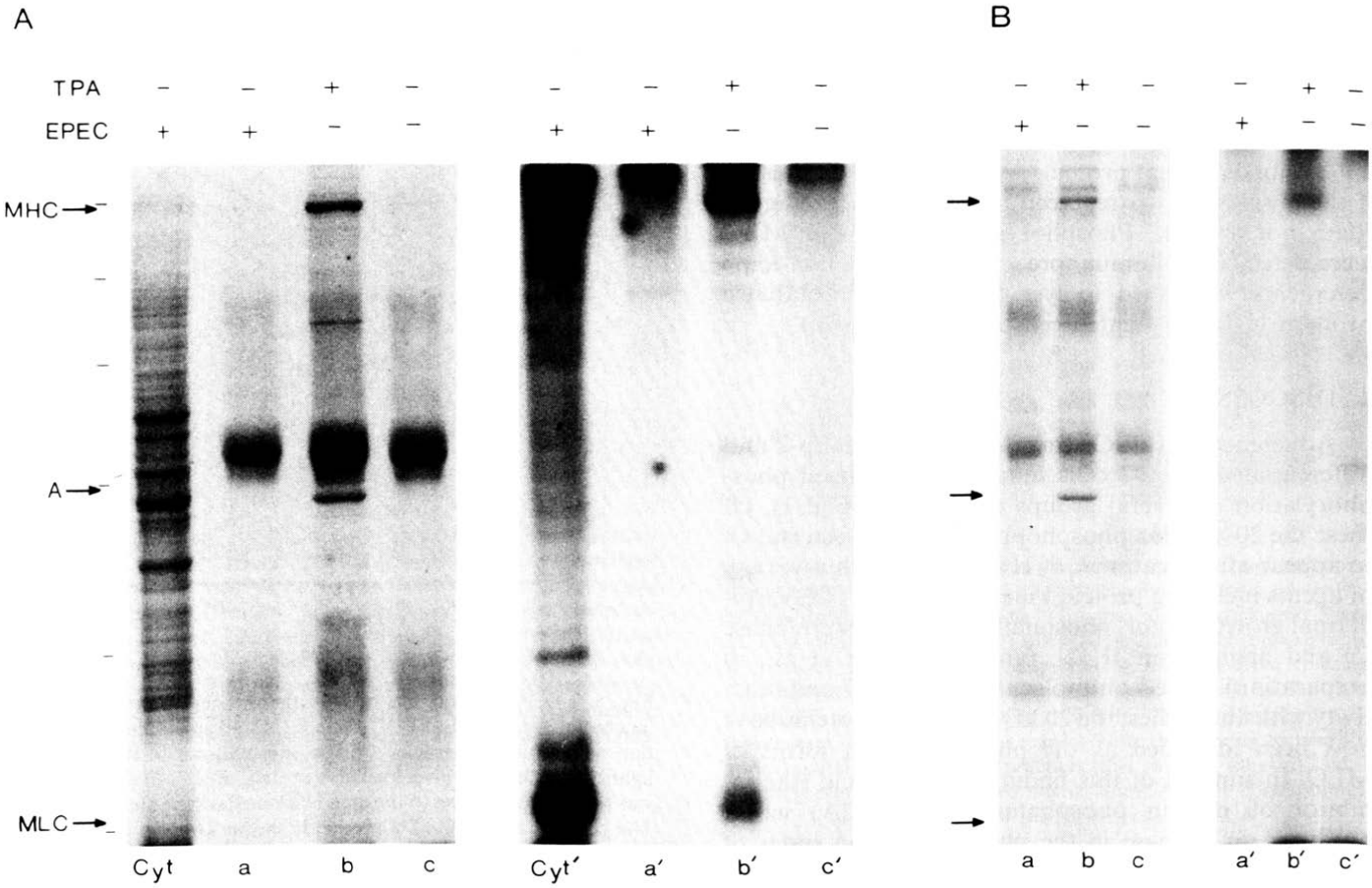

Fig. 6. 10% SDS-PAGE gels of immunoprecipitated cytosolic proteins from HEp-2 cells. Plus and minus indicate treatment with and without TPA or EPEC, respectively. (A) Cytosolic proteins before (Cyt and Cyt') and after immunoprecipitation with anti-M, and (B) with anti-MHC. A, actin; MHC, myosin heavy chain; MLC, myosin light chain (Cyt, a,b and c, Coomassie stain; Cyt', a',b' and c', autoradiograph). Positions of molecular weight markers are indicated (–) and are; myosin heavy chain (205 kDa), phospholipase b (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and β-lactoglobulin (18.4 kDa), from top to bottom, respectively.

occurs at different sites and is caused by different kinases, i.e. protein kinase C or MLC kinase, depending on the type of stimulation.

Hyperphosphorylation of MHC and MLC have been reported to occur in a variety of non-muscle cells in response to TPA [18]. It also has been shown that TPA induces reversible shape changes and synergic cytoskeletal reorganization in fibroblasts [19]. This suggests that MLC has an important function in a variety of physiological and pathological events. Elevated phosphorylation levels could be a consequence of protein phosphatase inhibition in addition to or as an alternative to the activation of several kinases through different transduction pathways.

Phosphorylation of the 20–21 kDa proteins in EPEC-mediated diarrhea only occurs on infection by strains of EPEC that induce localized degeneration of microvilli through cytoskeletal breakdown and membrane vesiculation [2]. It is tempting to speculate, therefore, that increased levels of phosphorylated MLC trigger events culminating in these so-called pedestal structures (accumulation of actin at the point of bacterial contract) [10]. This is consistent with the phosphorylation effect on MLC by angiotensin II, which also causes morphological changes in brush-border cells reminiscent of the lesion-forming effects of EPEC [20].

The nature of the protein kinases involved in the direct phosphorylation of MLC isoforms in response to various agonists, is as yet unclear. Non-muscle myosins have been shown to contain 5 different sites of phosphorylation [21] and in vitro are substrates for as many kinases [22]. In vivo, they have been shown to be phosphorylated by protein kinase C as well as myosin light chain kinase [21], with the existence of both mono- and di-phosphoproteins [23], a possible explanation for the two isolectric forms shown on two-dimensional gels in this study (Fig. 5).

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Table II

Amino acid analysis of the 20 kDa phosphoprotein

Residue	A	B
Asp/Asn	13.9	16.35
Glu/Gln	26.9	13.25
Ser	4.96	4.28
Gly	6.65	5.73
His	1.46	1.34
Arg	4.74	4.76
Thr	3.69	6.69
Ala	8.01	6.75
Pro	4.39	3.13
Tyr	1.49	2.25
Val	3.2	2.26
Ile	1.73	6.24
Leu	4.55	6.18
Phe	1.47	7.65
Lys	8.21	8.19
Met	–	5.07
Trp	–	0
Cys	–	0.58
	95.3%	100%

Values are expressed as mol percent. Met, Trp and Cys values were not determined. The amino acids are presented in the Table in their elution order drom the reverse-phase HPLC PTC column. Column A is the average of three separate determinations of the amino acid analysis of the 20 kDa protein. Column B is the average mol% of two mammalian smooth muscle light chains in the EMBL database and of human nonsarcomeric 20 kDa myosin regulatory light chain [24].

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