Antagonistic Regulation of Tight Junction Dynamics by Glucocorticoids and Transforming Growth Factor-\(\beta\) in Mouse Mammary Epithelial Cells*

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The synthetic glucocorticoid, dexamethasone, stimulated the transepithelial electrical resistance and suppressed the DNA synthesis of 31EG4 nontransformed mouse mammary epithelial cells. The addition of transforming growth factor-\(\beta\) 1 (TGF-\(\beta\)) to mammary cells simultaneously with or up to 24 h after dexamethasone treatment prevented the steroid induction of transepithelial electrical resistance and stimulated the incorporation of \(^3H\)thymidine. However, the TGF-\(\beta\) inhibition of tight junction formation did not require de novo DNA synthesis. Confocal microscopy revealed that the organized immunostaining pattern of the tight junction protein, ZO-1, and F-actin at the cell periphery was disrupted by TGF-\(\beta\), resulting in disorganized and diffuse staining patterns throughout the cell. Western blot analysis demonstrated that TGF-\(\beta\) did not alter the protein levels of ZO-1. In contrast to cells not treated or pretreated with steroid for up to 24 h, TGF-\(\beta\) had no effect on cells pretreated with dexamethasone for 48 h. Transfection of chimeric reporter genes containing promoters responsive to either glucocorticoid or TGF-\(\beta\) demonstrated that the mutual antagonism of tight junction dynamics by dexamethasone and TGF-\(\beta\) occurs in the presence of intact signaling pathways. Taken together, our results establish for the first time that glucocorticoids and TGF-\(\beta\) can antagonistically regulate tight junction formation in a nontransformed mammary cell line.

The precise regulation of cell-cell interactions is an essential feature of the development and function of the mammary gland. Three differentiated cell types, stromal, myoepithelial, and epithelial cells, exist in the mammary epithelium and/or mesenchyme, and reciprocal communication between these cell types occurs as part of mammary morphogenesis during postnatal development and puberty, as well as during pregnancy and lactation (1). Radioactive tracer studies and freeze fracture electron microscopy revealed that an increase in tight junction structural organization and a decrease in permeability of mammary epithelium correlates with the differentiation state of the mammary gland during the onset and establishment of lactation (2–4). Environmental cues such as systemic ovarian steroids and lactogenic hormones, locally acting growth factors, and the extracellular matrix control the normal differentiation and proliferation of mammary cells (5–7) and collectively have been proposed to regulate the dynamics of cell-cell interactions. Part of the complexity of understanding the hormonal pathways that control mammary cell-cell interactions is the likelihood that combinations of steroids, protein hormones, and growth factors contribute to this process in an additive, synergistic, or antagonistic fashion. Also, cellular targets of hormonal pathways that potentially regulate mammary cell-cell interactions are generally uncharacterized, although our in vitro evidence has shown that glucocorticoids regulate tight junctions in transformed and nontransformed mammary epithelial cells (8–10).

The tight junction or zonula occludens is the most apical member of a series of intercellular junctions known as the junctional complex with the adherens junction immediately below it and desmosomes further basal. Tight junctions form a continuous seal around the lateral membrane of adjacent cells and have a highly dynamic structure whose permeability, assembly, and/or disassembly can be altered by a variety of cellular and metabolic regulators (11–13). At intercellular contact points, where the membranes of adjacent cells come into close proximity, tight junctions serve as a selective barrier to the paracellular diffusion of solutes on the basis of size and charge across epithelial and endothelial cell monolayer (14, 15). Tight junctions also contribute to the maintenance of cellular polarity by physically defining the border between the apical and basolateral plasma membrane surfaces (16). To date, one transmembrane protein, occludin (17), and five cytoplasmic-residing peripheral membrane proteins, ZO-1 (18), ZO-2 (19), cingulin (20), the 7H6 antigen (21), and Rab13 (22) have been identified as being localized at tight junctions. Actin, which forms the characteristic perijunctional ring at the underlying adherens junction, has also been shown to associate with the tight junction, suggesting a direct interaction between the cytoskeleton and tight junctions (13, 23-26). The 220-kDa phosphoprotein ZO-1 (27), which has been shown to specifically bind to the related cytoplasmic protein, ZO-2 (19), and to the cytoskeletal protein, spectrin (28), is the only known cytoplasmic link to the transmembrane protein occludin (29). Moreover, ZO-1 is a member of a family of membrane-associated proteins containing potential SH3 and guanylate kinase domains, the first of which is the lethal(1) discs-large-1 (dlg) tumor suppressor gene located at the sepalate junction in Drosophila (30). This homology implicates ZO-1 as a potential component of signal transduction cascades through the plasma membrane (31).

