Glucocorticoid-induced Functional Polarity of Growth Factor Responsiveness Regulates Tight Junction Dynamics in Transformed Mammary Epithelial Tumor Cells*

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The synthetic glucocorticoid, dexamethasone, induces the "normal-like" differentiated property of tight junction formation and suppresses growth of the Con8 mammary epithelial tumor cell line, derived from a 7,12-dimethylbenz(a)anthracene-induced rat mammary adenocarcinoma. Characterization of the transepithelial electrical resistance of Con8 mammary tumor cells cultured on permeable supports revealed that a novel response to dexamethasone is the generation of a polarized cell monolayer with respect to epithelial growth factor receptor responsiveness. Administration of transforming growth factor-α (TGF-α) to the basolateral, but not the apical, plasma membrane compartment disrupted the glucocorticoid-stimulated tight junction barrier. Confocal immunofluorescence microscopy revealed that dexamethasone caused the ZO-1 tight junction-associated protein to localize exclusively to the apical border of laterally adjacent membranes of the cell periphery, whereas basolateral administration of TGF-α caused the redistribution of ZO-1 back to disorganized aggregates along the cell periphery. In contrast, TGF-α was able to exert its mitogenic effects equally on both sides of the cell monolayer independent of its polarized disruption of tight junction formation. Our results represent the first evidence for a functional polarization of the epidermal growth factor receptor and strongly implicated the glucocorticoid-regulated formation of tight junctions in policing the polarized responsiveness of mammary cells to growth factors.

Differentiation and proliferation of mammary gland tissue is stringently regulated by a dynamic balance of environmental cues that include systemic steroid and protein hormones, such as the lactogenic factors prolactin and glucocorticoids as well as estrogen and progestin ovarian steroids, locally acting growth factors, and extracellular matrix components (1–5). A distinguishing feature of transformed mammary epithelial cells is the dysregulation of cellular responsiveness to particular sets of extracellular signals and loss of normal interdependence between these signals, which can result in uncontrolled proliferation and loss of differentiated properties (6, 7). One mechanism by which transformed mammary epithelial cells lose their ability to be controlled by the microenvironment is by the inappropriate expression of growth factors and/or function of their cognate receptors (1, 2, 5, 6). For example, two particularly important mammary-derived growth factors regulated at different stages of normal mammary gland growth and development are transforming growth factor-α (TGF-α) and epidermal growth factor (EGF), which both act through the EGF receptor and have been directly implicated in the pathogenesis and growth of mammary tumors in rodents and humans (1, 2, 4, 5). However, virtually nothing is known about the role of cell-cell interactions in controlling access to growth factors and other signals from the microenvironment.

The regulation of tight junction permeability in normal mammary epithelia prevents the paracellular leakage of macromolecules, such as growth factors, from the apical and basolateral membranes during the onset of lactation (8). In addition to this "gate" function, tight junctions maintain cellular polarity by physically defining the border between the apical and basolateral plasma membrane compartments (9–11). Tight junction structure is highly dynamic in that permeability, assembly and/or disassembly can be controlled by a variety of cellular and metabolic regulators (9, 10, 12), which include calcium (13), phorbol esters (14), and members of the insulin-like growth factor-I family (15). Our recent studies have documented that glucocorticoids stimulate the in vitro formation of tight junctions in nontransformed mouse mammary epithelial cell line of ductal origin (16–18), which directly implicates glucocorticoids as one of the in vivo physiological modulators of tight junctions in the mammary gland. Given the biological importance of structurally distinguishing the luminal side from the blood or basolateral side of normal mammary epithelia, it is tempting to consider that tight junctions mediate the polarized availability of growth factor receptors and/or their signaling pathways in mammary cells.

