Involvement of the Helix-Loop-Helix Protein Id-1 in the Glucocorticoid Regulation of Tight Junctions in Mammary Epithelial Cells*

Received for publication, December 27, 1999, and in revised form, June 29, 2000
Published, JBC Papers in Press, June 30, 2000, DOI 10.1074/jbc.M910373199

Paul L. Woo‡§, Andrea Cercek‡, Pierre-Yves Desprez¶, and Gary L. Firestone‡¶

From the §Department of Molecular and Cell Biology and the Cancer Research Laboratory, University of California at Berkeley, Berkeley, California 94720-3200 and ¶Geraldine Brush Cancer Research Institute, California Pacific Medical Center, San Francisco, California 94115

Mammary epithelial cell-cell junctions undergo morphological and structural differentiation during pregnancy and lactation, but little is known about the transcriptional regulators that are involved in this process. In Con8 mammary epithelial tumor cells, we have previously documented that the synthetic glucocorticoid, dexamethasone, induces the reorganization of the tight junction and adherens junction and stimulates the monolayer transepithelial electrical resistance (TER), a reliable in vitro measurement of tight junction sealing. Western blots demonstrated that dexamethasone treatment rapidly and strongly stimulated the level of the Id-1 protein, which is a serum-inducible helix-loop-helix transcriptional repressor. The steroid induction of Id-1 was robust by 4 h of treatment and maintained over a 24-h period. Isopropyl-1-thio-β-D-galactopyranoside-inducible expression of exogenous Id-1 in Con8 cells was shown to strongly facilitate the dexamethasone induction of TER in the absence of serum without altering the dexamethasone-dependent reorganization of ZO-1, β-catenin, or F-actin. Ectopic overexpression of Id-1 in the SCp2 nontumorigenic mammary epithelial cells, which does not undergo complete dexamethasone-dependent tight junction reorganization, enhanced the dexamethasone-induced ZO-1 tight junction localization and stimulated the monolayer TER. Moreover, antisense reduction of Id-1 protein in SCp2 cells prevented the apical junction reorganization and dexamethasone-stimulated TER. Our results implicate Id-1 as acting as a critical regulator of mammary epithelial cell-cell interactions at an early step in the glucocorticoid-dependent signaling pathway that controls tight junction integrity.

A major determinant of epithelial cell differentiation is its capacity to form extensive intercellular interactions through adhesion and tight junction molecules. These contacts are critical in forming selectively permeable epithelial sheets, which are immobile, by virtue of being closely linked to the adjacent cell. Through a series of junctional complexes located along the lateral plasma membrane surfaces, epithelial cells are able to derive their characteristic morphology, linking tissue integrity with physiological functions such as adhesion, polarized secretion, and compartmentalized barrier (1, 2). The tight junction and the adherens junction are located at the apical region of the junctional complex (1, 3) and mediate paracellular permeability and adhesion, respectively (4). The combined capabilities of both junctions help to establish and maintain cellular polarity by separating the plasma membrane into two compositionally distinct domains that are involved in spatially restricted biochemical reactions.

Assembly and stability of the intercellular junction is dependent on the binding of calcium ions to the extracellular region of E-cadherin, thereby initiating homophilic interaction between cadherin molecules on neighboring cells (5). On the intracellular side, β-catenin or plakoglobin (γ-catenin) associates with the cytoplasmic region of E-cadherin, and binding of α-catenin to this adherens junction complex allows the interaction with the actin cytoskeleton (6). Subsequently, the formation of tight junctions is thought to occur through the recruitment and assembly of the highly related PDZ-containing proteins, ZO-1, ZO-2, and ZO-3, tethered to the integral membrane protein, occludin (1–3). The tight junction is also associated with the actin cytoskeleton through direct interaction with ZO-1 (7) or, potentially, through ZO-1-binding proteins, such as spectrin (8), AF-6/afadin (9), and cortactin (10). Recently, claudin proteins, of which 15 have been cloned (11–14), and JAM (junctional adhesion molecule) (15) have been identified as integral membrane proteins localized at the tight junctions, although their precise role or nature of their interactions have not been defined. The importance of the adherens junction and tight junction in the acquisition and maintenance of the differentiated phenotype is well established. However, little is known about the cell signaling networks that utilize transcriptional regulators to control the formation and integrity of epithelial cell junctions.

Basic helix-loop-helix transcription factors are important regulators of differentiation in various cell systems (16–20). Another class of helix-loop-helix proteins, the Id proteins, interfere with basic helix-loop-helix transcription factors (21). Id proteins lack the basic domain responsible for DNA binding but interact with basic helix-loop-helix transcription factors through their own helix-loop-helix region (22, 23). Thus, Id

---

* This work was supported by National Institutes of Health (NIH) Grant DK-42799 (to G. L. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1724 solely to indicate this fact.

§ Recipient of a predoctoral fellowship supported by NIH National Research Service Grant CA-09041. Portions of this work were submitted to fulfill the requirements for a doctorate of philosophy at the University of California at Berkeley.