Conceivably, the availability and combinatorial actions of systemic steroids and locally acting growth factors, which regulate particular stages of mammary gland growth and differentiation, concomitantly control tight junction dynamics. In vivo studies suggest that one or more of the lactogenic hor-
maternal tight junctions. The glucocorticoid-dependent stimulation of 31E G4 tight junctions occurs by a receptor-dependent process requiring normal levels of extracellular calcium (10) and functioning serine/threonine phosphorylation-dephosphorylation cascades (9). However, the role of other classes of extracellular signals on tight junction dynamics is unknown, and perhaps other mammogenic factors may concurrently modulate tight junction function and/or structure. One candidate mammogenic factor is transforming growth factor-β (TGF-β),1 which is produced within morphologically distinct areas of the mammary gland (34, 35) and signals through its cognate cell surface serine/threonine kinase receptors. TGF-β is a regulator of mammary branching morphogenesis and cell growth (36), yet relatively little is known about its effects on mammary cell-cell interactions (7, 37). In this study, we establish that glucocorticoids and TGF-β mutually antagonize each other’s actions to regulate tight junction dynamics, localization of the ZO-1 tight junction protein, and cell growth in nontransformed mammary epithelial cells.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium/Ham’s F-12 (50:50) was supplied by BioWhittaker (Walkersville, MD). Fetal bovine serum, insulin, dexamethasone, gentamicin sulfate, and the E-cadherin antibody (uvomorulin, clone DECCMA-1) were purchased from Sigma. Transforming growth factor-β1 was purchased from Life Technologies, Inc. Permeable supports were made by Nunc and distributed by Applied Scientific (San Francisco, CA), and [3H]thymidine (82.9 Ci/mmol) was obtained from NEN Products (Boston, MA). The ZO-1 antibody R40.76 (27) was a generous gift from Bruce Stevenson (Department of Anatomy and Cell Biology, University of Alberta). Fluorescein isothiocyanate-conjugated anti-rat IgG antibodies were supplied by Cappel Laboratories (Malvern, PA). Rhodamine-labeled phalloidin was purchased from Molecular Probes Inc. (Eugene, OR). GRE-CAT was a generous gift of Keith R. Yamamoto (Department of Biochemistry and Biophysics, UCSF). p3TP-Lux was a generous gift of J. San Massague (Cell Biology and Genetics Program, Memorial Sloan-Kettering Cancer Center), and pUC131–1 was a generous gift of Astar Winoto (Department of Biochemistry and Biophysics, University of Alberta). Western blot analysis for ZO-1 and E-cadherin protein production—Cell lysates were subjected to SDS-PAGE electrophoresis, were transferred to nitrocellulose membranes (Micron Separations Inc., Westboro, MA). The membranes were blocked overnight at 4°C in 3% NFDM and probed with ZO-1 monoclonal antibodies directed against ZO-1, R40.76 (1:1000), and primary polyclonal E-cadherin antibodies (1:1600) in TBS-TNFDFM overnight at 4°C; secondary antibody directed against rat IgG conjugated to HRPO (Cappel) was diluted 1:10,000 in TBS-TNFDFM and incubated for 1 h. The signal was detected by enhanced chemiluminescence on Hyperfilm-ECL from Amersham Corp. Parallel cell samples were electrophoretically fractionated, and equivalent protein loading was demonstrated by Coomassie Blue staining of the protein gel.

