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Protein Phosphorylation by Protein Kinase C in HEp-2 Cells Infected with Enteropathogenic *Escherichia coli*

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Infection of HEp-2 monolayers with enteropathogenic *Escherichia coli* 2036-80 (O119) stimulated phosphorylation of several target cell proteins, the most prominent of which had apparent molecular weights of 21,000 and 29,000. Proteins of the same size were phosphorylated in response to known activators of the calcium-phospholipid-dependent protein kinase C. Screening of clinical isolates of various O serogroups revealed that all strains able to form the characteristic attaching and effacing lesion of enteropathogenic *E. coli* showed elevated phosphorylation of 21,000- and 29,000-dalton protein species.

Escherichia coli isolates from cases of diarrhea are a heterogeneous group displaying several pathogenic mechanisms. Enterotoxigenic *E. coli* isolates promote fluid efflux from the intestinal mucosa by secreting toxins that modulate cyclic nucleotide monophosphate levels (11), while enteroinvasive *E. coli* isolates penetrate the intestinal epithelium to elicit an inflammatory response and extensive tissue breakdown (18). So-called enteropathogenic *E. coli* (EPEC) isolates, however, neither invade nor produce recognized enterotoxins but nevertheless cause severe persistent diarrhea in young children (20, 21).

A common feature of many EPEC isolates is the formation of a characteristic attaching and effacing (AE) lesion. This involves intimate contact between the bacterial envelope and the plasma membrane of the target cell, followed by rapid localized degeneration of microvilli through cytoskeletal breakdown and membrane vesiculation (17, 31) and by formation of the so-called pedestal structure. Similar surface distortions occur when EPEC isolates attach to cultured epithelial cell lines such as HEp-2 (15); actin microfilaments, presumably derived from localized cytoskeletal breakdown, accumulate at the point of bacterial contact and can be detected microscopically after being stained with fluorescein-conjugated phalloidin (a phallotoxin that specifically binds to actin filaments). This forms the basis of the rapid fluorescent actin staining (FAS) test for identifying EPEC (16).

Many features of the AE lesion are reminiscent of effects previously seen in brush border cells treated with calcium ionophores and with certain hormones that increase intracellular calcium concentrations (14, 25). Hormone binding to receptors activates phopholipase C, which breaks down membrane phosphatidylinositol lipids, generating the second messengers diacylglycerol (DAG) and 1,4,5-inositol triphosphate (3, 23). DAG in the membrane activates the calcium-phospholipid-dependent protein kinase C (PKC), while 1,4,5-inositol triphosphate induces release of calcium from internal stores (10); both PKC and cytosolic calcium affect the architecture of the cell by modulating assembly of cytoskeletal elements (25, 30, 34, 35). Ca^{2+}-calmodulin-dependent protein kinases activated by the elevation of intracellular calcium may also be involved in cytoskeletal rearrangements (7, 24). Activation of protein kinases, particularly the calcium-phospholipid-dependent PKC, has previously been shown to stimulate intestinal ion efflux by phosphorylation of specific membrane-associated ion transport proteins (12, 13; M. Donowitz, H. Cheng, and G. W. G. Sharp, Gastroenterology **88**:1367, 1985; J. D. Fondacaro, G. P. McCafferty, and L. S. Henderson, Gastroenterology, **88**:1386, 1985).

Using the human laryngeal carcinoma cell line HEp-2 as a model system for infection (15), we showed in the present study that EPEC induces specific alterations in host protein phosphorylation patterns comparable with those mediated by known activators of PKC, and we propose that this enzyme has an important role in the pathogenesis of EPEC.

MATERIALS AND METHODS

Bacterial strains. EPEC strain 2036-80 (O119), isolated from a case of severe infantile diarrhea, has been described previously (17). It harbors a number of plasmids (T. J. Baldwin, unpublished data), including one of about 90 kilobase pairs (kbp) containing sequences homologous with the EPEC adherence factor DNA probe (2, 29). This probe is a 1-kbp fragment of a 90-kbp plasmid (pMAR2) required for localized adherence (LA) of EPEC strain E2348 (O127) to HEp-2 cells (29). Strain 2036-80 also shows LA to HEp-2 cells; furthermore, it causes actin accumulation detectable by the FAS test (16) at sites of bacterial adherence to HEp-2 and other cultured cells, and it promotes localized effacement of microvilli characteristic of the EPEC AE lesion in human ileal biopsy material, as defined electron microscopically (17).