† To whom correspondence and reprint requests should be addressed: Dept. of Molecular and Cell Biology, 591 LSA, University of California at Berkeley, Berkeley, CA 94720-3200. Tel.: 510-642-8319; Fax: 510-643-6791; E-mail: gfire@uclink4.berkeley.edu.

1 The abbreviations used are: ZO-1, zona occludens-1; TER, transepithelial electrical resistance; Dex, dexamethasone; ECM, extracellular matrix; EHS-ECM, Englebreth Holm Swarm tumor extracellular matrix; IPTG, isopropyl-1-thio-β-D-galactopyranoside; HA, hemagglutinin.

This paper is available on line at http://www.jbc.org
proteins function as negative regulators of the basic helix-loop-helix transcription factors by forming heterodimers and blocking the binding to specific DNA sequences, E boxes, found in the regulatory regions of target genes. Four id (inhibitor of DNA binding) genes, id-1 to id-4, have been identified in mammals (22, 23, 31, 32). id genes belong to a class of immediate-early serum-responsive genes (33–35) and are thought to be highest in undifferentiated tissues and decreases in their differentiated serum-responsive genes (33–35) and are thought to be highest 

The vector containing the LacSwitch-inducible promoter system (21, 24–26), and other id genes have been described in fish (27), zebrafish (28), Xenopus (29), and Drosophila melanogaster (30). There is considerable evidence that Id proteins can regulate cell proliferation and apoptosis in many cell systems (22, 23, 31, 32). id genes belong to a class of immediate-early serum-responsive genes (33–35) and are thought to be highest in undifferentiated tissues and decreases in their differentiated counterparts. However, several reports have contradicted this viewpoint by showing id gene expression present or induced in differentiated cells (36–43). Interestingly, genetic studies of *Drosophila id*, extramacrochaetae (*emc*), suggest that *emc* interacts genetically with the Ras signaling pathway and is required in both cell proliferation and cell differentiation processes during wing morphogenesis (44). In addition, *emc* gene expression was identified as a target of an uncharacterized signaling pathway involving the Drosophila ZO-1 protein, TAMEOU, which is localized to epithelial cell junctions (45). Using mammary epithelial cells as a model system to study the hormonal control of cell-cell interactions, we have uncovered a novel role for the Id-1 protein in mediating the glucocorticoid regulation of tight junctions.

Our previous studies have shown that glucocorticoids can induce the reorganization of the tight junction and adherens junction in the Con8 mammary epithelial tumor cells, leading to enhanced cell-cell adhesion and decreased paracellular permeability (46, 47). The induction of the differentiated phenotype coincides with a G1 cell cycle arrest (46). Furthermore, the glucocorticoid induction of tight junction sealing requires functional Ras activity (48) and corresponds to a decrease in the actin-bundling protein, fascin (47). Forced expression of dominant-negative Ras or the fascin protein inhibits the glucocorticoid stimulation of transepithelial electrical resistance (TER) of cell monolayers grown on filter inserts. Here we show that Id-1 expression increased as the tight junction begins to differentiate in response to glucocorticoids and that the ectopic overexpression of Id-1 increased the capacity of glucocorticoid to trigger tight junction sealing. Also, in the SCp2 mammary epithelial cell line, antisense ablation of Id-1 protein expression inhibited the glucocorticoid stimulation of tight junction structure and function.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco's modified Eagle's medium/Ham's F-12 (50:50) and calf serum were supplied by BioWhittaker (Walkersville, MD). Permeable tissue culture supports/filter inserts were manufactured by Cron Separations Inc., Westboro, MA). Blots were blocked in TBST (5% nonfat dry milk) and incubated with primary antibodies against TAMEOU (1:100 dilution), N-cadherin (1:400) at room temperature and washed three times. For the secondary reaction, goat fluorescein- or Texas red-conjugated antibodies were incubated for 1 h at room temperature and washed as above. The blots were developed by NEN's chemiluminescence reagent kit.

**Immunoprecipitation of E-cadherin Complex**—To examine the E-cadherinβ-catenin complex formation, confluent SCp2, SCp2-Id-S, and SCp2-Id-A cells were cultured on six-well plates (Corning Glass, Corning, NY) in the presence or absence of 1 µM dexamethasone for 5 days. The cells were extracted in 2 ml of radioimmune precipitation buffer (150 mM NaCl, 0.1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris-Cl, pH 8.0) in the presence of protease inhibitors (5 mM phenylmethylsulfonyl fluoride, 5 µg/ml pepstatin A, 10 µg/ml leupeptin, 20 µg/ml aprotinin, and 2 mM benzamidine). Extraction was carried out on ice for 30 min. Co-immunoprecipitation was performed with protein A-Sepharose CL-4B (Amersham Pharmacia Biotech) in the presence of rat anti-E-cadherin antibodies at 4 °C for 2 h. Immunoprecipitates were washed five times in the same immunoprecipitation buffer, and co-immunoprecipitated proteins were solubilized in sample buffer.