Transfection Procedures—31E G4 mammary cells were cultured on permeable filter supports in Dulbecco’s modified Eagle’s medium/Ham’s F-12 supplemented with 2% fetal bovine serum, 50 μg/ml gentamicin sulfate, and 5 μg/ml insulin with daily changes of medium as described previously (9, 10). In appropriate experiments, dexamethasone was added to a final concentration of 1 μM, and TGF-β was added to a final concentration of 10 ng/ml. In order to inhibit DNA synthesis, cell cultures were exposed to 1 μM hydroxyurea. TER was measured on filter grown cells using the EVOM Epithelial VoltOhm meter (World Precision Instruments) as described previously (8–10). The EVOM provides an alternating square wave current of ±20 μA through the monolayer to measure the tightness of the tight junction. Daily resistance measurements were taken at room temperature with the electrode after alcohol sterilization. Calculations for ohms-cm² were made by subtracting a blank filter (159 ± 3) from multiplying by the area of the monolayer (0.49 cm²). Assay of DNA Synthesis by [3H]Thymidine Incorporation—DNA synthesis was quantitated by determining the incorporation of [3H]thymidine. Triplicate samples of cells grown on permeable filter supports under the indicated hormonal conditions were incubated with 6 μCi/ml of [3H]thymidine (82.9 Ci/mmol) for 2 h at 37°C in a humid atmosphere of air/CO₂ (95:5). The cells were washed three times with cold 10% trichloroacetic acid, and the filters were placed directly into scintillation vials with 300 μl of 0.3 N NaOH to lyse the cells. Radioactivity was quantitated on a Beckman LS 1801 liquid scintillation counter.

Immunofluorescence Microscopy—31E G4 mammary cells were plated at 100% confluency on 24-well filters and incubated with the indicated combinations and times of exposure to dexamethasone and/or TGF-β1 in serum-free media for 48 or 72 h. The cell monolayers were washed three times with PBS and then fixed with acetonemethanol (50:50) at −20°C for 5 min. Cells were allowed to air dry and then washed with TBST-3% NFDM (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20, and 3% nonfat dry milk). All subsequent incubations with antibodies and washes were performed with this buffer. Cells were incubated with ZO-1 monoclonal antibody (1:100 dilution) at room temperature for 1 h, and then washed three times. Fluorescein isothiocyanate-conjugated goat IgG anti-rat IgG was added to a 1:100 dilution for 1 h at room temperature and then washed three times. The filters were punched out and mounted on glass slides in 50% glycerol, 50 mM Tris, pH 8–9, and 0.4% n-propyl galactoside. Immunofluorescent images were photographed at 1600 ASA with Kodak Ektachrome 400 ASA film.

Conflonal Immunofluorescence Microscopy—For conflonal microscopy, filter-grown monolayers were incubated with combinations of dexamethasone and TGF-β for 72 h. After three washes, the monolayers were fixed with 1.75% formaldehyde in PBS for 15 min at room temperature. The cells were washed three more times and permeabilized with 0.5% Triton X-100 in PBS for 10 min. Following three washes, cells were blocked with 3% NFDM, 2% NFDM, and 0.3% NFDM. After two incubations with primary antibodies as described above. After the ZO-1 immunostaining, cells were stained for F-actin by incubating monolayers with 165 mM rhodamine phalloidin in PBS for 0.5 h. After three washes, filters were mounted on glass slides as described above.

Conflonal images were obtained from a Zeiss Axioplan epifluorescence microscope using a Zeiss 40× Plan-Neofluar multi-immersion objective (0.9 numerical aperture) and analyzed with a Bio-Rad MRC 600 system. A split screen of the double label images was obtained with a dual filter set for fluorescent and Texas red. A series of optical sections was collected for each specimen at ascending z levels beginning at the basal surface in 2-μm increments. Due to the increased thickness of the cell, the number of optical sections collected in the presence of TGF-β was approximately twice that of the amount that collected from monolayers cultured in the absence of TGF-β. In Fig. 2, only the most apical regions of ZO-1 are exhibited in order to investigate whether actin can colocalize with ZO-1 at the apical level. After contrast and zoom enhancements, images were assigned pseudocolors representing the original color of the label used for immunofluorescence and printed with a Kodak Colorseas PS printer.