To address whether EGF receptor signaling can be regulated by tight junction formation in mammary epithelial tumor cells, we have been utilizing the Con8 rat mammary tumor cell line which is derived from a 7,12-dimethylbenz(a)anthracene-induced rat mammary adenocarcinoma (19). The synthetic glucocorticoid, dexamethasone, strongly suppresses the growth of Con8 mammary tumor cells (20, 21), by inducing a G₀ block in cell cycle progression (22) and inhibiting the production of autocrine-acting TGF-α (23, 24). We have recently shown that glucocorticoids stimulate tight junction formation in confluent monolayers of Con8 cells and that constitutive expression and secretion of TGF-α reversed this process under conditions in

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1 The abbreviations used are: TGF-α, transforming growth factor-α; EGF, epidermal growth factor; TER, transepithelial electrical resistance; PBS, phosphate-buffered saline; araC, cytosine β-D-arabinofuranoside.
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which cellular proliferation is stimulated (25). This result suggests that exogenously added TGF-α may be capable of disrupting fully functioning tight junctions in glucocorticoid treated Con8 cell monolayers, depending on the cell surface availability of EGF receptors. By examining the responsiveness of dexamethasone-treated monolayers of mammary tumor cells to TGF-α, we uncovered the first evidence for a polarized responsivity of EGF receptors and provide direct support for steroid-stimulated intercellular junctions as crucial structures that police the ability of mammary tumor cells to respond to locally acting growth factors.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium (Ham’s F-12 medium (50/50) and calf serum were supplied by Whittaker (Walkersville, MD) and phosphate-buffered saline (PBS), trypsin-EDTA, araC, and dexamethasone were obtained from Sigma. [3H]Thymidine (5 Ci/mmol) was obtained from Amersham Corp. Permeable supports manufactured by Nunc were distributed by Applied Scientific (San Francisco, CA). Human recombinant TGF-α was purchased from Becton Dickinson (San Jose, CA). The ZO-1 monoclonal antibodies (R40.76) were a generous gift of Bruce R. Stevenson (Department of Anatomy and Cell Biology, University of Alberta, Edmonton) and fluorescein 5-isothiocyanate-conjugated goat anti-rat IgG antibodies were supplied by Cappel (Malvern, PA). All other reagents were of highest available purity.

Cells, Method of Culture on Permeable Supports, and Measurement of Transepithelial Electrical Resistance—Con8 is a single cell-derived epithelial subclone obtained after collagenase digestion of the 13762NF transplantable rat mammary adenocarcinoma (20, 21). This cell line was routinely grown to 100% confluency on Nunc permeable tissue culture supports in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium supplemented with 10% calf serum, at 37°C in a humid atmosphere of air/CO2 (95:5). Cell culture medium was routinely changed every 24 h. In appropriate experiments, dexamethasone was added to a final concentration of 1 μM, and human recombinant TGF-α was added to a final concentration of 10 ng/ml. In order to inhibit DNA synthesis, cells’ cultures were exposed to 10 μM araC. The transepithelial electrical resistance was measured on support-grown cells at room temperature, using the EVOM Epithelial Voltohmmeter (World Precision Instruments, Sarasota, FL) as described previously (16, 17, 25). Resistance measurements were taken aspirationally every 8–24 h. Calculations for ohm/cm² were determined by subtracting the resistance measurement of a blank filter and multiplying by the area of the monolayer (0.49 cm² for the 10-mm filters).

Assay of DNA Synthesis by [3H]Thymidine Incorporation—To quan-titate relative rates of DNA synthesis, triplicate samples of Con8 cells were grown to confluency on permeable supports and treated with indicated combinations of dexamethasone, TGF-α, and/or araC. The media were replaced with fresh media containing 1–4 μCi/ml of [3H]thymidine (5 Ci/mmol), and the cells were incubated for 1 h. The cells were washed four times with PBS and twice with 100% methanol and air-dried for 12 h. The filter inserts were placed directly into scintillation vials, and radioactivity was quantitated by liquid scintillation counting.