Also used in this work were 15 other clinical strains of various O serogroups (see Table 1); some have been described previously, and others are recent isolates from cases of infantile diarrhea in Great Britain. Strains were serotyped at the Central Public Health Laboratory, Colindale, United Kingdom, and all were screened for pattern of adherence to HEp-2 cells (29) and AE lesion development by the FAS test (16). Laboratory *E. coli* strain HB101 (5) carrying plasmid pMAR15 was included as a control strain in this study; pMAR15 is a 40-kbp derivative of the E2348 plasmid pMAR2, in which some of the sequences nonessential for adherence to cultured cells have been deleted (2, 15). Strain

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HB101(pMAR15) shows LA to HEp-2 cells but is unable to form the characteristic AE lesion (15).

For infection of HEp-2 cells, single colonies were inoculated into 10-ml portions of Luria broth and incubated overnight at 37°C without agitation. Cultures were diluted fivefold into fresh broth and incubated for a further 3 h to the mid-exponential phase before use.

Cell culture. HEp-2 cells were routinely grown in Dulbecco modified Eagle medium containing 10% (vol/vol) fetal calf serum at 37°C under 5% CO_2 in air. The medium was replaced every 2 days until monolayers were confluent, at which time they were transferred to Dulbecco modified Eagle medium containing 0.5% (vol/vol) fetal calf serum for 30 to 40 h prior to treatment.

Phosphoprotein preparation. Confluent HEp-2 cell monolayers were maintained for 4 h immediately before use in phosphate-free Dulbecco modified Eagle medium (2 ml) containing 0.5% fetal calf serum and 100 μCi of carrier-free ^{32}P to label intracellular ATP pools. After treatment with bacterial suspensions (10^8 CFU in 0.3 ml) or with other agents (as described in the appropriate figure legends) for the times indicated, the cells were rapidly washed three times in ice cold phosphate-buffered saline to remove unincorporated ^{32}P. They were then suspended in 35-μl aliquots of 50 mM Tris hydrochloride (pH 5.0) buffer containing 50 mM benzamidine hydrochloride, 50 mM sodium fluoride, 2.5 mM sodium PP_i, 5 mM β-glycerophosphate, 2 mM EDTA, 20 μg of leupeptin per ml, 20 μg of pepstatin A per ml, 1.5% (vol/vol) Triton X-100, and 0.1% (wt/vol) sodium dodecyl sulfate (SDS) and rapidly frozen. Thawed cell suspensions were disrupted by sonication on ice, and the proteins were solubilized by the addition of an equal volume of 62.5 mM Tris hydrochloride buffer (pH 6.8) containing 4% (wt/vol) SDS, 20% (vol/vol) glycerol, 10% (vol/vol) 2-mercaptoethanol, and 0.001% (wt/vol) bromophenol blue and boiling for 5 min.

Analysis of phosphoproteins. Cell debris was pelleted by centrifugation, and solubilized phosphoproteins were analyzed by electrophoresis on 10 or 12.5% (wt/vol) polyacrylamide gels containing SDS by the method of Laemmli (19). The gels were subsequently stained with Coomassie brilliant blue, destained, and dried. Radiolabeled proteins were detected by autoradiography.

Chemicals and reagents. Cell culture media were purchased from GIBCO Ltd, Paisley, Scotland, and carrier-free $^{32}P_i$ (10 mCi/ml) was supplied by Amersham International plc., Amersham, England. Human angiotensin II (acetate salt), calcium ionophore A23187 (calimycin), N^6,2′-*O*-dibutyryl-cyclic AMP (sodium salt), N^2,2′-*O*-dibutyryl-cyclic GMP (sodium salt), dioleoyl L-α-phosphatidic acid (sodium salt), and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were all obtained from Sigma Chemical Co. Ltd., Poole, England. We are grateful to Martin Low (Columbia University, New York, N.Y.) for a gift of purified phospholipase C from *Staphylococcus aureus*.