Western Blot Analysis for ZO-1 and E-Cadherin Protein Production—Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes (Micron Separations Inc., Westboro, MA). The membranes were blocked overnight at 4°C with TBST-5% NFDM and incubated with a mixture of primary rat monoclonal antibodies directed against ZO-1, a primary polyclonal E-cadherin antibodies (1:1600) in TBS-TNFDFM overnight at 4°C; secondary antibody directed against rat IgG conjugated to HRPO (Cappel) was diluted 1:10,000 in TBS-TNFDFM and incubated for 1 h. The signal was detected by enhanced chemiluminescence on Hyperfilm-ECL from Amersham Corp. Parallel cell samples were electrophoretically fractionated, and equivalent protein loading was demonstrated by Coomassie Blue staining of the protein gel.

Transfection Procedures—31E G4 mammary cells from a logarithmically growing culture were transfected by electroporation. Briefly, cells were harvested with trypsin-EDTA, washed twice with sterile Ca²⁺- and Mg²⁺-free PBS, and resuspended in sucrose buffer containing 270 mM sucrose, 7 mM sodium phosphate buffer, pH 7.4, 0.5 mM MgCl₂, 250 μl of cell suspension and plasmid DNA (15–25 μg total including 10–15 μg of either GRE-CAT or p3TP-lux) were mixed, and 5 electric pulses (400 V square wave pulse for 99 μs) were delivered to the sample using a BTX 800 Transfector apparatus (BTX Inc., San Diego, CA). The samples were incubated for 10 min on ice and cultured at 37°C for 16 h in a 5% CO₂ incubator. The cells were treated for 24 h with TGF-β and/or dexamethasone in fresh media and harvested for CAT or luciferase assays. Cells harvested for CAT assays were washed three times in PBS, resuspended in 0.1 mM Tris-HCl, pH 7.8, and prepared by four cycles of freeze-thawing. The cell lysates were heated at 68°C for 15 min and centrifuged at 12,000 × g for 10 min, and the supernatants were recovered. Cells for the luciferase assay were washed three times with PBS and lysed with 1 ml of reporter lysis buffer (Promega) according to the manufacturer’s instructions.

CAT and Luciferase Assays—CAT activity in the cell extracts con-
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Fig. 1. Transforming growth factor-β reversibly inhibits the dexamethasone stimulation in transepithelial electrical resistance. 31E4G mammary epithelial cells were cultured on permeable supports with the indicated combinations of 1 μM Dex and 10 ng/ml TGF-β for 120 h. In one set of cultures, TGF-β was withdrawn from the medium of cells treated with dexamethasone and TGF-β for 48 h (large arrow) by incubating the cells with medium supplemented with dexamethasone alone for an additional 72 h (Dex/TGF-β Withdrawal). Throughout the 120-h time course, the TER was determined at the indicated times, and the ohms-cm² were calculated as described in the text. TER measurements were performed in triplicate, and the results are the averages of three separate experiments.

RESULTS

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methasone and TGF-β (Fig. 2, g and h), there is slightly more specific junctional staining of ZO-1 and actin. In the upper left portion, ZO-1 and actin staining is detected only in the apical plane (Fig. 2, g), indicating that certain areas of cells are raised higher than others. ZO-1 and actin staining in the mammary cells treated with glucocorticoids and TGF-β appear to be heterogeneous in that although most cells have no tight junctional staining, some cells have both specific junctional staining as well as diffuse cytoplasmic staining and still others have only the characteristic cell peripheral staining. Similar to cells treated with TGF-β alone, actin colocalizes with ZO-1 even in the most apical plane (Fig. 2, g). Thus, under conditions in which TGF-β prevents dexamethasone from stimulating TER, a remodeling of cell morphology occurs with a cellular redistribution of junctional associated proteins.

TGF-β Does Not Regulate ZO-1 Protein Levels—To determine whether TGF-β treatment alters the level of ZO-1 protein expression, 31EG4 mammary epithelial cells were cultured on permeable supports with the indicated combinations of 1 μM Dex and 10 ng/ml TGF-β for 72 h. Western blots of electrophoretically fractionated whole cell extracts were incubated simultaneously with monoclonal antibodies to ZO-1 and the adherens junction protein, E-cadherin. As shown in Fig. 3, TGF-β treatment did not affect either the basal levels or the dexamethasone-induced expression of ZO-1 previously characterized in these cells (9). Approximately equivalent gel loading was shown by similar amounts of E-cadherin in each sample. Importantly, under all hormonal conditions, the monoclonal antibodies to ZO-1 recognized a single protein band of approximately 220-kDa proteins. Thus, under conditions in which the transepithelial electrical resistance fails to be induced by glucocorticoids, the pattern of ZO-1 distribution in mammary cells but not its expression is significantly altered by exposure to TGF-β.

