Journal of Cell Science 108, 1735-1742 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

Epidermal growth factor induces tyrosine phosphorylation and reorganization of the tight junction protein ZO-1 in A431 cells

Christina M. Van Itallie*, Maria S. Balda† and James Melvin Anderson

Departments of Internal Medicine and Cell Biology, Yale University School of Medicine, New Haven, CT 06520-8019, USA

*Author for correspondence
†Present address: Department de Biologie Cellulaire, Sciences III, Geneva, Switzerland

SUMMARY

Addition of epidermal growth factor (EGF) to A431 human epidermal carcinoma cells results in actin reorganization and phosphorylation of several cytoskeletal proteins. In the present study, we found that EGF treatment of this cell line also results in the redistribution and tyrosine phosphorylation of ZO-1. In normal polarized epithelial cells, ZO-1 is restricted to the cytoplasmic surface of the most apical of the intercellular junctions, the tight junction. In contrast, ZO-1 in the majority of unstimulated A431 cells in small subconfluent islands colocalizes with actin along the lateral cell membranes and in rare microspikes and membrane ruffles. Exposure to EGF results in a transient redistribution of actin into an apically positioned ring. ZO-1 becomes highly focused at apical sites of cell contact and co-localizes with the newly formed band of perijunctional actin. Coincidently, ZO-1 and another tight junction protein, ZO-2, become transiently phosphorylated on tyrosine residues, as determined by anti-phosphotyrosine immunoblotting. Pretreatment of A431 cells with cytochalasin D, which disrupts normal microfilament organization, does not affect EGF-dependent phosphorylation of the EGF receptor. However, cytochalasin D pretreatment blocks both the EGF-induced ZO-1 rearrangement and tyrosine phosphorylation, suggesting that these responses are dependent on an intact actin microfilament system. We speculate that the transient tyrosine phosphorylation of ZO-1 in response to EGF treatment may be involved in remodeling of intercellular junctions in A431 cells.

Key words: ZO-1, ZO-2, epidermal growth factor, A431, actin, tyrosine phosphorylation, tight junction

INTRODUCTION

The cellular effects of epidermal growth factor (EGF) are mediated through a specific 180 kDa receptor with intrinsic tyrosine kinase activity. Binding of EGF to its receptor results in multiple cellular responses, including autophosphorylation of the receptor and tyrosine phosphorylation of several cytoplasmic proteins, generation of inositol phosphates and an increase in intracellular free Ca^{2+}, changes in transcription of specific genes, and in some cells, initiation of cell proliferation (reviewed by Hernandez-Sotomayor and Carpenter, 1992). In addition, EGF has dramatic effects on polymerization and organization of actin and these changes have been well documented in the A431 human epidermal epithelial cell line. In this cell line induction of surface microspikes is observed within 30 seconds, followed by membrane ruffling within 2-5 minutes and then by cell rounding after 10-20 minutes of continuous EGF exposure (Chinkers et al., 1979). Additional changes include disappearance of basal stress fibers and relocalization of filamentous actin into a ring adjacent to apical cell-cell contacts (Rijken et al., 1991). This perijunctional actin network is typical of polarized epithelial cells and, together with the reported effects of EGF on reorganization and phosphorylation of several cytoskeletal proteins (Bretscher, 1989), led us to investigate the effects of EGF on the tight junction protein ZO-1 in this cell line.

Normal epithelial cells have at their apical border a series of intercellular junctions (Farquhar and Palade, 1963). The most apical of these contacts is the tight junction, which seals the paracellular space. Immediately adjacent to the tight junction is the adherens junction. In electron microscopic images both junctions appear to have direct connections to actin (Hirokawa and Tilney, 1986). Although no actin-binding proteins are yet known in the cytoplasmic plaque of proteins under the tight junctions, adherens junctions contain the actin-binding proteins α-actinin and vinculin (reviewed by Tsukita et al., 1993). In addition, it has recently been demonstrated that two members of the catenin family associated with adherens junctions are tyrosine phosphorylated in A431 cells in response to EGF (Shibamoto et al., 1994; Hoschuetzky et al., 1994). A functional link between actin organization and the tight junction is supported by observations that disruption of perijunctional actin with either cytochalasin D (Madara et al., 1986) or the ZO-toxin of cholera (Fasano et al., 1991) breaks the paracellular barrier.

ZO-1 is a 220 kDa protein component of epithelial and endothelial cell tight junctions (Stevenson et al., 1986). Both ZO-1 and a second tight junction protein, ZO-2, that coimmunopre-

cipitates with ZO-1 (Gumbiner et al., 1991), have recently been identified as members of a family of putative signalling proteins (Willott et al., 1993; Jesaitis and Goodenough, 1994) called MAGUKs (for membrane-associated guanylate kinase homologs; P. Bryant and D. Woods, Irvine CA, personal communication). All members of this family have an SH3 domain, a region of homology to guanylate kinase and a 90 amino acid motif of unknown function termed the PDZ domain (for PSD-95/discs-large/ZO-1; M. Kennedy, Pasdena, CA). In addition they all share a conserved tyrosine residue (position 621 in human ZO-1) followed by EXV, a putative SH2 domain recognition sequence. MAGUK family members are associated with the plasma membrane, predominantly at specialized sites of cell-cell contact. The defining member of this family is the product of the discs-large tumor suppressor gene of *Drosophila* (Woods and Bryant, 1991), the protein product of which localizes to septate junctions, the invertebrate equivalent of tight junctions. Deletion or mutation of the discs-large gene leads to overgrowth of the imaginal disc epithelia (Woods and Bryant, 1991), suggesting that this protein may have a role in regulation of cell growth. Erythrocyte p55, another member of this family, has been shown to bind two proteins, protein 4.1 and dematin, which regulate actin organization in the red blood cell membrane cytoskeleton (Alloisio et al., 1993).