**RESULTS**

**Glucocorticoids Stimulate Expression of the Helix-Loop-Helix Protein Id-1**—In Con8 mammary tumor cells, glucocorticoids induce functional differentiation as evident by the reorganization of the apical junction, which is composed of the tight junction and the adherens junction. We have previously shown that the modeling of the apical junction results in tight junction sealing as measured by the TER of the monolayer and paracellular flux studies using mannitol or inulin as extracellular tracers (46, 49). To begin to characterize the early regulatory processes within the steroid-mediated cascade that controls cell-cell interactions, we examined whether glucocorticoids can regulate the production of the transcriptional regulatory protein Id-1. Con8
cells were treated with or without the synthetic glucocorticoid dexamethasone over a 72-h time course, and the level of Id-1 protein was determined by Western blot analysis. By 4 h of dexamethasone treatment, Id-1 protein levels were significantly stimulated compared with the untreated control, and this induction was sustained within a 24-h period (Fig. 1A). After 24 h, the basal level of Id-1 protein increased, which is probably due to the actions of growth factors that are secreted from these mammary tumor cells. In this regard, serum, which contains a variety of growth factors including those secreted by mammary tumor cells (50, 51), has been shown to rapidly stimulate Id-1 expression in several other cell systems (23). Therefore, the magnitude of the glucocorticoid- and serum-responsive Id-1 expression was compared in mammary tumor cells. Serum-starved Con8 cells treated for 24 h with either dexamethasone or serum induced significantly higher and approximately equivalent levels of Id-1 protein, while a combination of dexamethasone and serum did not produce any additional effects on Id-1 accumulation (Fig. 1B). These results suggest that the lack of any further glucocorticoid response on Id-1 levels after 24 h in culture is probably due to the basal level having reached a near maximal rate of Id-1 production. Taken together, our results demonstrate that the stimulation of Id-1 production is a relatively early response to glucocorticoids that occurs within the 24-h time lag observed before the formation of tight junctions (46, 47, 52).

**IPTG-inducible Id-1 Enhances Tight Junction Sealing in Con8 Cells**—To address the functional consequence of Id-1 expression on tight junction formation, we established Con8 cell lines expressing murine Id-1 under the control of the IPTG-inducible promoter. Stable cell lines expressing the Lac repressor protein were obtained by transfecting the lac repressor vector containing the hygromycin resistance gene and selecting for their resistance to the cytotoxic effects of hygromycin. The lac-4 clone was selected for its high expression of the Lac repressor protein and subsequently transfected with the lac operator vector containing the neomycin-resistant gene and the murine Id-1 cDNA with a C-terminal HA epitope tag. Subclones that survived an additional selection process in hygromycin and G418-containing media were screened for the expression of the conditionally expressed Id-1 protein in the presence of 1 mM IPTG for 24 h. Shown in Fig. 2A, Western blotting was performed using a monoclonal antibody to the HA epitope and revealed four clones that inducibly express the HA-tagged Id-1 protein in response to IPTG for a 24-h period. Moreover, ectopic Id-1 protein accumulated even after 5 days of IPTG treatment (data not shown).

In order to functionally assess whether Id-1 expression can affect tight junction sealing induced by dexamethasone, the TERs of the IPTG-inducible Id-1 subclones cultured on filter inserts were monitored in the absence of serum to circumvent the contribution of endogenous Id-1 on tight junction formation during the initial 24 h of glucocorticoid treatment. The cells

![Fig. 1. Dexamethasone stimulation of Id-1 protein levels in mammary tumor cells.](image)

A, confluent monolayers of Con8 mammary tumor cells were grown on filter inserts in the absence (−) or presence (+) of 1 μM dexamethasone for 24 h, and the medium was changed daily. Cell lysates were analyzed by Western blot for Id-1 expression at the indicated time points. B, postconfluent cells were serum-starved for 1 day and cultured in the absence (−) or presence (+) of 1 mM IPTG. Equal amounts of whole-cell extracts were analyzed using a Western blot with a monoclonal antibody directed against the HA epitope fused to the C terminus of murine Id-1. Each subclone expressed high levels of IPTG-inducible Id-1. B, Filter-grown lac-4 and IPTG-inducible Id-1 cells were serum-starved for 1 day and cultured in the absence (−) or presence (+) of 1 μM dexamethasone and/or 1 mM IPTG for 5 days. Serum-free medium was changed daily. TER was measured in triplicate cultures for each condition.

![Fig. 2. IPTG-inducible expression of Id-1 in Con8 mammary tumor cells enhances the glucocorticoid induction of transepithelial electrical resistance.](image)

A, Con8 cells expressing constitutive levels of the Lac repressor protein (lac-4 cells) were transfected with the pOP3-Id1 construct. Four independent subclones (Id-22.5, Id-22.3, Id-44.0, and Id-42.7) and the control lac-4 cells were cultured in the absence (−) or presence (+) of 1 mM IPTG. Equal amounts of whole-cell extracts were analyzed using a Western blot with a monoclonal antibody directed against the HA epitope fused to the C terminus of murine Id-1. Each subclone expressed high levels of IPTG-inducible Id-1. B, Filter-grown lac-4 and IPTG-inducible Id-1 cells were serum-starved for 1 day and cultured in the absence (−) or presence (+) of 1 μM dexamethasone and/or 1 mM IPTG for 5 days. Serum-free medium was changed daily. TER was measured in triplicate cultures for each condition.
were plated at confluency and serum-starved for 1 day prior to the addition of combinations of 1 μM dexamethasone and/or 1 mM IPTG for 3 days. Similar to the lac-4 clone, the four clones (Id-22.5, Id-22.3, Id-44.0, and Id-42.7) isolated for their ability to express the IPTG-inducible Id-1, exhibited a dexamethasone-dependent induction of TER compared with untreated cells (Fig. 2b). Interestingly, in these clones, treatment with both dexamethasone and IPTG stimulated TER induction in a synergistic manner as compared with dexamethasone or IPTG added alone. In fact, treatment with IPTG alone does not appear to significantly alter basal TER values in untreated cells. Although the Id-42.7 cells appeared to partially respond to the actin cytoskeleton in rat mammary tumor cells.