Pretreatment with Dexamethasone Precludes TGF-β from Disrupting Tight Junction Integrity or Altering ZO-1 Distribution—When added simultaneously with dexamethasone, TGF-β precludes glucocorticoids from inducing the sealing of tight junctions. To determine whether TGF-β can disrupt tight junctions once they are well sealed due to glucocorticoid treatment, TGF-β was added for 24 h to 24 or 48 h dexamethasone-pretreated cells, and the monolayers were assayed for changes in TER or ZO-1 localization. As previously mentioned, dexamethasone rapidly induces the TER of 31EG4 mammary cells after an initial time lag of 24 h. As shown in Fig. 4, TGF-β addition at 48 h (Dex/TGF-β 48–72) has only a minimal effect on dexamethasone-stimulated TER. In contrast, similar to cells simultaneously treated with TGF-β and dexamethasone (Dex+TGF-β), TGF-β completely inhibited tight junction formation when added at 24 h of glucocorticoid treatment (Dex/TGF-β 24–48). The addition of TGF-β at any time to dexa-
Dexamethasone-treated cells after the stimulation in TER had no effect on the maintenance of well established tight junctions. 

Indirect immunofluorescence microscopy revealed that the addition of TGF-\(\beta\) to steroid-treated cells with an induced TER had no effect on the cellular distribution of ZO-1 protein. TGF-\(\beta\) was added to mammary cells at various times (0, 24, or 48 h or no addition) during exposure to 1 \(\mu\)M dexamethasone and then incubated for up to 48 or 72 total hours. A control set of cultures were treated with TGF-\(\beta\) for the same time points in the absence of steroid. Under conditions in which the monolayer TER is stimulated by dexamethasone for 72 h (no TGF-\(\beta\) or TGF-\(\beta\) treatment 48–72 h), ZO-1 protein was localized to the tight junction as a sharp continuous band of immunostaining at the cell periphery (Fig. 5). The 48 h dexamethasone-treated and untreated cells displayed the same peripheral ZO-1 staining as mammary cells exposed to steroid for 72 h. In contrast, when TGF-\(\beta\) was added to 24 h steroid-treated cells (TGF-\(\beta\) 24–48 h) or simultaneously with dexamethasone (0–72 h), TGF-\(\beta\) caused a disruption of the overall ZO-1 staining pattern with most of the immunostaining being diffused throughout the cell (Fig. 5). A similar TGF-\(\beta\) effect on ZO-1 immunostaining was also observed in the absence of glucocorticoids (Fig. 5). Taken together, these observations demonstrate that glucocorticoid or TGF-\(\beta\) receptor signaling pathways can regulate tight junction dynamics of 31EG4 cells.

Mutual Antagonism of Tight Junction Integrity by Dexamethasone and TGF-\(\beta\) Is Not Due to Inhibition of Receptor Signaling—One possible mechanism through which dexamethasone and TGF-\(\beta\) treatment can mutually exclude the actions of the other signal is by directly inhibiting receptor function. To test this possibility, 31EG4 mammary cells were transfected with either the glucocorticoid-responsive GRE-CAT chimeric reporter plasmid or the TGF-\(\beta\)-responsive p3TP-Lux reporter plasmid by electroporation and then cultured for 72 h with the indicated combinations of 1 \(\mu\)M Dex and 10 ng/ml TGF-\(\beta\). In one set of cultures, TGF-\(\beta\) was included in the medium only during the last 24 h of dexamethasone treatment (48–72 hr). Cell extracts were assayed for either CAT-specific activity (top panel) or luciferase specific activity (bottom panel) as described in the text. The results are the averages of two independent sets of triplicate samples.