The presently incomplete information on the MAGUK family suggests members may participate in membrane signalling events and conceivably regulate the organization of cortical actin (reviewed by Anderson et al., 1993). However, the nature of the signalling events are presently undefined. The conserved tyrosine residue within MAGUK proteins and the recent report that ZO-1 tyrosine phosphorylation occurs during experimentally-induced collapse of tight junctions between glomerular epithelial cells (Kurihara et al., 1995) has suggested that this modification may play a role in tight junction assembly or regulation. Here we report that treatment with EGF of A431 cells in subconfluent islands results in tyrosine phosphorylation of ZO-1 and redistribution with actin into a focused apical ring of intercellular contact. In addition, we demonstrate that disruption of the actin microfilament system with cytochalasin D prevents both ZO-1 reorganization and EGF-induced phosphorylation.

MATERIALS AND METHODS

Cell culture and EGF treatment

A431 cells were maintained in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (JRH Biosciences, Lexena, KS). Subconfluent cells were used for all experiments; ZO-1 immunoprecipitation required pooling of two 60 mm plates for each point. For each experiment, cells were washed once and then incubated in filter-sterilized ABS (6.4 g/l NaCl; 0.3 g/l $CaCl_2$-$2H_2O$; 0.4 g/l KCl; 4 g/l $NaHCO_3$; 0.2 g/l $MgSO_4$-$7H_2O$; 1 g/l glucose; 0.1 g/l Na pyruvate; 2.13 g/l BES, pH 7.4 with NaOH) buffer for 60 minutes at 37°C, as originally described by Bretscher (1989). Unless otherwise noted, EGF (Collaborative Research Inc., Bedford MA, Culture Grade) was added to a final concentration of 100 ng/ml and cells were collected after 5 minutes of EGF treatment at 37°C. Cells were washed once with ice cold PBS and scraped into PBS containing 1 mM PMSF, 0.5 mM benzamidine, 0.4 mM Na_3VO_4 and 10 mM NaF. Cells were collected by centrifugation and extracted into 1 ml of extraction buffer as described previously (Balda et al., 1993).

Cytochalasin D treatment

A431 cells were maintained as described above, but after washing with ABS buffer, 1.2 μM cytochalasin D (Sigma Chemical Co., St Louis, MO) was added to some wells; cells were incubated for 60 minutes at 37°C before addition of EGF.

Immunoprecipitation and western blotting

ZO-1 was immunoprecipitated from cell extracts using a rabbit polyclonal anti-ZO-1 antibody (Zymed Laboratories, South San Francisco, CA); EGF receptor was immunoprecipitated with a sheep polyclonal antibody (UBI, Lake Placid, NY). Because A431 cells over-express the EGF receptor, only 1-5% of the cell extract was used for the EGF receptor immunoprecipitations; the remainder was used for ZO-1 immunoprecipitations. Cell extracts plus antibodies were incubated overnight on an end-over-end shaker at 4°C; Protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) was added for the final 1-3 hours. After washing 4 times with extraction buffers, immunoprecipitates were analyzed on an 8% polyacrylamide-SDS gel by the method of Laemmli (1970) and transferred to nitrocellulose (Towbin et al., 1979). Immunoblotting with ZO-1 antibodies, including those specifically recognizing two products of alternative RNA splicing, has been described (Balda and Anderson, 1993). Phosphotyrosine-containing proteins were detected using the mouse monoclonal anti-phosphotyrosine antibody 4G10 (UBI, Lake Placid, NY); rabbit anti-ZO-2 antibodies were generously provided by Lynne Jesaitis and Daniel Goodenough, Harvard Medical School, Boston, MA. In all cases horseradish peroxidase-coupled secondary antibodies were used with the ECL chemiluminescence kit (Amersham, Rockford, IL) and light emission detected by Kodak X-O-Mat AR X-ray film (Kodak, Rochester, NY). Where necessary, the primary and secondary antibodies were stripped from the filter per manufacturer's instructions and the filters reprobed with additional antibodies.

Immunofluorescence microscopy

A431 cells were plated on glass coverslips and treated with EGF as described above. In some cases, cells were pretreated with cytochalasin D as described above. Cells were washed quickly with PBS and fixed for 15 minutes on ice in 4% paraformaldehyde in PBS, rinsed with PBS and stored at 4°C in PBS. Methods for indirect immunolocalization of ZO-1 and visualization of filamentous actin by binding rhodamine-labeled phalloidin have been described (Balda et al., 1993).

RESULTS

Actin and ZO-1 are redistributed to sites of apical cell-cell contact after EGF treatment of A431 cells

Because EGF treatment of A431 cells induces a series of rapid morphologic changes accompanied by the rearrangement of actin and other cytoskeletal proteins, we chose to examine the subcellular distribution of actin and ZO-1 in these cells after the addition of EGF. In commonly studied epithelial models like the MDCK cell line or tight junctions in vivo, ZO-1 staining has a very discrete and continuous junctional location (for example see Stevenson et al., 1986). However, initial immunolocalization of ZO-1 in confluent cultures of A431 cells revealed multiple cellular localizations (Fig. 1). A431 cells form multiple layers during prolonged culture and we routinely observed that cells in monolayers growing on top of another layer of cells showed a strikingly continous apical ring of ZO-1 (Fig. 1). On the other hand, a significant fraction of cells in lower layers showed a diffuse and discontinuous localization of ZO-1 at regions of cell-cell contact. Subconfluent