RESULTS

Phosphorylation of HEp-2 cell proteins in response to infection with EPEC strain 2036-80. Incubation of HEp-2 monolayers for various times with strain 2036-80 caused increasing phosphorylation of several proteins compared with those in uninfected cells (Fig. 1a). The most prominent phosphorylated species were proteins with apparent molecular weights of 21,000 and 29,000 (21K and 29K proteins); the 21K species formed a broad band and may therefore represent a multiply phosphorylated protein or a glycoprotein. Minor proteins of 23K and 27K and several between 30K and 45K were also observed (Fig. 1a). In addition, dephosphorylation of some proteins (for example, one with a molecular weight of 45,000) was evident 1 to 2 h after infection. It is unlikely that increased labeling of the 21K band simply represents proteolysis of larger proteins; not only was the initial intensity of the 45K band considerably less than that of the 21K protein but dephosphorylation always occurred more rapidly than increased phosphorylation after EPEC infection. Varying the dose of infecting EPEC had little effect on the degree of phosphorylation of the 21K and 29K proteins (data not shown), probably because infection is a dynamic process in which bacteria are continually growing and colonizing new cells.

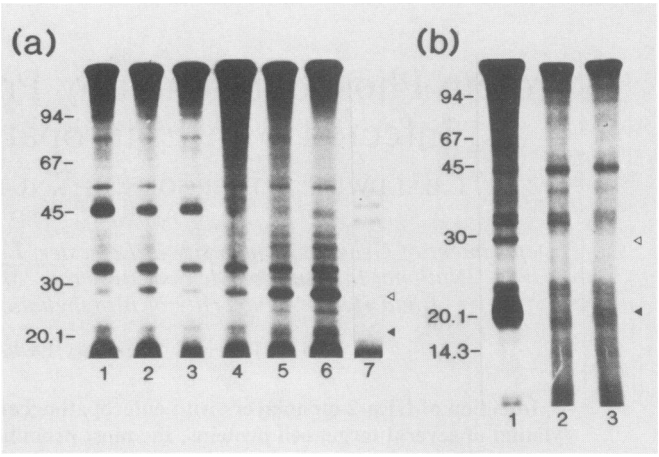

FIG. 1. EPEC-induced phosphorylation of HEp-2 cell proteins. (a) SDS-polyacrylamide (10% [wt/vol]) gel electrophoresis of ^{32}P-labeled total-cell phosphoproteins prepared from HEp-2 monolayers after incubation with bacteria. Lane 1, Uninfected; lane 2, infected with strain HB101(pMAR15) for 3 h; lanes 3 through 6, infected with EPEC strain 2036-80 for 0.5, 1, 2, or 3 h, respectively; lane 7, phosphorylated species from strain 2036-80 similarly labeled for 3 h in the absence of HEp-2 cells. (b) SDS-polyacrylamide (12.5% [wt/vol]) gel electrophoresis of ^{32}P-labeled total-cell phosphoproteins prepared from HEp-2 monolayers incubated for 3 h with live strain 2036-80 (lane 1), heat-killed (65°C, 7 min) strain 2036-80 (lane 2), or filter-sterilized culture supernatants of strain 2036-80 (lane 3). Molecular weight markers were phosphorylase *b* (94,000), albumin (67,000), ovalbumin (45,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and lactalbumin (14,300). Arrowheads indicate the 21K and 29K phosphoproteins described in the text.

Incubation of killed HEp-2 cells with EPEC resulted in no significant enhancement of phosphorylation of these proteins (data not shown), nor was there significant enhancement when HEp-2 cells were incubated with killed bacteria or with the culture medium in which the bacteria had been grown (Fig. 1b). Phosphorylated species from the EPEC strain itself showed a ladder appearance (Fig. 1a) characteristic of molecules with regular-size increments, such as lipopolysaccharide, but none of these coincided with the major phosphoproteins detected in EPEC-infected HEp-2 cells. Infection with the nondiarrheagenic laboratory strain HB101 (pMAR15), which carries a plasmid encoding the initial localized nonintimate adherence of EPEC to HEp-2 cells (2, 15), caused no changes in phosphorylation of proteins compared with that in uninfected cells (Fig. 1a).