Fig. 5. Pretreatment with dexamethasone for 48 h prevents transforming growth factor-\(\beta\) from disrupting the cellular distribution of ZO-1. 31EG4 mammary cells were cultured on permeable supports with (+ Dex) or without (− Dex) 1 \(\mu\)M dexamethasone. Cells were treated with 10 ng/ml TGF-\(\beta\) simultaneously with dexamethasone during an entire 72-h time course (0–72 hr), for the last 24 h of a 48 h time course (24–48 hr), or for the last 24 h of a 72-h time course (48–72 hr). A control set of cell cultures were not treated with TGF-\(\beta\) (none). The cells were fixed and analyzed for ZO-1 localization by indirect immunofluorescence using a ZO-1 monoclonal primary antibody as described in the text.

Fig. 6. Effects of hormone treatment on glucocorticoid receptor and transforming growth factor-\(\beta\) receptor signaling. 31EG4 mammary cells were transfected with either the glucocorticoid-responsive GRE-CAT reporter plasmid or the TGF-\(\beta\)-responsive p3TP-Lux reporter plasmid. The cells were cultured for 72 h in the presence of TGF-\(\beta\) for 72 h, in which the monolayer TER remains low, and in the presence of TGF-\(\beta\) only during the last 24 h (48–72 h) of a 72-h dexamethasone treatment, during which the monolayer TER remains high at the steroid-induced level. Similarly, TGF-\(\beta\) stimulates the activity of p3TP-Lux in 31EG4 cells not treated with steroids in cells incubated simultaneously with
Transforming growth factor-β (TGF-β) stimulates incorporation of \[^{3}H\]thymidine in 31EG4 mammary epithelial cells under conditions that inhibit tight junction formation. 31EG4 mammary cells were cultured on permeable supports in the presence or the absence of \[^{3}H\]thymidine after a 2-h pulse label as described in the text. The results are the averages of triplicate samples.

A graphical representation of the incorporation of \[^{3}H\]thymidine in the presence or absence of dexamethasone is shown. The graph displays the incorporated counts per minute (cpm) for cells treated with dexamethasone (DEX) and TGF-β (TGF-β) alone or in combination for 48 hr or 72 hr. The graph shows that TGF-β, in the presence of dexamethasone, inhibits the incorporation of \[^{3}H\]thymidine in a dose-dependent manner.

**DISCUSSION**

Our results using a nontransformed mammary epithelial cell line represent the first evidence that glucocorticoids, an important systemic lactogenic steroid (49), and TGF-β, a locally produced factor, can antagonistically regulate tight junction dynamics. This observation implicates these two distinct extracellular signals as playing important roles in controlling cell-cell interactions in vivo. The mammary gland undergoes a progression of morphological and functional changes during pregnancy, lactation, and involution, including temporal regulation of tight junction permeability. For instance, colostrum, the milk secreted by the mammary gland a few days before or after parturition, contains more protein, immunoglobulins, s-
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**Fig. 8.** Transforming growth factor-β disrupts the glucocorticoid-stimulated formation of tight junctions in the presence of a DNA synthesis inhibitor. 31E64 mammary cells were cultured on permeable supports in the presence of the indicated combinations of 1 μM Dex, 10 ng/ml TGF-β, and/or 1 μM hydroxyurea for 48 h; one set of control cultures were not treated with either hormone or hydroxyurea (No Addition). Top panel, the TER was monitored, and the ohms/cm² were calculated as described in the text. Each assay was performed in triplicate, and the results are the averages of three separate experiments. Lower panel, the rate of DNA synthesis was monitored by determining the incorporation of [3H]thymidine after a 2-h pulse label as described in the text. The results are the averages of triplicate samples.

...cellular DNA content to a profile consistent with a growing population of cells. The ability of TGF-β to preclude the steroid induction of tight junction formation occurred in the presence of hydroxyurea, a DNA synthesis inhibitor, demonstrating that this disruptive effect on tight junction integrity is not an indirect consequence of an increase in DNA synthesis. This observation suggests that the tight junction machinery is a selective target of TGF-β receptor signaling. However, this evidence does not exclude the possibility that TGF-β-mediated stimulation in [3H]thymidine incorporation and cell growth may be a consequence of the disruption in tight junction structure and ZO-1 redistribution.