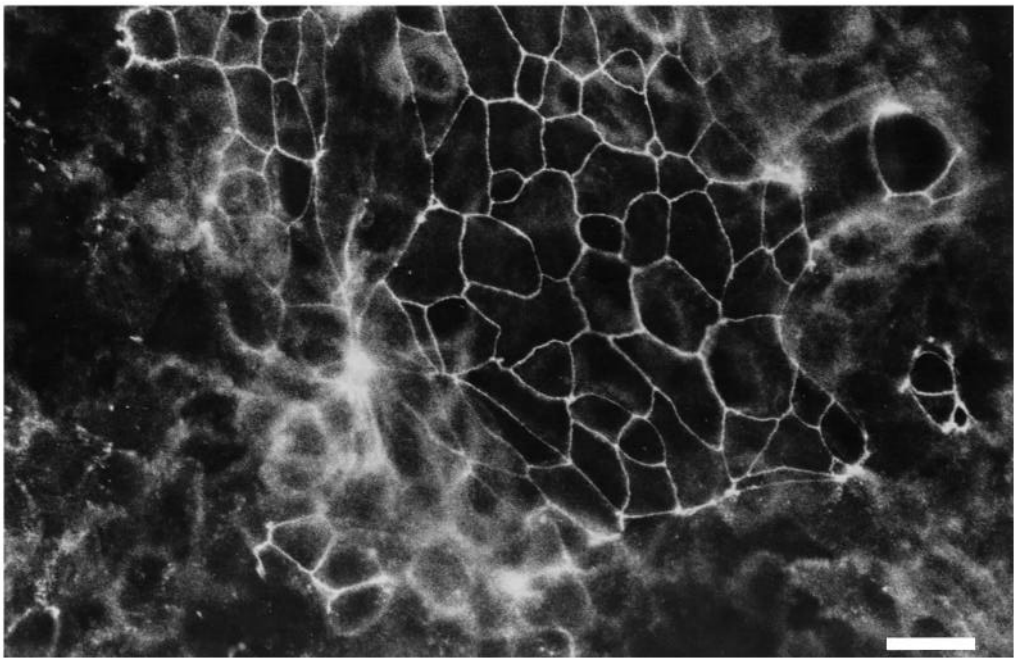

Fig. 1. Immunolocalization of ZO-1 in confluent cultures of A431 cells. Cells in central region of the field are growing as a small monolayer on top of a lower layer of cells. ZO-1 in these cells localizes to a continuous apical band. Cells around the edge of the field are a single cell thick and ZO-1 is diffusely and discontiuously localized to broad surfaces of cell-cell contact. Bar, 20 μm.

cultures of A431 cells showed this latter diffuse staining pattern in all cells. Because of this complex and variable localization of ZO-1 in confluent cells, we chose to study the effects of EGF on subconfluent cultures where ZO-1 distribution was more homogeneous and nonjunction-like.

In control A431 cells, when cells are in small islands, actin is primarily seen in stress fibers and diffusely at areas of cell-cell contact. ZO-1 is localized diffusely on the plasma membrane but is concentrated with actin in a broad and discontinuous fashion along surfaces of cell-cell contacts (Fig. 2a and b). Both microspikes and membrane ruffles are occasionally observed in unstimulated cells and ZO-1 colocalizes with actin in these non-junctional sites. In contrast, ZO-1 is never observed along actin stress fibers (data not shown). Within 5 minutes after the addition of EGF, the actin staining at the sites of cell-cell contact is stronger and most of the ZO-1 staining has moved into a continuous focused line positioned at the apical end of cell-cell contacts, as determined by focusing from the base to apex of cells. However, the response to EGF is not uniform and some cells still show considerable cytoplasmic staining after EGF treatment. After 20 minutes of EGF exposure, most of the cells become round and actin is diffusely distributed around the cortex of the cells and at cell-cell contacts. ZO-1 staining appears to remain predominantly in regions of cell-cell contact but is no longer focused in a discrete line.

ZO-1 becomes phosphorylated after EGF treatment of A431 cells

Activation of the EGF receptor is known to induce tyrosine phosphorylation of the cytoskeletal protein ezrin coinciding with enhanced colocalization of ezrin with actin in microspikes and ruffles (Bretscher, 1989). Thus, we investigated whether ZO-1 was also a tyrosine kinase substrate during EGF-induced redistribition. A431 cells were treated with EGF for varying periods of time, and the ZO-1 was immunoprecipitated, subjected to SDS-PAGE, transferred to nitrocellulose and protein phosphotyrosine detected using an antibody to phosphotyrosine. A protein doublet of approximately 220 kDa is phosphorylated in a time-dependent fashion (Fig. 3A), with phosphorylation obvious as soon as 1 minute after EGF exposure, maximal at 2-5 minutes and declining after 20 minutes of EGF exposure. Tyrosine phosphorylation of unstimulated A431 cells was never observed, either in sparse or dense cultures. Similar kinetics were observed in multiple experiments. Blots were stripped and reprobed with an antibody to ZO-1 to confirm the doublet as ZO-1 and that equal amounts of total ZO-1 were present in each gel lane (Fig. 3B). Identity of the doublet bands as two isoforms of ZO-1 which arise from alternative RNA splicing (Willott et al., 1992; Balda and Anderson, 1993) was confirmed by blotting with antibodies specific for each isoform (data not shown). In addition to the ZO-1 bands, a prominent tyrosine phosphorylated band at about 165 kDa is also co-immunoprecipitated with the anti-ZO-1 antibody. ZO-2, a 165 kDa protein, has been reported to coprecipitate with ZO-1 under the buffer conditions employed in our experiments (Gumbiner et al., 1991). ZO-2 has recently been cloned and antibodies are available (Jesaitis and Goodenough, 1994) which allowed us to confirm the identity of this band as ZO-2 (Fig. 3C). A fuzzy band intermediate in size between ZO-1 and ZO-2 is also visible in the blot probed with the antiphosphotyrosine antibody. This band is likely the EGF receptor, since immunoblotting for the EGF receptor shows that a small amount of the receptor co-immunoprecipitates with the anti-ZO-1 antibody, but not with Protein A beads alone. This band is co-immunoprecipitated with ZO-1 in a non-tyrosine phosphorylation-dependent fashion and is present to a variable extent in the immunoprecipitates.