Fig. 3. Co-staining of ZO-1 and β-catenin in the control lac repressor-expressing cells (lac-4) and the Id-22.5 IPTG-inducible Id-1 cell line. Filter-grown cells were serum-starved for 1 day and cultured in serum-free medium containing 1 μM dexamethasone (+ Dex), 1 mM IPTG (+ IPTG), dexamethasone and IPTG (Dex + IPTG), or medium alone (Unt). At the end of the 5-day treatment, the cells were fixed, permeabilized, and incubated with polyclonal ZO-1 antibodies and monoclonal β-catenin antibodies as described under “Experimental Procedures.” Double immunofluorescence analysis revealed that ZO-1 and β-catenin colocalized only in dexamethasone-treated cells.

Co-localization of ZO-1 and β-Catenin in IPTG-inducible Id-1 Cells—In the parental Con8 cell line, the glucocorticoid induction of TER corresponds to a reorganization of the apical junction, consisting of the tight junction and the adherens junction (47). To determine whether the ectopic expression of Id-1 is capable of altering the glucocorticoid-induced tight junction and adherens junction formation, the subcellular localization of the tight junction protein, ZO-1, and the adherens junction protein, β-catenin, was analyzed in the lac-4 control cells and the Id-22.5 cells by indirect immunofluorescence microscopy. After 5 days of treatment with combinations of dexamethasone and/or IPTG as described above, the cells were fixed, and the junction proteins were visualized simultaneously using a polyclonal antibody directed against ZO-1 and a monoclonal β-catenin antibody (Fig. 3). In both the untreated (Unt) and IPTG-treated (IPTG) lac-4 and Id-22.5 cells, ZO-1 staining appeared as punctate aggregates with occasional staining present at cell-cell contacts. ZO-1 localization appears to be slightly more punctuated in lac-4 cells in comparison with Id-22.5 cells, although this difference is minor and probably due to cell clone variation. Concurrent with the disorganized distribution of ZO-1, β-catenin resided primarily in the nucleus with little or no junctional staining. In sharp contrast, treatment with dexamethasone (Dex) or dexamethasone and IPTG (Dex + IPTG) resulted in a dramatic redistribution and co-localization of ZO-1 and β-catenin to the cell junction in both the lac-4 and Id-22.5 cell lines. Both proteins exhibited an intense and uniform staining pattern in the region of the apical junctional complex in mammary cells, concurrent with a glucocorticoid growth-inhibited state (data not shown). Importantly, treatment of the Id-22.5 cells with IPTG did not affect the dexamethasone-induced translocation of ZO-1 and β-catenin to the apical junction, despite bolstering the dexamethasone induction of TER.

F-actin Organization in IPTG-inducible Id-1 Mammary Tumor Cells—The organization of the F-actin was also examined by phalloidin staining to determine the effects of dexamethasone and ectopic Id-1 expression on the cytoskeleton. As shown in Fig. 4, F-actin staining was irregularly arranged with diffuse cytoplasmic and junctional distribution in both lac-4 and Id-22.5 cells untreated (Unt) or treated with IPTG (IPTG). In the presence of dexamethasone alone (Dex) or in combination with IPTG (Dex + IPTG), the actin cytoskeleton reorganized into a network of continuous circumferential belts along the apical junction of each cell. Taken together, these results suggest that Id-1 functions downstream of the dexamethasone regulation of the actin cytoskeleton in rat mammary tumor cells.

Morphology and ZO-1/β-Catenin Co-localization in Nonconfluent SCp2-derived Mammary Epithelial Cell Lines—Previous reports have described Id-1 as an inhibitor of mammary differentiation in the SCp2 mammary epithelial cells (19). In subconfluent cell cultures, ectopic overexpression of Id-1 in the SCp2 cells inhibited the hormonal induction of β-casein mRNA but did not prevent the growth-suppressive effects induced by the differentiating stimulus, glucocorticoids, prolactin, insulin, and Englebreth Holm Swarm tumor extracellular matrix (EHS-ECM). Although the cells that overexpressed Id-1 grew in a dispersed manner, these cells still maintained their epithelial characteristics by expressing keratin and forming three-dimensional alveolar structures in response to the lactogenic hormones and EHS-ECM. Because the formation of tight junctions is another aspect of mammary differentiation (53), we explored to what extent the constitutive overexpression or reduction by antisense transfection of Id-1 alters the glucocorti-
Glucocorticoid-regulated Id-1 and Tight Junctions