It has been proposed that junctional plaque proteins, such as ZO-1, help orchestrate the interactions of integral membrane proteins with cytoplasmic signaling components and, as a result, may serve as regulators of cell proliferation, cell adhesion, and cell-cell interactions. In this context, ZO-1 is homologous to the lethal(1)discs-large-1 (dlg) tumor suppressor gene of Drosophila, whose mutation resulted in neoplastic overgrowth of

addition, TGF-β can suppress the synthesis and secretion of milk caseins from mammary explants in pregnant mice (53).

During functional differentiation of the mammary gland, the key hormonal regulators of mammary cell-cell interactions must be selective and reversible in their actions and in some instances, counteract each other depending on the stage of differentiation. Consistent with these biological properties, glucocorticoids and TGF-β antagonistically control tight junction dynamics in vitro in a temporally regulated manner. In our studies of mammary cells pretreated with glucocorticoids for 48 h, TGF-β failed to reduce the monolayer TER or alter ZO-1 localization. In contrast, the addition of TGF-β simultaneously with or up to 24 h after glucocorticoid treatment disrupted the structural organization and function of tight junctions. Activation of glucocorticoid-responsive or TGF-β-responsive reporter plasmids demonstrated that the mutual antagonism displayed by dexamethasone and TGF-β targets signaling pathways that regulate cell-cell interactions and growth control rather than having direct effects on receptor function. The time lag required for TER induction suggests that this process is mediated by steroid-regulated events of about 24 h. Because exposure to TGF-β during but not after this lag period precludes the ability of dexamethasone to induce tight junction formation, the timing of glucocorticoid-induced gene expression and/or function is critical for regulating TGF-β responsiveness. Given the known transcriptional mechanism of action of glucocorticoid receptors (54, 55), it is tempting to consider that expression and/or activity of the immediate early steroid-regulated gene products that initiate the tight junction response can be reversed by TGF-β receptor signaling. Conceivably, the delayed dexamethasone-responsive gene products, which are responsible for the increase in TER and development of well sealed tight junctions, are relatively unaffected by exposure to TGF-β. Alternatively, components of the TGF-β receptor signaling pathway that directly or indirectly target the tight junction may not be functional or adequately expressed in glucocorticoid-treated mammary cells. Identification of the signaling components that regulate tight junction assembly, disassembly, and integrity will be important to clarify the precise mechanism of hormonal control.

Dexamethasone coordinately induced tight junction formation and suppressed DNA synthesis, whereas the TGF-β disruption of ZO-1 localization and prevention of steroid-induced TER were accompanied by a stimulation in [3H]thymidine incorporation. Flow cytometry analysis revealed that TGF-β also induced a shift in cellular DNA content to a profile consistent with a growing population of cells. The ability of TGF-β to preclude the steroid induction of tight junction formation occurred in the presence of hydroxyurea, a DNA synthesis inhibitor, demonstrating that this disruptive effect on tight junction integrity is not an indirect consequence of an increase in DNA synthesis. This observation suggests that the tight junction machinery is a selective target of TGF-β receptor signaling. However, this evidence does not exclude the possibility that TGF-β-mediated stimulation in [3H]thymidine incorporation and cell growth may be a consequence of the disruption in tight junction structure and ZO-1 redistribution.