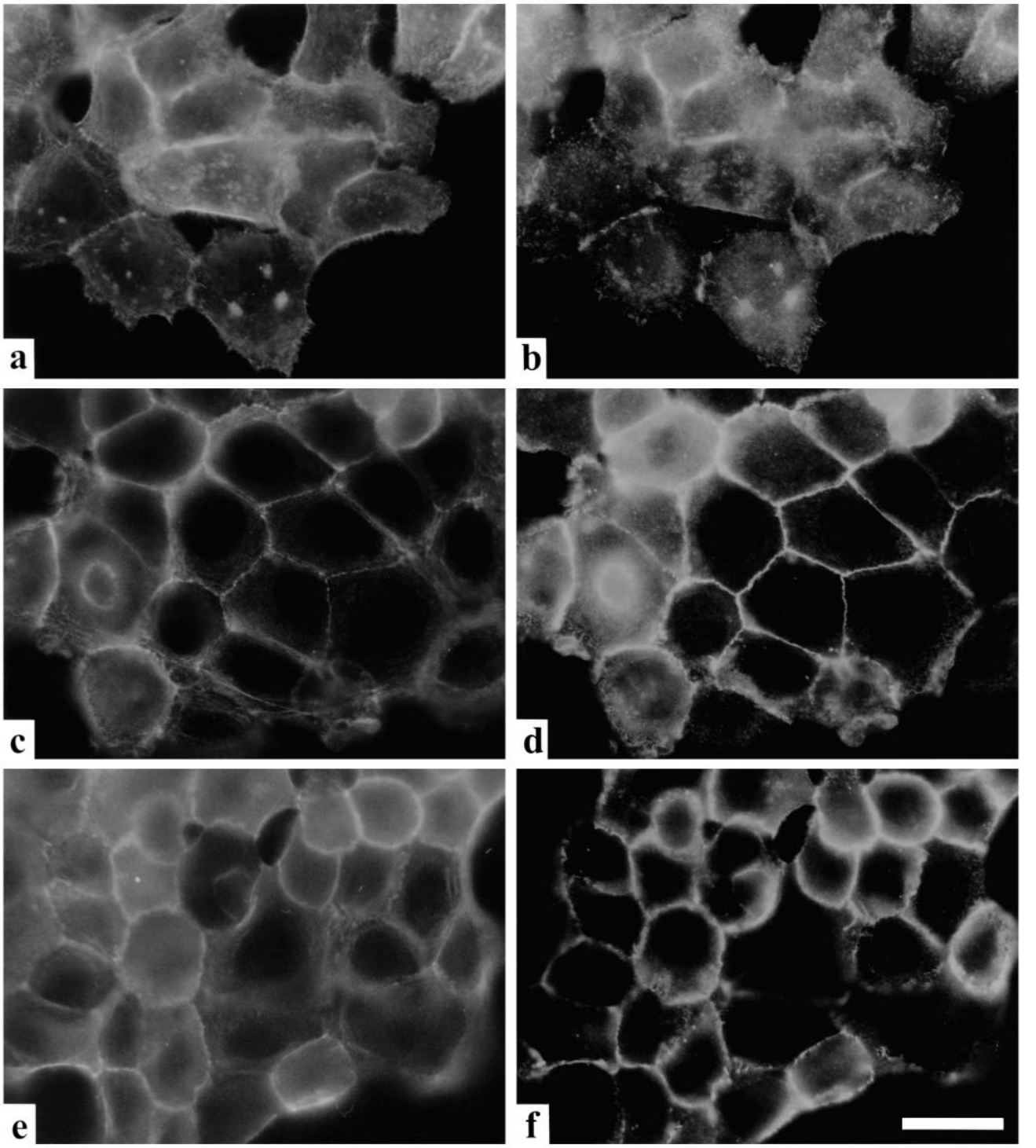

Fig. 2. EGF-induced redistribution of actin and ZO-1 in A431 cells. A431 cells at 20% confluence were treated with 100 ng/ml EGF for 0 (a,b), 5 (c,d) and 20 (e,f) minutes, fixed and stained for f-actin (a,c,e) using rhodamine-phalloidin, and immunostained for ZO-1 (b,d,f). Bar, 10 μm.

Aliquots of the same samples used for ZO-1 immunoprecipitation were immunoprecipitated with an anti-EGF-receptor antibody and the pelleted protein probed with antibodies to phosphotyrosine. The EGF receptor appears as a very prominent band at 180 kDa and shows a similar onset of tyrosine phosphorylation, however, unlike ZO-1, phosphorylation was maintained over the entire 20 minute period (Fig. 3D).

The EGF dose dependance for ZO-1 and ZO-2 tyrosine phosphorylation at 5 minutes was also determined. Maximal tyrosine phosphorylation is observed for ZO-1, ZO-2 (Fig. 4A) and the EGF receptor (Fig. 4C) at a dose between 10 and 50 ng/ml, in several experiments. In addition, scanning densitometry demonstrates no consistent differences between tyrosine phosphorylation of the two ZO-1 isoforms in either EGF dose- and time-dependance (Figs 3A and 4A).