Confocal Immunofluorescence Staining for ZO-1—Con8 cells were grown to 100% confluency on permeable 24-well filters and incubated with the indicated combinations of dexamethasone and TGF-α. TGF-α was added to either the apical or basolateral surface of the monolayer. The monolayers were washed three times with Dulbecco’s PBS (BioWhit-taker, Walkersville, MD), and the cells were fixed with 1.75% formaldehyde in PBS at room temperature. After three washes with PBS, the monolayers were permeabilized with TX-100 extraction buffer (0.5% Triton X-100, 10 mM Tris-HCl, pH 7.5, 120 mM NaCl, 25 mM KCl, 2 mM EDTA, 2 mM EGTA, 0.1% dithiothreitol, 0.5 mM phenyl-methylsulfonyl fluoride). The primary and secondary antibodies were diluted in TBST (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20) with 3% BSA. The cells were washed three more times with TBST and then blocked with 3% nonfat dry milk in PBS. ZO-1-specific antibody R40.76 was used at a dilution of 1:400. Cells were incubated at room temperature for 1 h and then washed three times with TBST with 3% nonfat dry milk. The secondary antibody was a fluorescein 5-isothiocyanate-conjugated goat IgG fraction to rat IgG and was used at a dilution of 1:100. Cells were incubated with the antibody for 1 h in the dark at room temperature. The cell monolayers were then washed three times with TBST containing 3% nonfat dry milk. The supports were punched out and mounted on glass slides in 50% glycerol, 50 mM Tris, pH 8–9, and 0.4% n-propyl gallate. Confocal images were obtained from a Zeiss Axiosplan epifluorescence microscope using a Zeiss 40X Plan-Neofluar multi-immersion objective (0.9 numerical aperture) and processed with a MRC 600 system. A series of digitized optical sections were collected starting from the basal side of the monolayer and stepping toward the apical membrane in 1 μm steps. The sections representing the plane of ZO-1 were printed on a Kodak Dye Transfer printer.

Western Blot Analysis for ZO-1 Protein Production—For Western blot analysis, whole cell samples in lysis buffer were normalized for protein content with the Bio-Rad Bradford protein assay. Cell lysates were subjected to 6% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose (nitro ME, Milan Separations Inc, Westboro, MA). Western blot signals were incubated with rat anti-ZO-1 monoclonal antibodies (R40.76), and horseradish peroxidase-conjugated goat anti-rat IgG secondary antibody, and the signal was detected by enhanced chemiluminescence as described previously (16). Parallel cell samples were electrophoretically fractionated, and equivalent protein loading was demonstrated by Coomassie Blue staining of the protein gels.

RESULTS

Tight junctions define the distinct boundary between the apical and basolateral plasma membrane surfaces (9, 10), and the in vitro formation of tight junctions in glucocorticoid-treated Con8 mammary tumor cells (25) provides the opportunity to examine the polarized responsiveness of mammary tumor cells to environmental signals. A key issue is to determine whether mamogenic growth factor receptor responsiveness is polarized, since polarity of the normal epithelia is critical to the function of the mammary gland (2, 8, 26). To test this notion, confluent monolayers of Con8 mammary tumor cells were cultured on permeable supports and tight junctions formed by treatment with 1 μM dexamethasone. The trans-well permeable support system allows the apical or basolateral surfaces of the cells to be selectively manipulated once tight junctions are formed since cell surface acting agents, such as TGF-α, can be added to either the basolateral (via the “outer compartment”) or apical (via the “inner compartment”) plasma membrane of the cell monolayer. Consistent with our previous results (25), dexamethasone induced a significant increase in transepithelial electrical resistance (TER) of the cell monolayer over 42 h time period, which remained essentially unchanged in the absence of steroid (Fig. 1, upper panel; +DEX versus −DEX). To test for the polarized distribution and/or function of EGF receptors, TGF-α was added to either the apical or basolateral surfaces of dexamethasone-treated mammary tumor cells, and the effects on monolayer TER were monitored over 42 h. When TGF-α was added to the apical side of a Con8 mammary tumor cell monolayer treated with dexamethasone for 24 h, TER continued to increase at a rate slightly greater than that of cells treated only with dexamethasone (Fig. 1, upper panel; +DEX/ TGF-α Ap versus +DEX) which indicates that the tight junctional machinery was intact. In striking contrast, when TGF-α was added to the basolateral surface of the cells, TER was reduced to basal levels within 18 h of growth factor treatment (Fig. 1, upper panel; +DEX/TGF-α BI versus −DEX). Similarly, when both the apical and basolateral surfaces of the monolayer were exposed to TGF-α (Fig. 1, upper panel; +DEX/TGF-α Ap/B1), the monolayer electrical resistance was quickly restored to basal levels. Basolateral, but not apical, addition of TGF-α reversed the dexamethasone-inhibited paracellular leakage of small radiolabeled tracers, such as [3H]linulin (data not shown), further confirming that TGF-α disrupts the “gate” function and increases monolayer permeability in a polarized manner.