Constitutive Overexpression of Id-1 in SCp2 Cells Enhances the Glucocorticoid Stimulation of Tight Junction Sealing—Although junctional localization of ZO-1 has been correlated with well developed tight junctions, the tight junction can only be functionally assessed under confluent conditions, in which the capacity of an epithelial monolayer to form an impermeable barrier is measured by its ability to resist electrical pulses passed across the epithelium. High density conditions also favor differentiation by forming stable and mature cell contacts. Moreover, by not allowing available free surface for cell migration, cell confluence has been implicated in promoting growth inhibition through cellular signals transduced by E-cadherin that ultimately regulates cell cycle events (54). We therefore investigated the effects of confluence on cell morphology and tight junction sealing in the SCp2-derived cell lines and examined whether glucocorticoids can enhance tight junction formation in the SCp2 cells as it does in the Con8 mammary tumor cells. To test this directly, control SCp2, SCp2-Id-S, and SCp2-Id-A cell lines were plated at confluence on filter inserts and grown in serum-free media in the presence or absence of dexamethasone for 5 days (Fig. 6A). TER measurements were taken daily throughout the time course. At the end of the time course, the control SCp2 cells responded to dexamethasone treatment with a 2-fold induction of TER (Fig. 6A, SCp2 + Dex versus SCp2 – Dex), whereas the SCp2-Id-S cells, which ectopically express full-length Id-1, exhibited a 4-fold dexamethasone induction of TER (Fig. 6A, SCp2-Id-S + Dex versus SCp2-Id-S – Dex). In contrast, the TER of untreated SCp2-Id-A cells, which express Id-1 antisense sequences, was consistently lower than that of the control SCp2 and SCp2-Id-S and was not affected by the addition of dexamethasone (Fig. 6A, SCp2-Id-A ± Dex). These data are consistent with our observations in the Con8 cells expressing IPTG-inducible Id-1 (Fig. 2B), supporting the model that ectopically expressed Id-1 can help promote glucocorticoid-stimulated tight junction sealing and that Id-1 expression is required for the glucocorticoid-regulated stimulation of tight junction formation.

As shown in Fig. 6B, confluent cultures of the control, SCp2-Id-S, and SCp2-Id-A cells all exhibited typical cuboidal morphology, characterized by a cohesive and cobblestone monolayer. In addition, treatment with dexamethasone did not overtly affect the epithelial phenotype of any of the confluent SCp2 cell lines. Expression of Id-1 protein was subsequently assessed in these untreated (−Dex) or dexamethasone-treated (+Dex) cells (Fig. 6C). Similar to previous observation (19), the SCp2-Id-S cells expressed high levels of Id-1, while the SCp2-Id-A cells expressed lower levels of Id-1 protein compared with the parental SCp2 cells. Moreover, a small increase of Id-1 was also observed in the SCp2 cells in response to dexamethasone, although the expression was considerably lower than that of the SCp2-Id-S cells. These results indicate that the fibroblastic-like morphology displayed by the SCp2-Id-S cells, due to the overexpression of Id-1 under nonconfluent conditions, is not observed in confluent cultures.

Although all three cell lines are capable of undergoing growth arrest in response to the lactogenic hormones and EHS-ECM (19), it is not known whether one signal or a combination of the differentiating signals can elicit the growth suppression response alone. Our previous studies have shown that glucocorticoids are potent growth-inhibiting agents in tumorigenic and nontumorigenic mammary epithelial cells (46, 52, 55, 56). We therefore tested whether glucocorticoids can inhibit the growth of all three of the SCp2-derived cell lines. As judged by [3H]thymidine incorporation, the SCp2-Id-A cells were growth-inhib-
ited by 43% by 12 h of dexamethasone treatment, whereas the control SCp2 and SCp2-Id-S cells were growth-inhibited by 18 and 22%, respectively (Fig. 6D). By 36 h of dexamethasone treatment, the three cell lines tested exhibited 40–50% dexamethasone-dependent growth suppression, demonstrating that, regardless of the amount of Id-1 protein present in each cell line, glucocorticoids are still capable of inhibiting DNA synthesis.

ZO-1 and β-Catenin Distribution in Dexamethasone-treated SCp2-derived Cells—We next examined whether changes in tight junction sealing induced by glucocorticoids in the SCp2 cells correlated with alterations in the localization of the tight junction protein, ZO-1. In the control SCp2 cells, ZO-1 antibodies revealed staining along the cell-cell boundaries in untreated and dexamethasone-treated samples (Fig. 7A). Similar to the control cells, SCp2-Id-S cells also exhibited jagged circumferential ZO-1 organization in the absence of dexamethasone, while staining in dexamethasone-treated cells revealed sharp and continuous localization of ZO-1 at the cell junction. Consistent with the TER data showing a greater dexamethasone induction in the SCp2-Id-S cells than in the control SCp2 cells, the continuity of ZO-1 staining was more prominent in dexamethasone-treated SCp2-Id-S cells than in the control cells. Immunostaining of ZO-1 in the SCp2-Id-A cells showed ZO-1 distribution at the cell-cell boundaries and the cytoplasm. These cells featured scant and discontinuous ZO-1 staining, which, unlike the Con8 cells, was not dramatically affected by dexamethasone treatment. These results indicate that the ability of the SCp2-Id-A cells to form an electrically tight seal is hindered by the lack of continuous localization of ZO-1 at the cell junction. Moreover, constitutive overexpression of Id-1 appears to prime the SCp2 mammary cells for a heightened activation of dexamethasone-dependent tight junction that is corroborated by an enhanced definition of junctional ZO-1.