Exposure of confluent cultures of A431 cells to EGF also

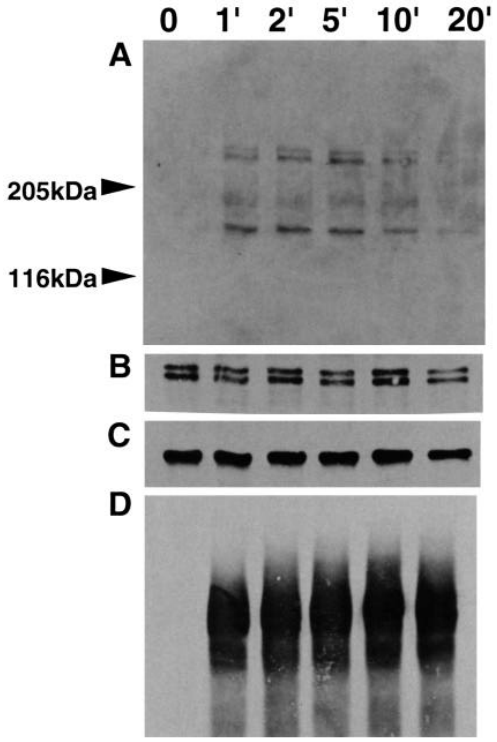

Fig. 3. Time-dependent EGF-induced tyrosine phosphorylation of ZO-1 and ZO-2 in A432 cells. Cells were exposed to EGF for 0, 1, 2, 5, 10 and 20 minutes. Equal amounts of cell lysates were immunoprecipitated with anti-ZO-1 (A-C) or anti-EGF-receptor antisera (D). The ZO-1 immunoprecipitates were resolved using SDS-PAGE, transferred to nitrocellulose and the blots stained with a monoclonal antibody to phosphotyrosine (A), stripped and reprobed with an anti-ZO-1 antiserum (B), stripped and reprobed with an anti-ZO-2 antiserum. The EGF-receptor immunoprecipitates were resolved and transferred as above and stained with the antiphosphotyrosine monoclonal antibody (D).

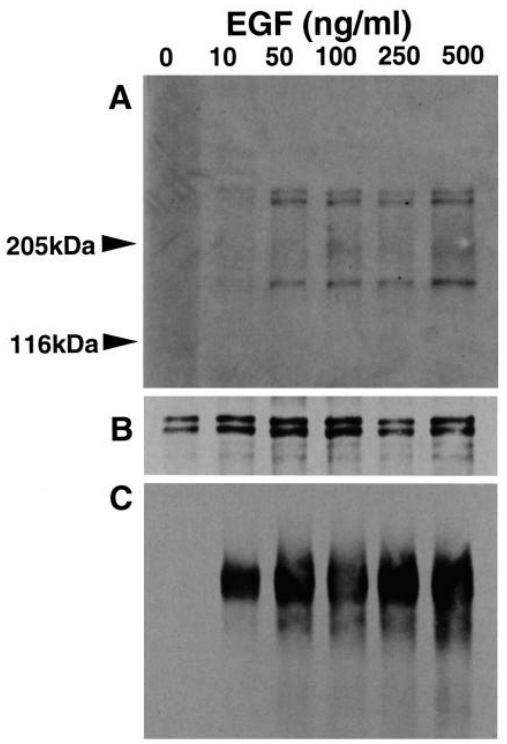

Fig. 4. Dose response of EGF-induced tyrosine phosphorylation of ZO-1 and ZO-2. A431 cells were treated with either 0, 10, 50, 100, 250 or 500 ng/ml EGF for 5 minutes. Cell lysates were immunoprecipitated with ZO-1 or EGF-receptor antibodies, subjected to SDS-PAGE and transferred to nitrocellulose. ZO-1 immunoprecipitates were probed with antiphosphotyrosine antibody (A) and stripped and reprobed with anti-ZO-1 antibody (B). EGF receptor immunoprecipitates were probed with antiphosphotyrosine antibody (C).

resulted in tyrosine phosphorylation of ZO-1 and ZO-2 with the same kinetics and dose dependance as subconfluent cells (data not shown).

Cytochalasin treatment disrupts actin organization and inhibits EGF-dependent ZO-1 tyrosine phosphorylation

Because the reorganization of ZO-1 following EGF treatment parallels actin reorganization, we next determined if disrupting actin with cytochalasin D would inhibit ZO-1 tyrosine phosphorylation. We used the D analog of cytochalasin whose cellular effects appear to be most specifically limited to disrupting actin filament formation (Cooper, 1987). A431 cells were pretreated for 60 minutes with 1.2 μM cytochalasin D and then treated with EGF for 5 minutes. At this dose and time, cytochalasin D induced aggregates of actin around the cell cortex; ZO-1 colocalized with actin in these aggregates (Fig. 5). Addition of EGF to these cells induced no obvious additional changes in the localization of actin or ZO-1. EGF-induced tyrosine phosphorylation of ZO-1 and ZO-2 was significantly decreased in cytochalasin D treated cells, while autophosphorylation of the EGF receptor was only slightly affected (Fig. 6). Cytochalasin D treatment, even without EGF, resulted in a small reproducible increase in tyrosine phosphorylation of the EGF receptor (Fig. 6C).

DISCUSSION

In this study, we describe the change in distribution and tyrosine phosphorylation of ZO-1 after EGF treatment of A431 cells. The major morphologic observation is that EGF induces ZO-1 to become transiently focused into a continuous apical position typical of the tight junction in normal polarized epidermal cells, and that actin simultaneously moves into a typical perijunctional location. At the same time that ZO-1 assumes a junctional location, both ZO-1 and ZO-2 become phosphorylated on tyrosine residues.