To determine whether the observed polarized disruption of tight junction integrity may simply be due to differences in EGF receptor ligand responsiveness, 42-h dexamethasone-stimulated cells were incubated for an additional 72 h with various concentrations of TGF-α (0–20 ng/ml) added to either
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The apical or basolateral sides of the monolayer. Cells treated with dexamethasone without TGF-α displayed a 6-fold stimulation in TER (defined as 0 point in lower panel in Fig. 1). Basolateral addition of TGF-α dose-dependently reduced the TER to basal levels, whereas apical addition of TGF-α further increased the TER (Fig. 1, lower panel). Although most dramatic at the higher doses of TGF-α, the polarized effects are observed at the lower concentrations of TGF-α corresponding to the physiological levels of growth factor. The similarities in each dose response suggest that the EGF receptor affinities are similar on both sides of the monolayer and supports the hypothesis that TGF-α activates different components of the EGF-receptor signal transduction pathways linked to the apical versus the basolateral compartments.

To further investigate the dynamic steroid and growth factor control of tight junction structure, we tested whether the localization or expression of ZO-1, an intracellular peripheral membrane protein highly associated with tight junctions in epithelial cells (10, 27) may be a target of glucocorticoid or TGF-α signaling in Con8 mammary tumor cells. The images shown in Fig. 2 illustrate sections taken in the apical plane of ZO-1 at the top through the basolateral plane at the bottom. The distance between each optical section is 2 μm. In the absence of steroid (Fig. 2, Panels a–c), ZO-1 staining displayed a spotty, discontinuous pattern, which appeared as apparent aggregates along the cell periphery, especially at the junctions between several cells. In contrast, ZO-1 at the middle and basolateral plane (Fig. 2, Panels b and c) in certain cells of the untreated monolayer were visualized as intense, linear series of accumulations at the cell border. When cells were treated with dexamethasone (Fig. 2, Panels d–f), ZO-1 localization exhibited a very discrete and continuous band at the lateral membrane of adjacent cells in all three planes. Consistent with the effects on TER, addition of TGF-α to the apical side of a glucocorticoid-induced polarized monolayer (Fig. 2, Panels g–i) had no effect on ZO-1 localization which was maintained as a distinct and continuous band surrounding the peripheries of the cells. In contrast, when cells were treated with TGF-α at the basolateral surface of the monolayer (Fig. 2, Panels j–l), ZO-1 distribution reverted back to a disorganized and discontinuous pattern of staining, analogous to that seen in control cells without drug treatment. Although these cells retained the peripheral ZO-1 localization, the more intense staining was at the junctions shared by numerous cells. A similar disorganized ZO-1 immunostaining was also observed after the addition of TGF-α to both the apical and basolateral sides of the monolayer (data not shown). This growth factor alteration of ZO-1 localization is indicative of the disruption in the apical/basolateral boundary mediated by the loss of tight junctions. Under conditions in which glucocorticoids or TGF-α modulates the pattern of ZO-1 immunostaining, Western blot analysis revealed that there were no detectable differences in ZO-1 protein levels (Fig. 3). Thus, the coordinate and opposing regulation of the cellular distribution of ZO-1 correlates with the regulation of monolayer transepithelial electrical resistance of mammary tumor cells by glucocorticoids and basolateral addition of TGF-α. These results demonstrate for the first time a polarity of EGF receptor responsiveness in epithelial cells and strongly implicates the glucocorticoid induction of tight junction formation in mammary tumor cells as playing a role in governing this process.