Correlation of phosphorylation with EPEC lesion-forming ability. To determine the generality of the effects observed with EPEC strain 2036-80, we analyzed phosphoprotein

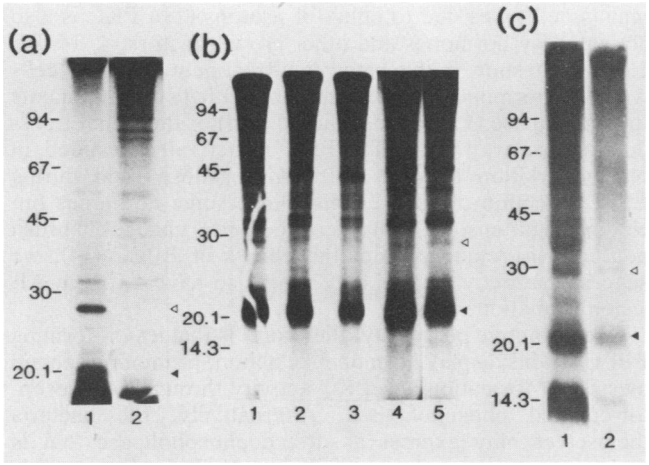

FIG. 2. Screening of EPEC isolates for ability to phosphorylate HEp-2 cell proteins. SDS-polyacrylamide (12.5% [wt/vol]) gel electrophoresis of ^{32}P-labeled total-cell phosphoproteins prepared from HEp-2 monolayers incubated for 3 h with FAS test-positive clinical isolates 2036-80 (lane 1), E2348 (lane 2), E1621-1 (lane 4), E57107 (lane 5), E57106 (lane 7), E851 (lane 10), BCH2/82 (lane 11), and 182/83 (lane 12). HEp-2 cells were also incubated with FAS test-negative clinical isolates DP095 (lane 3), 135 (lane 6), and 469-3 (lane 9). Lanes 13 and 8, Protein profiles of uninfected HEp-2 cells and of cells incubated for 3 h with the laboratory strain HB101(pMAR15), respectively. Molecular weight markers were as described in the legend to Fig. 1. Arrowheads indicate the 21K and 29K phosphoproteins described in the text.

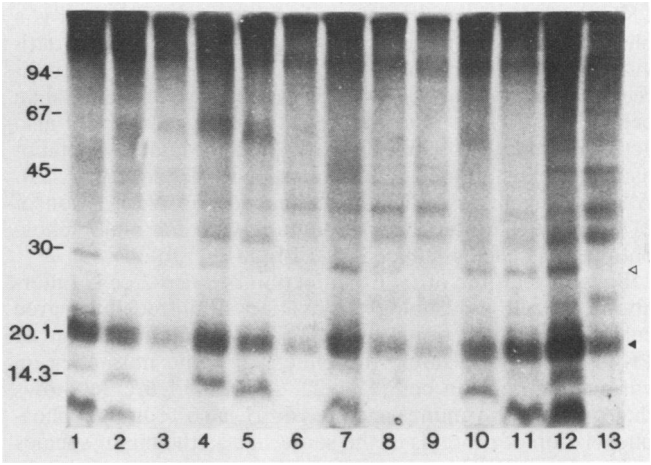

FIG. 3. Effect of known activators of protein kinases on HEp-2 cell protein phosphorylation. (a) SDS-polyacrylamide (10% [wt/vol]) gel electrophoresis of ^{32}P-labeled total-cell phosphoproteins prepared from HEp-2 monolayers incubated for 3 h with EPEC strain 2036-80 (lane 1) or treated for 1 h with 12.5 μM A23187 (lane 2). (b) SDS-polyacrylamide (12.5% [wt/vol]) gel electrophoresis of ^{32}P-labeled total-cell phosphoproteins prepared from HEp-2 monolayers incubated with EPEC strain 2036-80 for 3 h (lane 1), TPA (10 nM) for 1 h (lane 2), lipid A (20 ng/ml) for 4 h (lane 3), phosphatidic acid (10 nM) for 15 min (lane 4), or phospholipase C (1 μg/ml) for 1 h (lane 5). (c) SDS-polyacrylamide (12.5% [wt/vol]) gel electrophoresis of ^{32}P-labeled total-cell phosphoproteins prepared from HEp-2 monolayers incubated for 3 h with EPEC strain 2036-80 (lane 1) or for 3 h with angiotensin II (1 μg/ml) (lane 2). Molecular weight markers were as described in the legend to Fig. 1. Arrowheads indicate the 21K and 29K phosphoproteins described in the text.