In all three SCp2-derived cell lines, β-catenin was mainly restricted to the plasma membrane when cultured at confluency (Fig. 7B). Control SCp2 and SCp2-Id-S cells exhibited prominent β-catenin staining at the cell junction, which became more defined in the SCp2-Id-S cells in the presence of dexamethasone. Similar to E-cadherin localization in the SCp2-Id-A cells, β-catenin was distributed in a discontinuous

**Fig. 6.** Overexpression of Id-1 enhances the glucocorticoid stimulation of transepithelial electrical resistance in confluent SCp2-derived cell lines. A, filter-grown SCp2, SCp2-Id-S, and SCp2-Id-A cells were serum-starved for 1 day and cultured in the absence (−) or presence (+) of 1 μM dexamethasone for 5 days. Serum-free medium was changed and TER was measured daily. The experiment was performed in triplicates, and the results are representative of three separate experiments. B, confluent cultures of filter-grown SCp2 cells transfected with Id-1 sense (SCp2-Id-S) or Id-1 antisense (SCp2-Id-A) vectors were grown in the absence or presence of 1 μM dexamethasone for 36 h. The cells were fixed, and the morphology was analyzed by phase-contrast microscopy. C, Id-1 protein expression was analyzed in a parallel set of cultures by Western blot. D, confluent SCp2-derived cells were cultured in the absence (−) or presence (+) of dexamethasone for 12, 24, and 36 h. DNA synthesis was determined by incorporation of [3H]thymidine, and the results are shown as the percentage by which dexamethasone inhibits DNA synthesis.
manner along the cell-cell boundaries.

**Co-staining of E-cadherin and F-actin in Dexamethasone-treated SCp2-derived Cell Lines**—Organization of the actin cytoskeleton into thick cortical actin bundles at the adherens junction has been proposed to coordinate the physiological changes in tight junction plasticity. Co-immunostaining of E-cadherin and F-actin was analyzed in confluent monolayers treated in the absence or presence of dexamethasone (Fig. 8). In the absence of glucocorticoids, actin can be observed at the junctional belt in control SCp2 and Id-1-expressing SCp2-Id-S mammary cells. In response to dexamethasone, F-actin organized into an intensely stained cortical belt at the apical region of the junctional complex in the control SCp2 and SCp2-Id-S cells. Stress fibers were more apparent in the SCp2-Id-S cells than either of its counterparts and disappeared upon dexamethasone treatment. E-cadherin distribution also became more enriched along the cell-cell boundaries in the control SCp2 and SCp2-Id-S cells, closely mirroring F-actin organization. Although relatively minor differences are observed in actin and E-cadherin staining between glucocorticoid-treated control SCp2 cells and SCp2-Id-S cells, the pronounced TER differences are probably due to the variations in ZO-1 localization (shown in Fig. 7). In contrast, E-cadherin and F-actin staining was chaotically distributed in the SCp2-Id-A cells, which express the Id-1 antisense sequences. F-actin accumulated in aggregates with some peripheral staining in response to dexamethasone. E-cadherin was distributed diffusely within the cytoplasm and intermittently at cell-cell contact points, which became more apparent with dexamethasone treatment.

**Glucocorticoid and Id-1 Regulation of E-cadherin Protein Levels and Interaction with β-Catenin in the SCp2-Derived Cell Lines**—Acquisition of epithelial differentiation has been correlated with alterations in the expression of adherens junction or tight junction proteins. Our previous studies in mammary epithelial cell lines have shown that the enhanced cell-cell interactions induced by glucocorticoids are associated with either an up-regulation of ZO-1 or a down-regulation of the actin-bundling protein, fascin, in the 31EG4 and Con8 cell lines, respectively (47, 57). Although neither ZO-1, occludin, β-catenin, nor fascin is regulated by 5 days of dexamethasone treatment in any of the SCp2-derived cell lines (Fig. 9A), the ratio of E-cadherin/occludin increased in both the control SCp2 and SCp2-Id-S cells by 30 and 16%, respectively (Fig. 9B). Although this increase is modest, this reproducible result suggests a functional relationship between E-cadherin levels and tight junction formation in mammary epithelial cells that express Id-1. In contrast, an alteration in E-cadherin expression was not detected in the SCp2-Id-A cells in response to dexamethasone when normalized to occludin. Comparison of E-cadherin in the untreated samples of the three cell lines also shows that the SCp2-Id-S cells exhibited higher amounts of E-cadherin than either the control or SCp2-Id-A cells (Fig. 9B). ZO-1 and β-catenin protein content was also less in the SCp2-Id-A cells compared with the control or SCp2-Id-S cells, while occludin and fascin levels remained unchanged. Collectively, these data suggest that the stimulation of adhesive and/or tight junction structures by glucocorticoids may be provoked by alterations of junctional proteins, such as E-cadherin in the SCp2 cells. Furthermore, reduced expression of Id-1 in the SCp2-Id-A cells resulted in dedifferentiation of the mammary epithelial cells, which prevented the regulation of E-cadherin by glucocorticoid stimulation.

