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Phosphorylation of Myosin Light Chain at Distinct Sites and Its Association with the Cytoskeleton during Enteropathogenic *Escherichia coli* Infection

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Myosin light chain, the most prominent host cell phosphoprotein during adhesion of enteropathogenic *Escherichia coli* to cultured HEp-2 cells, was shown to be distributed between cytosolic and cytoskeletal cell fractions; its association with the cytoskeletal fraction increased with time of enteropathogenic *E. coli* incubation. Phosphopeptide mapping indicated cytosolic and cytoskeletal myosin light chain phosphorylation at different sites by protein kinase C and myosin light chain kinase.

Enteropathogenic *Escherichia coli* (EPEC) remains an important cause of infantile diarrhea in developing countries. EPEC colonizes the small bowel and exploits host cell signal transduction pathways to produce an attaching-and-effacing lesion in which enterocyte microvilli are destroyed; bacteria adhere intimately to the apical enterocyte surface (12); and cytoskeletal proteins, including actin and myosin, accumulate in a dense plaque beneath adherent bacteria (11, 15). Similar lesions are produced when EPEC adheres to cultured HEp-2 cells (10). We previously showed that EPEC adhesion to HEp-2 cells caused localized elevation of calcium levels by release from intracellular stores (2) and stimulated phosphorylation of several proteins, the most prominent of which was myosin light chain (MLC) (16); MLC phosphorylation was also observed in Caco-2 cells and in human small intestinal mucosal organ cultures infected with EPEC (15). Reversible phosphorylation of MLC controls actin-myosin interaction in invertebrate smooth muscle and nonmuscle cells (6, 20) and is related to changes in actin organization and cell shape in nonmuscle cells (3); it is likely, therefore, to be important in the actomyosin cytoskeletal rearrangements which occur during EPEC attaching-and-effacing lesion formation. We previously suggested that protein kinase C (PKC) was implicated in the phosphorylation of MLC, since treatment of HEp-2 cells with direct or indirect activators of PKC (12-*O*-tetradodecanoylphorbol-13-acetate [TPA], phosphatidic acid, or phospholipase C) caused an increase in phosphorylation levels similar to those observed during EPEC infection of the same cells (1). Phosphorylated MLC in HEp-2 cells (16) comprises two forms with isoelectric points (pI) and molecular weights (20 to 21 kDa) consistent with observations made by Burnham et al. (4). These may be attributable to different extents of phosphorylation or some other charge isoform at the protein sequence level. Notably, the pI values of the phosphorylated MLC isoforms are unusually acidic (pI = 3.8 to 4.1) compared with the pI values of those found in pancreatic acini (pI = 4.8 [4]), platelets (4.7 to 4.9, [9]), and vascular smooth muscle (4.6 to 5.0 [21]). Mono- and diphosphorylated forms of the MLCs have been found in smooth muscle (19) and nonmuscle cells (9) after PKC activation.

Protein phosphorylation in intact cells infected with EPEC strain E2348/69 (O127:H6) or treated with TPA was carried out as previously described (1, 16). In order to investigate the nature and extent of MLC phosphorylation and its cellular distribution, cells were fractionated into Triton X-100-insoluble (cytoskeletal) and -soluble (cytosolic) fractions according to the method of Fox and Phillips (5) and phosphorylated proteins were detected by autoradiography following analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1, 16). Relative quantities of ^{32}P-labelled MLC were assessed by scintillation counting of excised bands. Two-dimensional phosphoamino acid and phosphopeptide mapping of phosphorylated MLC was carried out according to the methods of Hunter and Sefton (8) and Naka et al. (17), respectively. Fractionation of TPA-treated HEp-2 cells (Fig. 1) indicated that all the phosphorylated 20-kDa protein was located in the cytosol. Phosphoamino acid analysis of this protein showed 82.6% phosphothreonine and 17.4% phosphoserine, consistent with PKC phosphorylation of MLC as demonstrated for smooth muscle MLC (18).

In contrast, significant amounts of phosphorylated 20-kDa MLC in HEp-2 cells infected with EPEC strain E2348/69 were observed in the pellets of fractionated cells (Fig. 1), especially after prolonged infection. Thus, after 1 h of EPEC infection, 90% of phosphorylated 20-kDa MLC was in the supernatant fraction (cytosol), while at 4 h 75% was associated with the cytoskeleton. Phosphoamino acid analysis showed 69.7% phosphoserine and 30.3% phosphothreonine, strongly suggesting the involvement of an additional kinase (other than PKC) in the phosphorylation of MLC in response to EPEC-mediated events.