patterns of HEp-2 cells infected with several other isolates of various O serogroups from cases of severe infantile diarrhea (Table 1 and Fig. 2). All strains were characterized for pattern of adherence to HEp-2 cells (LA or diffuse adherence [DA] [29]) and for lesion-forming ability as assessed by the FAS test (16). Figure 2 shows a representative autoradiogram of a gel in which seven FAS test-positive strains and three negative strains were analyzed; the former induced HEp-2 cell phosphoprotein profiles comparable with that caused by strain 2036-80, while the FAS test-negative strains showed no appreciable elevation of phosphorylation above that in uninfected cells or cells treated with the control strain HB101(pMAR15). These results are summarized in Table 1, which also includes data for five additional FAS test-positive strains, all of which also caused the phosphorylation pattern characteristic of strain 2036-80.

TABLE 1. Correlation between AE lesion formation and phosphorylation of HEp-2 cell proteins among clinical isolates from cases of diarrhea

Strain	Serogroup	Reference(s)[a]	Adherence pattern[b]	FAS test	Phosphorylation
DPO95	O18ac	16	DA	−	−[c]
469-3	O21	16	DA	−	−[c]
660/79	O55	16, 17	LA	+	+
135	O86	16, 17	DA	−	−[c]
BCH2/82	O111	17	LA	+	+[c]
1923/77	O111	17	LA	+	+
E57104	O114		LA	+	+
QEH1/86	O114	16	LA	+	+
E1621-1	O119		LA	+	+[c]
E2348	O127	15–17	LA	+	+[c]
E57108	O127		LA	+	+
E57107	O127a		LA	+	+[c]
E57106	O127ab		LA	+	+[c]
182-83	O128ac	16, 17	LA	+	+[c]
E851	O142	16, 17	LA	+	+[c]
HB101(pMAR15)		2, 15, 16	LA	−	−[c]

[a] Strains for which no references are given are newly described in this study.
[b] DA, Diffuse adherence; LA, localized adherence.
[c] Data shown in Fig. 2.

Effects of known activators of mammalian protein kinases on HEp-2 cells. To identify the nature of the observed changes in phosphoprotein profiles induced by EPEC strains, we attempted to mimic the effects with a variety of agents known to stimulate protein kinase activity. The observation that EPEC infection of HEp-2 cell monolayers causes elevation of cytosolic calcium concentrations (T. J. Baldwin, unpublished data) suggested that a calcium-dependent protein kinase(s) might be involved. However, phosphoprotein profiles from HEp-2 cells treated with the calcium ionophore A23187 were markedly different from those of cells infected with lesion-forming EPEC (Fig. 3a). Elevated phosphorylation of proteins only in the range of 30,000 to 45,000 corresponded to some of the EPEC-induced minor phosphoproteins; several additional larger phosphoprotein species (>75,000) were also observed. Incubation of HEp-2 cells with lipophilic dibutyryl derivatives of cyclic AMP and cyclic GMP caused little observable change in phosphorylation profiles (data not shown), indicating that cyclic nucleotide-dependent kinases were not involved in the major phosphorylation events.

PKC was implicated as the cause of the major EPEC-induced phosphorylated species by comparison of the effects of several known activators of this enzyme. TPA is a powerful tumor-promoting agent which directly activates

PKC by substituting for DAG (1, 6). Phosphatidic acid is a phosphorylated derivative of DAG which also directly activates PKC (4, 27). Phospholipase C stimulates PKC indirectly by generating DAG in the membrane of mammalian cells (3, 23). Lipid A (in bacterial lipopolysaccharide) also acts indirectly, probably by providing, through its metabolism, the phospholipid requirement of PKC activity (9, 33). All these agents significantly enhanced phosphorylation of 21K and 29K proteins corresponding with the major phosphoprotein species induced by EPEC (Fig. 3b).