Although the SCp2-Id-A cells, which express Id-1 antisense sequences, exhibited a disorganized apical junction that was less responsive to glucocorticoids, these cells were still able to assemble into compacted colonies, which is an indication of strong adhesive function. Conceivably, the adhesion complex between E-cadherin and β-catenin may still be intact despite their disordered distribution. To this end, normalized amounts of immunoprecipitated E-cadherin were analyzed from the three SCp2-derived cell lines to detect differences in the E-cadherin and β-catenin interaction. Shown in Fig. 9C, approximately equal amounts of β-catenin were detected in the E-cadherin immunoprecipitates in all three cell lines, regardless of whether the cells were treated with dexamethasone. Taken together, these results indicate that antisense reduction of Id-1 disrupted the E-cadherin-cytoskeletal organization at the adherens junction, without altering its assembly or adhesive function. Moreover, proper cytoskeletal structures present at the cell junction are essential for the glucocorticoid induction of tight junction organization and function, suggesting that the Id-1 protein plays a key role downstream of glucocorticoid signaling that targets adhesive and permeability properties of mammary epithelial cells.

**DISCUSSION**

Regulation of epithelial cell-cell interactions is a fundamental process that is essential for normal tissue development, yet the mechanisms that control this process are not clearly understood. We have used mammary epithelial cells as a system to study the regulation of junctional plasticity. Mammary cells are able to dynamically modulate intercellular interactions during normal mammary gland development, pregnancy, lac-
Glucocorticoid-regulated Id-1 and Tight Junctions

Fig. 8. Co-staining of E-cadherin and F-actin in SCP2-derived cell lines. Filter-grown SCP2, SCP2-Id-S, and SCP2-Id-A cells were serum-starved for 1 day and cultured in the absence (−) or presence (+) of 1 μM dexamethasone for 5 days. At the end of the 5-day treatment, the cells were fixed, permeabilized, and incubated with monoclonal E-cadherin (E-cad) antibodies and Texas Red-conjugated phalloidin.

Fig. 9. Glucocorticoids and Id-1 regulate E-cadherin protein levels but not its interaction with β-catenin. A, SCP2, SCP2-Id-S, and SCP2-Id-A cells were serum-starved for 1 day and cultured in the absence (−) or presence (+) of 1 μM dexamethasone for 5 days. Western blots of total cell extracts were probed with antibodies directed against ZO-1, occludin, E-cadherin, β-catenin, and fascin. B, the band intensities of E-cadherin and occludin protein for each condition were quantitated, and E-cadherin protein levels were normalized to occludin protein levels by dividing the band intensities of E-cadherin by the band intensity for occludin. C, a parallel set of cultures was immunoprecipitated using monoclonal E-cadherin antibodies. The immunoprecipitates were normalized for E-cadherin protein and analyzed for the presence of β-catenin by Western blot analysis.

Between mammary epithelial cells (46, 58). Studies in dedifferentiated rat hepatoma cells have also shown that the glucocorticoid induction of the epithelial phenotype, characterized by tight junction formation, involves the cooperativity of the glucocorticoid receptor and hepatocyte nuclear factor-4 (59). Given the known transcriptional mechanism of the glucocorticoid receptor, it is likely that one or more glucocorticoid-regulated genes are responsible for its effects on cell-cell interactions in mammary cells. In this study, we provide evidence that the Id-1 helix-loop-helix protein is a potential regulator of transcription that mediates the glucocorticoid-dependent stimulation of tight junction sealing.

Through our work, as well as the work of others, it is well established that the regulation of cell-cell interactions occurs at multiple levels and by diverse sets of signal transduction pathways (2, 5, 46–49, 52, 57, 58, 60). We have demonstrated that the helix-loop-helix protein, Id-1, accumulates within 4 h after treatment of serum-starved Con8 mammary epithelial tumor cells with dexamethasone and that ectopic production of Id-1 facilitates the ability of glucocorticoids to induce a highly sealed tight junction. The expression of Id proteins has been shown to be stimulated by several extracellular growth factors and mitogens, including serum, and serves a general function to activate cell proliferation and apoptosis, while inhibiting differentiation in diverse cell types (22, 23). Consistent with the involvement of Id-1 in regulating tight junction dynamics, we have observed that the dexamethasone stimulation of tight junction sealing is enhanced in the presence of serum.