Conceivably, the polarized manner in which TGF-α disrupts the dexamethasone-stimulated formation of tight junctions may be due to the selective localization of the EGF receptor protein or due to the polarized function of the receptor-signal transduction pathway either at the apical or basolateral side of the cell. To test this possibility, TGF-α’s mitogenic activity on Con8 cells was examined in monolayers exposed at the apical and/or basolateral surfaces to this growth factor. Dexamethasone strongly inhibited the DNA synthesis of Con8 cell monolayers grown on permeable supports (Fig. 4). In contrast to the polarized effects on monolayer TER, the addition of TGF-α to either the apical, basolateral, or both sides of the monolayer strongly restimulated [3H]thymidine incorporation of dexamethasone treated mammary tumor cells to approximately the same extent (Fig. 4). This result demonstrates that EGF receptors are distributed to and are functional at both the apical or basolateral cell surfaces in glucocorticoid-treated cells; however, the signal transduction events triggering the disruption of tight junctions appear to be distinct for the apical versus the basolateral compartments of the mammary tumor cells.

To examine whether the basolateral-mediated disruption of tight junction formation by TGF-α is independent of the proliferative response, the glucocorticoid and growth factor regulation of [3H]thymidine incorporation and TER of Con8 cell monolayers were analyzed after treatment with araC, an inhibitor of DNA synthesis. As shown in Table I, dexamethasone stimulated the monolayer TER to the same extent in the presence or absence of 10 μM araC. Under conditions in which
of tight junctions in Con8 mammary tumor cells is the forma-
tion of a polarized monolayer. This process involves activation of the mitogen-activated protein kinase (MEK) signal transduction pathways via the EGF receptor tyrosine kinase (29), and we are currently attempting to uncover the basolateral specific components which selectively transduce the TGF-α disruption of cell-cell interactions.

A key consequence of the glucocorticoid stimulated formation of tight junctions in Con8 mammary tumor cells is the formation of a polarized cell monolayer, since TGF-α reduces monolayer electrical resistance when added to the basolateral membrane compartment but not when exposed to the apical or luminal compartment. Growth factor addition to either compartment of a dexamethasone-treated monolayerrestimulated DNA synthesis, demonstrating for the first time that TGF-α, which binds to the EGF receptor (28), appears to trigger differential signal transduction cascades or activate polarized intermediates depending on the compartmental localization of its cognate receptor (Fig. 4). The proliferative pathway of TGF-α involves activation of the mitogen-activated protein kinase and protein kinase C signal transduction pathways via the EGF receptor tyrosine kinase (29), and we are currently attempting to uncover the basolateral specific components which selectively transduce the TGF-α disruption of cell-cell interactions.

**FIG. 2.** Glucocorticoid induced localization of ZO-1 and the polarized disruption of ZO-1 distribution by transforming growth factor-α. Con8 mammary tumor cells were treated with no hormones (−Dex, Panels a–c), 1 μM dexamethasone (−Dex, Panels d–f), dexamethasone for 24 h followed by 10 ng/ml human recombinant TGF-α applied on the apical surface of the monolayer for 18 h (+Dex+/TGF-α Ap, Panels g–i) or dexamethasone for 24 h follow by 10 ng/ml TGF-α added on the basolateral surface of the monolayer for 18 h (+Dex+/TGF-α Bl, Panels j–l). The cells were fixed and analyzed for ZO-1 localization by confocal microscopy. The upper panels display the apical sections, and the bottom panels display the basolateral sections. The bar represents 25 μm.

**FIG. 3.** Western blot analysis of ZO-1 expression levels. Con8 mammary tumor cells were treated with no hormones (−Dex) or 1 μM dexamethasone (+Dex) for 72 h. 10 ng/ml human recombinant TGF-α was applied to 24-h dexamethasone-treated cells for an additional 48 h on the apical surface of the monolayer, +Dex+/TGF-α (Ap), basolateral surface, +Dex+TGF-α (Bl), or simultaneously to the apical and basolateral surface, +Dex+TGF-α (Ap+Bl). Cell lysates were normalized for total protein (confirmed by Coomassie Blue staining of a parallel polyacrylamide gel), electrophoretically fractionated, blotted onto nitrocellulose filters, and analyzed for ZO-1 protein as described under “Experimental Procedures.” The protein molecular mass standards are myosin (200 kDa) and phosphorylase b (97.4 kDa).