Further support for the involvement of a second kinase is provided by two-dimensional phosphopeptide mapping of tryptic digests of MLC, which indicates that the cytosolic and cytoskeletal forms of the protein observed after EPEC infec-

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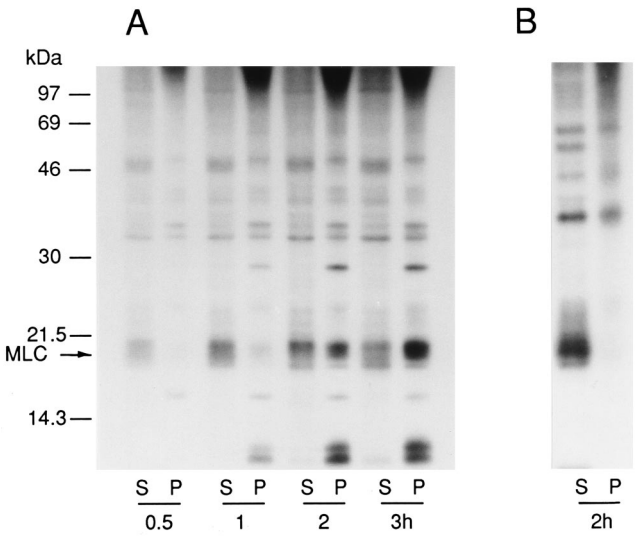

FIG. 1. An autoradiograph comparing the distribution of phosphorylated proteins between the cytosolic (S) and cytoskeletal (P) fractions induced by treatment of HEp-2 cells with EPEC strain E2348/69 over the time course shown (A) and following a 2-h treatment with TPA (B).

tion are phosphorylated at distinct sites (Fig. 2). Thus, MLC from the supernatant fraction of EPEC-infected cells contains a phosphopeptide that is also found in MLC phosphorylated in response to TPA treatment (i.e., by PKC). On the other hand, the 20-kDa phosphoprotein in the cytoskeleton-associated fraction of EPEC-infected cells contains a different phosphopeptide (Fig. 2).

We propose, therefore, that the site phosphorylated by both TPA and EPEC (phosphopeptide 1) in some way reduces the association of MLC with the cytoskeleton, while the site induced by EPEC alone (phosphopeptide 2) is compatible with such an association. Phosphopeptide 2 could be a substrate site for a Ca^{2+}-dependent kinase such as the narrow specificity Ca^{2+}-calmodulin-dependent MLC kinase (MLCK). Consistent with this, it has been shown that intracellular free calcium levels are increased by release from IP_3-sensitive stores (2) and that the calmodulin inhibitor 48/80 reduced the phosphorylation of MLC during EPEC infection (15).

There is comparatively little information on nonmuscle MLC phosphopeptide patterns, although Higashihara et al. (7) reported that platelet MLC is phosphorylated by both MLCK and PKC but only phosphorylation by MLCK shifts the equilibrium from the inactive (10S) MLC conformation towards the active (6S) conformation. Moreover, PKC phosphorylation decreased the affinity of myosin for actin.

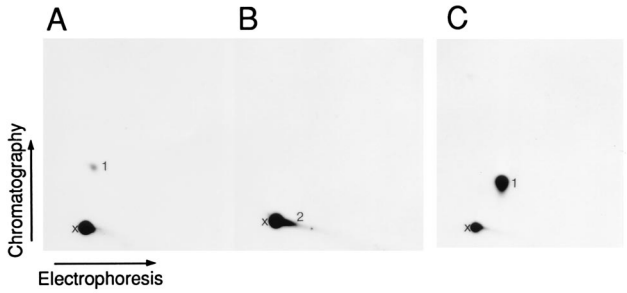

FIG. 2. Two-dimensional phosphopeptide maps of tryptic peptides of the phosphorylated 20-kDa MLC from HEp-2 cells in cytosolic (A) and cytoskeletal (B) fractions after EPEC infection and the cytosolic fraction after TPA treatment (C). Spots 1 and 2 represent peptides 1 and 2, respectively; x indicates the origin.

The physiological significance of differential phosphorylation of MLC in EPEC-infected cells is not clear, but since dephosphorylation of MLC results in a loss of actin microfilament bundles (13), we suggest that the phosphorylation state of MLC may play a key role in the control of actin microfilament polymerization-depolymerization and thus in the formation of the attaching-and-effacing lesion. A further physiological consequence of EPEC-induced phosphorylation of MLC could be to contribute to diarrhea through an effect on the regulation of paracellular water and electrolyte movement. Opening of tight junctions by contraction of the tight-junction-associated actomyosin cytoskeleton is one mechanism whereby paracellular transport is thought to be regulated (14). EPEC is known to increase paracellular conductance of polarized monolayers of intestinal cells, and the permeability defect has been localized to the tight junctions (22). Paracellular permeability has also been shown to be increased by overexpression of constitutive MLCK (22) and prevented in EPEC-infected cells by inhibition of MLCK (23). Thus, an important physiological consequence of EPEC-induced phosphorylation of MLC (possibly mediated by MLCK) could be to increase the permeability of tight junctions and thus to contribute to diarrhea by affecting transport processes and passive water absorption as a consequence of the dissipation of electrochemical gradients.

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