EGF effects on actin organization in A431 cells have been described in a number of studies (Schlessinger and Geiger, 1981; Bretscher, 1989; Rijken et al., 1991); these changes include concentration at the apical cell-cell borders (Rijken et al., 1991). In subconfluent control cells, ZO-1 is found along the lateral surfaces of cells and in microspikes and ruffles. After EGF treatment, ZO-1 staining is more obvious in microspikes, but most of the protein is now concentrated with the actin at the apical perijunctional area. ZO-1 in confluent cells showed variable ratios of junction-like and nonjunction-

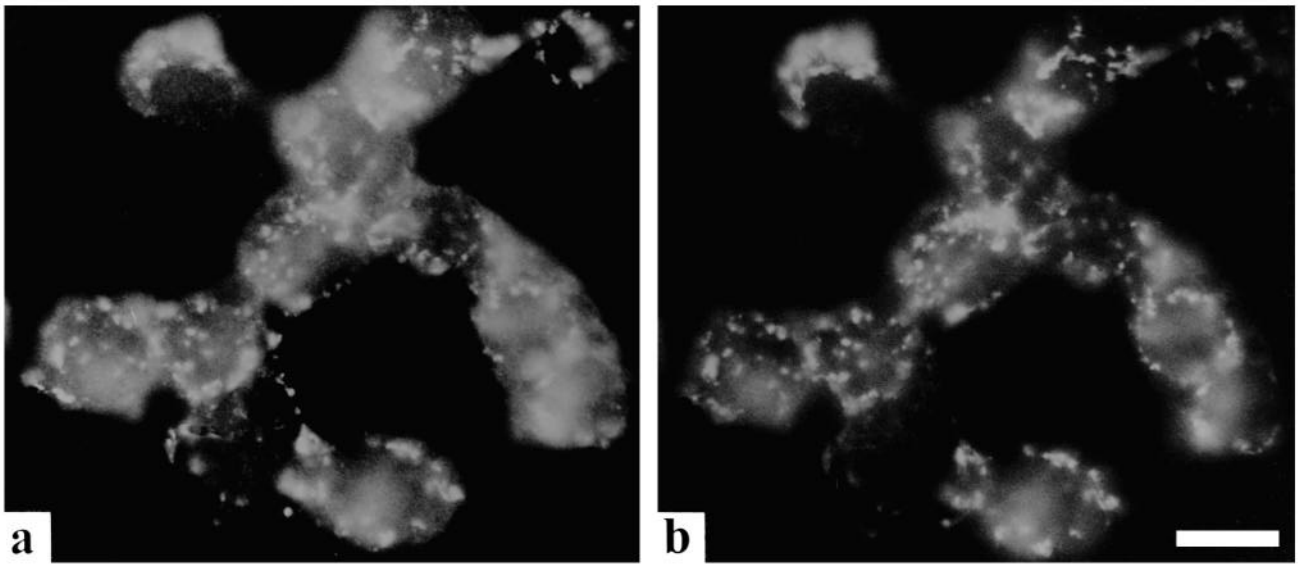

Fig. 5. Effect of cytochalasin D on actin and ZO-1 distribution in A431 cells. A431 cells were pretreated with 1.2 μM cytochalasin D for 60 minutes and then fixed and stained for f-actin (a) or ZO-1 (b). Bar, 20 μm.

like localization in the absence of EGF and we could not reliably document any change in its distribution in response to EGF. In contrast, ZO-1 in subconfluent cells was almost uniformly nonjunction-like in the absence of growth factor and EGF treatment transiently induced a junction-like organization.

The significance of actin remodeling for the activity of EGF is not well understood. The EGF receptor itself is an actin binding protein (den Hartigh et al., 1992). Cellular enzymes which are activated by EGF such as the phosphatidylinositol kinases, diacylglycerol kinase and phospholipase C are also associated with detergent insoluble actin filaments in A431 cells (Payrastre et al., 1991). In addition, EGF treatment of A431 cells results in the phosphorylation of a number of cytoskeletal components, including p35/annexin I, ezrin, spectrin and microtubule-associated protein 2 (Fava and Cohen, 1984; Akiyama et al., 1986; Bretscher, 1989). Both ezrin and spectrin are actin-binding proteins and both these proteins also change cellular distribution after EGF treatment. There is also recent data demonstrating that two members of the catenin family, proteins which mediate the linkage of cadherins with actin microfilaments, are also tyrosine phosphorylated in response to EGF in A431 cells (Shibamoto et al., 1994; Hoschuetzky et al., 1994). Synthesis of these observations has led to the suggestion (van Bergen en Hengouwen et al., 1992) that actin filaments may serve as a cortical membrane matrix serving to localize key proteins involved in the transduction of responses to EGF.

How ZO-1 is associated with actin is unclear, as is whether its redistribution in response to EGF is the cause or effect of the redistribution of actin. We have no evidence that ZO-1 is itself an actin-binding protein. Therefore, like p55, its homolog in the red cell cytoskeleton (Alloisio et al., 1993), it seems reasonable to suggest ZO-1 might bind an actin-binding protein which moves into a junctional location in response to EGF. Alternatively, ZO-1 could transduce a signal downstream of the EGF receptor, which induces perijunctional actin polymerization and accumulation.