It has been proposed that junctional plaque proteins, such as ZO-1, help orchestrate the interactions of integral membrane proteins with cytoplasmic signaling components and, as a result, may serve as regulators of cell proliferation, cell adhesion, and cell-cell interactions. In this context, ZO-1 is homologous to the lethal(1)discs-large-1 (dlg) tumor suppressor gene of Drosophila, whose mutation resulted in neoplastic overgrowth of
the imaginal discs as well as a loss of cell polarity and adhesion. Although a role for ZO-1 in intercellular signaling is unknown, several studies have attempted to ascertain the nature of its regulation. For example, ZO-1 was shown to be phosphorylated only on serine residues under normal conditions in MDCK cells (27) and tyrosine residues during the formation of tight junctions in the slit diaphragms of glomerular epithelial cells (56). We have shown that glucocorticoid stimulation of TER in 31E4G cells did not alter the localization, phosphate content, or phosphopeptide digest pattern of ZO-1 but did increase protein levels (9). Recent evidence has shown that the PKC agonist 1,2-dioctanoylglycerol can promote the assembly of tight junctions, as evidenced by the translocation of ZO-1 and actin filaments to the cell periphery (57), and PKC can in vitro phosphorylate ZO-1, which may play a role in the formation of tight junctions (58). Previous observations demonstrating that ZO-1 is detected at the tight junction as well as adherens junction zone only in cells with leaky tight junctions (59) may explain the high colocalization of actin and ZO-1 in 31E4G cells treated with TGF-β. Clearly, the modification and regulation of ZO-1 are complicated processes that are dependent on the physiological environment, cell type, and integrity of the tight junction. Conceivably, the TGF-β-mediated stimulation in 

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\text{\textsuperscript{3}H} \text{thymidine incorporation and tight junction disassembly may involve modification and redistribution of the ZO-1 protein.}
\]

TGF-β is a potent growth inhibitor of many epithelial-derived cells but generally stimulates proliferation of many fibroblast cells (60). The 31E4G mammary cells used in our study are epithelial in nature because they are polarized and express epithelial cell markers but generally stimulate proliferation of many fibroblast cells (61). For example, highly proliferative mammary tumors contain an increased level of TGF-β transcripts compared with their normal human mammary cell counterparts (62). The mechanism by which TGF-β promotes cell growth is not well understood, although the stimulation in proliferation of a fibroblast cell line is accompanied by an activation of cyclin E-Cdk2 kinase and down-regulation of the p27/Kip1 cell cycle inhibitor (63). Another mechanism by which TGF-β may be stimulating mammary epithelial cell growth is by inducing transdifferentiation to a nonepithelial, mesenchymal-like phenotype (37). Whatever the precise mechanism of growth regulation of mammary epithelial cells, the novel antagonistic regulation of tight junction dynamics by TGF-β and glucocorticoids may have important implications for understanding hormonal contributions to controlling differentiation processes of the mammary gland, as well as cell-cell interactions involved with invasiveness and metastasis of mammary tumors.

It is tempting to consider that the selective antagonism between glucocorticoid and TGF-β signaling observed in vitro parallels that of in vivo cellular events associated with the control of mammary cell-cell interactions. Transgenic mice expressing TGF-β-1 targeted to the pregnant mammary gland showed inhibited alveolar development and lactation (64). We propose that TGF-β, which is developmentally regulated during mammary gland differentiation, plays a critical role in maintaining a relatively leaky paracellular pathway, and that during lactation, dexamethasone is the predominant regulator of tight junction integrity. In an analogous manner, TGF-β has been shown to alter the synthesis of extracellular matrix components (65–67), which can potentially affect extracellular signaling and cell function. For instance, tenascin-C, which inhibits fibronectin-mediated adhesion, is stimulated by TGF-β-1 and down-regulated by glucocorticoids (68, 69). The downstream targets of TGF-β- and/or glucocorticoid receptor-activated signaling cascades in mammary epithelial cells are mostly unknown. Conceivably, ZO-1 or other tight junction proteins could serve as potential targets for steroid or growth factor signaling pathways. We are currently attempting to elucidate the cellular events underlying the mutual antagonism of tight junction dynamics by glucocorticoids and TGF-β that operate in mammary epithelial cells. Such pathways could potentially represent an important cross-talk between growth factor and steroid receptor signal transduction cascades that is necessary to guide the functional relationships between particular sets of environmental cues that control mammary cell-cell interactions.

Acknowledgments—We thank Patricia Buse, Anita C. Maiyar, and Kay-Ming Toon for constructive comments during the course of the work and for critical comments of this manuscript. We also express our appreciation to Jerry Kapler for skillful photography, Anna Fung for preparation of this manuscript, and Richard D. Fetter, William J. Melander, Marina Chin, Ritu Patel, Vinh Trinh, and Thai Truong for technical support.

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J. Biol. Chem. 1996, 271:404-412.
doi: 10.1074/jbc.271.1.404

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