[^3H]thymidine incorporation remains strongly inhibited by araC, basolateral exposure to TGF-α rapidly reduced the monolayer TER back to basal levels (Table I). The monolayer TER remained at approximately the steroid-induced level after apical addition of TGF-α in the presence or absence of araC. This result suggests that the basolateral specific signaling pathway leading to the disruption of cell-cell interactions by TGF-α is distinct from and independent of the proliferative pathway mediated by this growth factor.

**FIG. 4.** Nonpolarized stimulation of Con8 mammary tumor cell DNA synthesis by transforming growth factor-α. Con8 mammary tumor cells were cultured on permeable supports in the presence of 1 μM dexamethasone (DEX), and one control culture was incubated in the absence of steroid. After 24 h in dexamethasone, 10 ng/ml human recombinant TGF-α was added to either the apical side of the monolayer (Ap), the basolateral side of the monolayer (Bl), or to both sides of the monolayer (Ap/Bl). After an additional 18-h incubation, DNA synthesis was determined by the incorporation of[^3H]thymidine. The results are an average of quadruplicate samples.
Polarized disruption of tight junction formation by TGF-α is independent of growth factor-stimulated DNA synthesis

Conα mammary tumor cells were cultured on permeable supports in the presence of 1 μM dexamethasone (DEX) for 48 h, while one set of control cultures were not treated with steroid. Dexamethasone-treated cells were incubated with the indicated combinations of 10 ng/ml TGF-α and 10 μM araC for an additional 48 h. The final monolayer TER was determined, and DNA synthesis was monitored by the incorporation of [3H]thymidine as described in the text. The results are averages from triplicate samples.

| Cell culture conditions | TER [3H]Thymidine incorporation |
|------------------------|---------------------------------|
|                        | DEX-TGF-α-araC                  |
|                        | ohms cm² | qpm                   |
| − − −                  | 213 ± 38 | 8,823 ± 1,412         |
| + − −                  | 634 ± 43 | 825 ± 20              |
| − + −                  | 594 ± 36 | 807 ± 222             |
| + Ap −                 | 842 ± 71 | 3,363 ± 662           |
| + Ap +                 | 881 ± 233 | 1,451 ± 274          |
| + BI                   | 38.6 ± 4.4 | 9,795 ± 1,660       |
| + BI +                 | 5.7 ± 2.3 | 2,526 ± 934          |
| + Ap/BI                | 44.1 ± 2.1 | 10,267 ± 24        |
| + Ap/BI                | 5.2 ± 2.7 | 2,671 ± 521          |

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Glucocorticoids can exert their effects on gene expression by specific binding of the steroid receptor complex to DNA transcriptional enhancer elements, which are present in promoters of steroid-regulated genes, or by interfering with the action of steroid-regulated genes, or by interfering with the action of specific binding of the steroid receptor complex to DNA transcriptional enhancer elements, which are present in promoters of steroid-regulated genes, or by interfering with the action of specific binding of the steroid receptor complex to DNA transcriptional enhancer elements, which are present in promoters of steroid-regulated genes, or by interfering with the action of specific binding of the steroid receptor complex to DNA transcriptional enhancer elements, which are present in promoters of steroid-regulated genes, or by interfering with the action of specific binding of the steroid receptor complex to DNA transcriptional enhancer elements, which are present in promoters of steroid-regulated genes, or by interfering with the action of specific binding of the steroid receptor complex to DNA transcriptional enhancer elements, which are present in promoters of steroid-regulated genes, or by interfering with the action of specific binding of the steroid receptor complex to DNA transcriptional enhancer elements, which are present in promoters of steroid-regulated genes, or by interfering with the action of