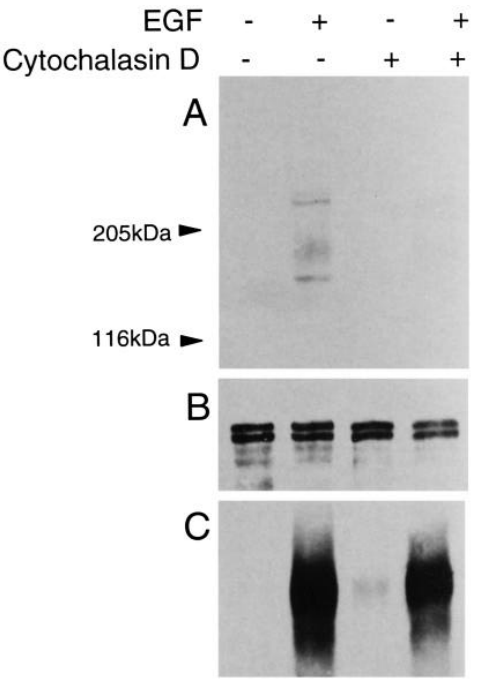

Fig. 6. Effect of cytochalasin D on ZO-1 and EGF receptor phosphorylation induced by EGF in A431 cells. A431 cells were pretreated with 1.2 μM cytochalasin D for 60 minutes and then treated with 0 or 100 ng/ml EGF for 5 minutes. Cell lysates were immunoprecipitated with ZO-1 or EGF-receptor antibodies, subjected to SDS-PAGE and transferred to nitrocellulose. ZO-1 immunoprecipitates were probed with antiphosphotyrosine antibody (A) and stripped and reprobed with anti-ZO-1 antibody (B). EGF receptor immunoprecipitates were probed with antiphosphotyrosine antibody (C).

The present studies do not allow temporal discrimination between tyrosine phosphorylation and the redistribution of ZO-1, but the fact that tyrosine phosphorylation occurs at the same time as accumulation of this protein in the perijunctional area is

consistent with a role for this modification during transient ZO-1 targeting or in junctional rearrangements. The time course of this response is similar to ezrin (Bretscher, 1989) but different from the EGF effect on the catenins, since phosphorylation of these proteins is maintained over at least the first 30 minutes of EGF exposure (Hoschuetzky et al., 1994). Since protein tyrosine phosphorylation creates a sequence specific binding site for the SH2 domain of proteins typically involved in signal transduction pathways (Songyang et al., 1993), we can hypothesize that ZO-1 associates transiently with an SH2-domain containing proteins during EGF-induced relocalization. It does not appear that phosphorylation is required to maintain a junctional localization, since at 20 minutes, ZO-1 tyrosine phosphorylation is reduced, while ZO-1 is still concentrated at cell borders. In spite of the temporally coincident redistribution of ZO-1 in subconfluent islands and its tyrosine phosphorylation, it is possible that phosphorylation is not required for relocation since an indistinquishable phosphorylation response was observed in confluent cells in which ZO-1 is already in a junction-like location in some cells prior to adding EGF. In addition, in MDCK cells where ZO-1 distribution is normally restricted to junctions, EGF treatment has no effect on tyrosine phosphorylation of this protein (data not shown).

Pretreatment of A431 cells with cytochalasin D, which disrupts the actin cytoskeleton, has only a minor effect on the EGF-induced phosphorylation of the EGF receptor. However, cytochalasin D prevents redistribution of ZO-1 and dramatically diminishes the EGF-induced tyrosine phosphorylation of ZO-1 and ZO-2. These data suggest that the integrity of the actin cytoskeleton is essential not only for ZO-1 rearrangement but also for ZO-1 to come into proximity with its tyrosine kinase.

We suggest that EGF-induced phosphorylation of ZO-1 may be related to EGF-induced ZO-1 redistribution to the perijunctional area of A431 cells, and that this signal and redistribution are dependent on an intact actin network. Understanding the nature of the causal relationship between tyrosine phosphorylation and ZO-1 rearrangements is presently under investigation.

This study was supported in part by grants from National Institutes of Health (PO1-DK38979, DK45134) and the Yale Liver Center (DK34989). JMA is a Lucille P. Markey Scholar and this work was supported in part by a grant from the Lucille P. Markey Chartible Trust.

REFERENCES

Akiyama, T., Kadowaki, T., Nishida, E., Kadooka, T., Ogawara, H., Fukami, Y., Sakai, H., Takaku, F. and Kasuga, M. (1986). Substrate specificities of tyrosine-specific protein kinases toward cytoskeletal proteins in vitro. *J. Biol. Chem.* **261**, 14797-14803.

Alloisio, N. Dalla Venezia, N., Rana, A., Andrabi, K., Texier, P., Gilsanz, F., Carton, J. P., Delawney, J. and Chishti, A. H. (1993). Evidence that red blood cell protein p55 may participate in the skeleton-membrane linkage that involves protein 4. 1 and glycophorin c. *Blood* **82**, 1323-1327.

Anderson, J. M., Stevenson, B., Jesaitis, L. A., Goodenough, D. and Mooseker, M. (1988). Characterization of ZO-1, a protein component of the tight junction from mouse liver and Madin-Darby canine kidney cells. *J. Cell Biol.* **106**, 1141-1149.

Anderson, J. M., Balda, M. S. and Fanning, A. S. (1993). The structure and regulation of tight junctions. *Curr. Opin. Cell Biol.* **5,** 772-776.

Balda, M. S. and Anderson, J. M. (1993). Two classes of tight junctions are revealed by ZO-1 isoforms. *Am. J. Physiol.* **264**, C918-924.

Balda, M. S., Gonalez-Mariscal, L., Matter, K., Cereijido, M. and Anderson, J. M. (1993). Assembly of the tight junction: the role of diacylglycerol. *J. Cell Biol.* **123**, 293-302.

Bretscher, A. (1989). Rapid phosphorylation and reorganization of ezrin and spectrin accompany morphological changes induced in A-431 cells by epidermal growth factor. *J. Cell Biol.* **108**, 921-930.

Chinkers, M. McKanna, J. and Cohen, S. (1979). Rapid induction of morphological changes in human carcinoma A431 cells by epidermal growth factor. *J. Cell Biol.* **83**, 260-265.

Cooper, J. A. (1987). Effects of cytochalasin and phalloidin on actin. *J. Cell Biol.* **105,** 1473-1478.

den Hartigh, J. C., van Bergen en Henegouwen, P. M., Verkleij, A. J. and Boonstra, J. (1992). The EGF receptor is an actin-binding protein. *J. Cell Biol.* **119,** 349-355.