Several features of EPEC infection of HEp-2 cells (alterations in cell morphology, elevation of intracellular free calcium concentration, and the proposed stimulation of PKC) strongly resemble the effects of some hormones on various mammalian cells (14, 22, 26, 32). Figure 3c shows that one such hormone, angiotensin II, also promotes phosphorylation of proteins of the same size as the major species induced by EPEC, suggesting similar receptor-coupled indirect activation of PKC.

DISCUSSION

Adherence of EPEC strains to HEp-2 cell monolayers stimulated phosphorylation of several proteins, the most prominent of which had apparent molecular weights of 21,000 and 29,000. As far as we are aware, this is the first report of extensive phosphorylation of mammalian cell proteins as a direct result of infection with bacteria. There is some evidence that soluble enterotoxins may activate cyclic nucleotide-dependent protein kinases in target cells (8). However, none of the EPEC strains used in our study elaborated detectable soluble enterotoxins; moreover, exogenously added membrane-permeable cyclic nucleotides did not mimic the major phosphorylation events observed with bacterial infection. Artificial elevation of intracellular free calcium concentrations to stimulate calcium-dependent protein kinases (7, 24) caused elevated phosphorylation of several proteins, some of which were similar in size to minor phosphoproteins seen with EPEC infection. However, convincing evidence that PKC plays a significant role in EPEC-induced phosphorylation and indeed is an important component of the pathogenic mechanism of the group comes from the observation that several direct and indirect activators of this enzyme also promoted phosphorylation of 21K and 29K protein species in HEp-2 cells. Activation of PKC by EPEC probably requires contact of live bacteria with the plasma membrane. However, bacterial adherence alone seems not to be sufficient for the observed effects, since some strains that adhere intimately to HEp-2 cells did not stimulate PKC-like activity. Significant protein phosphorylation was a feature only of EPEC strains which promoted gross cytoskeletal changes in HEp-2 cells, detectable as actin accretion at the point of bacterial contact by the FAS test.

PKC is pivotal in the control of normal ion fluxes across mammalian cell membranes (12, 13; Donowitz et al., Gastroenterology; Fondacaro et al., Gastroenterology); prolonged stimulation of PKC activity in response to EPEC infection may perturb the system sufficiently to cause exaggerated ion efflux from the intestinal mucosa. In support of this model we note that intestinal hypersecretion and diarrhea of comparable severity to that induced by cholera toxin were seen after oral administration of TPA, a potent activator of PKC, to conscious rats (12, 13). TPA also increased chloride ion secretion from isolated rabbit intestinal tissue (Donowitz et al., Gastroenterology; Fondacaro et al., Gastroenterology) and raised intracellular pH in various mammalian cell types due to efflux of protons (28). PKC is also activated by hormones and other growth factors (4, 14, 22, 26, 32). We show in this paper that treatment of HEp-2 cells with the hormone angiotensin II or with two components (phospholipase C and phosphatidic acid) of the signal transduction pathway by which PKC is activated resulted in phosphorylation patterns identical to those seen during EPEC infection of HEp-2 cells. Since some hormones (including angiotensin II) cause morphological changes in brush border cells reminiscent of the effects of EPEC (14), we suggest that activation of PKC may also have a role in AE lesion formation.

One attractive possibility, therefore, is that lesion-forming EPEC strains display hormonelike action, promoting second messenger formation and PKC activity through host receptor-coupled phospholipases. Alternatively, the bacteria themselves may express a surface phospholipase that is active on contact with mammalian cells. It is also possible that adherence of EPEC presents the lipid A moiety of lipopolysaccharide in a particularly effective orientation for processing and intercalation into the target cell membrane to stimulate PKC. The genetic and biochemical basis of PKC induction by bacteria and the identity of the major phosphoproteins observed in HEp-2 and other cell types are areas of current investigation in our laboratories.

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