Farquhar, M. G. and Palade, G. E. (1963). Junctional complexes in various epithelia. *J. Cell Biol.* **17,** 375-412.

Fasano, A., Baudry, B., Pumplin, D. W., Wasserman, S. S., Tall, B. D., Ketley, J. M. and Kaper, J. B. (1991). Vibrio cholerae produces a second enterotoxin, which affects intestinal tight junctions. *Proc. Nat. Acad. Sci. USA* **88,** 5242-5246.

Fava, R. A. and Cohen, S. (1984). Isolation of a calcium-dependent 35-kilodalton substrate for the epidermal growth factor receptor kinase from A431 cells. *J. Biol. Chem.* **259**, 2636-2645.

Gumbiner, B., Lowenkopf, T. and Apatira, D. (1991). Identification of 160-kDa polypeptide that binds to the tight junction protein ZO-1. *Proc. Nat. Acad. Sci. USA* **88,** 3460-3464.

Hernandez-Sotomayor, S. M. T. and Carpenter, G. (1992). Epidermal growth factor receptor: elements of intracellular communication. *J. Membr. Biol.* **128**, 81-89.

Hirokawa, N. and Tilney, L. G. (1986). Interactions between actin filaments and between actin filaments and membranes in quick-frozen and deeply etched hair cells of the chick ear. *J. Cell Biol.* **95,** 249-261.

Hoschuetzky, H., Aberle, H. and Kemler, R. (1994). β-Catenin mediates the interaction of the cadherin-catenin complex with epidermal growth factor receptor. *J. Cell Biol.* **127**, 1375-1380.

Jesaitis, L. A. and Goodenough, D. A. (1994). Molecular characterization and tissue distribution of ZO-2, a tight junction protein homologous to ZO-1 and the Drosophila discs-large tumor suppressor protein. *J. Cell Biol.* **124,** 949-962.

Kurihara, H., Anderson, J. M. and Farquhar, M. G. (1995). Tyrosine phosphorylation of ZO-1 and other proteins occurs during modification of tight junctions between foot processes of glomerular epithelial cells. *Am. J. Physiol. (Renal)* (in press).

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Madara, J. L., Barenberg, D. and Carlson, S. (1986). Effects of cytochalasin D on occluding junctions of intestinal absorptive cells: further evidence that the cytoskeleton may influence paracellular permeability and junctional charge selectivity. *J. Cell Biol.* **102**, 2125-2136.

Payrastre, B., Van Bergen en Henegouwen, P. M. P., Breton, M., Den Hartigh, J. C., Plantavid, M., Verkleij, A. J. and Boostra, J. (1991). Phosphoinositide kinase, diacylglycerol kinase, and phospholipase C activities associated to the cytoskeleton: effect of epidermal growth factor. *J. Cell Biol.* **115**, 121-128.

Rijken, P. J., Hage, W. H., Van Bergen en Henegouwen, P. M. P, Verkleij, A. J. and Boonstra, J. (1991). Epidermal growth factor induces rapid reorganization of the actin microfilament system in human A431 cells. *J. Cell Sci.* **100**, 491-499.

Schlessinger, J. and Geiger, B. (1981). Epidermal growth factor induces redistribution of actin and α-actinin in human epidermal carcinoma cells. *Exp. Cell Res.* **134**, 273-279.

Shibamoto, S., Hayakawa, M., Takeeuchi, K., Hori, T., Oku, N., Miyazawa, K., Kitamura, N., Takeichi, M. and Ito, F. (1994). Tyrosine phosphorylation of β-catenin and plakoglobin enhanced by hepatocyte growth factor and epidermal growth factor in human carcinoma cells. *Cell Adhesion Commun.* **1**, 295-305.

Small, J. V. (1988). The actin cytoskeleton. *Electron Microsc. Rev.* **1**, 155-174.

Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., King, F., Roberts, T., Ratnofsky, S., Birge, R. B., Fajardo, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B. and Cantley, L. C. (1993). SH2 domains recognize specific phosphopeptide sequences. *Cell* **72,** 767-778.

Stevenson, B. R., Siliciano, J. D., Mooseker, M. and Goodenough, D. A.

(1986). Identification of ZO-1: A high molecular weight polypeptide associated with the tight juction (zonula occludens) in a variety of epithelia. *J. Cell Biol.* **103**, 755-766.

Towbin, H., Staelin, T. and Gordon, J. (1979). Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Nat. Acad. Sci. USA* **76**, 4350-4354.

Tsukita, S., Itoh, M., Nagafuchi, A., Yonemura, S. and Tsukita, S (1993). Submembranous junctional plaque proteins include potential tumor suppressor molecules. *J. Cell Biol.* **123,** 1049-1053.

van Bergen en Henegouwen, P. M. P., den Hartigh, J. C., Romeyn, P., Verkleij, A. J. and Boonstra, J. (1992). The epidermal growth factor receptor is associated with actin filaments. *Exp. Cell Res.* **199**, 90-97.

Willott, E., Balda, M. S., Heintzelman, M., Jameson, B. and Anderson, J. M. (1992). Localization and differential expression of two isoforms of the tight junction protein ZO-1. *Am. J. Physiol.* **262:C,** 1119-1124.

Willott, E., Balda, M. S., Fanning, A. S, Jameson, B., Van Itallie, C. and Anderson, **J. M.** (1993). The tight junction protein ZO-1 is homologous to the *Drosophila discs-large* tumor suppressor protein of septate junctions. *Proc. Nat. Acad. Sci. USA* **90**, 7834-7838.

Woods, D. F. and Bryant, P. J. (1991). The discs-large tumor suppressor gene of Drosophila encodes a guanylate kinase homolog localized at septate junctions. *Cell* **66,** 451-464.

(Received 23 November 1994 - Accepted 20 January 1995)