At a molecular level, Id proteins function as dominant-negative antagonists of basic helix-loop-helix transcription factors through protein-protein interaction, mediated by the helix-loop-helix region. The formation of the inactive protein complex would presumably prevent the transcription of genes thought to negatively regulate growth (61) or activate differentiation. We now show that Id-1 protein levels significantly rise prior to the onset of the glucocorticoid induction of tight junction formation that occurs after 24 h of dexamethasone treatment. At later time points, the glucocorticoid up-regulation of Id-1 protein over basal levels is no longer observed, in part due to a significant increase in basal Id-1 between 24 and 48 h. Given the 24-h time lag required to detect the glucocorticoid increase in TER formation (46, 47, 52), it is conceivable that the activation and coordination of early and late glucocorticoid-regulated events are necessary to induce the tight junction sealing response. Interestingly, id genes have been reported to increase during preadipocyte differentiation by 24 h and decline by 8 days of hormone treatment when maturation of adipose cells is complete (43). In this system, dexamethasone has been implicated in the early induction of the id genes, prior to differentiation, which occurs over a period of 4–7 days (62, 63). After 24 h, when the glucocorticoid stimulation of tight junction formation was most obvious, Id-1 levels were unchanged over

2 P. L. Woo and G. L. Firestone, unpublished result.
the high basal levels in Con8 mammary epithelial cells, suggesting that Id-1 acts early in this process and that additional regulation occurs at later time points to maintain tight junction sealing. In fact, our previous studies have shown that the down-regulation of the actin-bundling protein, fascin, is a late but essential glucocorticoid-regulated event that controls cell-cell interactions (47). Fascin protein decreases after at least 24 h of dexamethasone treatment. Therefore, we propose that the glucocorticoid-stimulation of Id-1 is an early event that would serve to ensure the proper initiation and completion of downstream events involved in the glucocorticoid induction of proper cell-cell interactions.

The functional consequence of Id-1 on tight junction formation was investigated using both tumorigenic and nontumorigenic rodent mammary epithelial cell lines. We have utilized the lac operator-repressor system to regulate the expression of the mouse Id-1 cDNA by the addition of IPTG in the Con8 rat mammary tumor cells. Clones, which expressed Id-1 in response to IPTG, exhibited an enhanced potential to establish a tight electrical seal in response to dexamethasone, directly correlating ectopic Id-1 expression to the glucocorticoid-induced transepithelial electrical resistance. However, the conditionally expressed Id-1 alone did not mimic the glucocorticoid stimulation of tight junctions, suggesting that Id-1 is not sufficient for, but supportive of, signaling to the tight junction. Moreover, the enhanced effects of Id-1 on transepithelial electrical resistance did not correlate with changes in the glucocorticoid-induced tight junction and adherens junction organization, suggesting that Id-1-regulated events target the tight junction after the glucocorticoid-stimulated junctional rearrangement in Con8 cells. ZO-1 and F-actin were reorganized to the cell periphery in response to dexamethasone, regardless of whether Id-1 was ectopically overexpressed. In addition, the glucocorticoid-induced recruitment of β-catenin from a nuclear location to the plasma membrane, in the vicinity of ZO-1, was also unaffected by inducible Id-1. Nuclear β-catenin has been associated with undifferentiated and exponentially growing cells (64). In the case of the Con8 rat mammary tumor cells, nuclear β-catenin most likely contributes to the undifferentiated profile, since these cells were cultured in the absence of serum and under confluent conditions for over 5 days. It appears that the capacity of glucocorticoids to induce a more differentiated profile with regard to ZO-1, β-catenin, and actin cytoskeletal organization is sufficient alone, whereas Id-1 would act to maintain a higher degree of tight junction sealing, rather than directly stimulate tight junction formation.

Id-1 has been previously described as an inhibitor of mammary differentiation in the SCp2 mammary epithelial cell line (19). Stable overexpression of Id-1 prevented the hormonally regulated production of the milk protein, β-casein, whereas diminished Id-1 expression in an antisense-transfected population of cells did not affect β-casein expression. Consistent with these results, the morphology of SCp2 cells overexpressing Id-1 (SCP2-Id-S) appeared more fibroblastic than the control SCp2 cells or the Id-1 antisense cell population (SCP2-Id-A) under low density conditions. On the other hand, at confluent conditions, differences in the morphology of the SCP2-derived cell lines were less obvious. In fact, even under nonconfluent conditions, the SCP2-Id-S appeared to form tight junctions and adherens junctions, as evident by the presence of ZO-1 and β-catenin in regions where neighboring cells physically interacted. Maturation of the cell junction has been reported to affect the responsiveness of signaling pathways that control junction formation. For instance, inhibition of the Rac and Rho GTPases removes E-cadherin from keratinocyte cell-cell contacts in newly formed junctions but not in mature junctions (65). We have also documented that the ability of TGF-β to disrupt junction organization in a nontransformed mammary epithelial cell line is dependent on the maturation status of the glucocorticoid-stimulated tight junction (52). Considering the growing number of signaling molecules residing at cell-cell junctions (66–70), it is conceivable that glucocorticoids, as well as confluency, can induce stable cell-cell contacts and polarity that is necessary for the specificity and precision of spatially restricted signaling cascades.

The SCp2-Id-S cells, which overexpress Id-1 protein, were growth-inhibited by glucocorticoids and formed more developed tight junctions than the parental cells or the SCp2-Id-A cells in which Id-1 production was ablated. The enhanced function of the tight junction correlated with a more defined and continuous localization of ZO-1 at the cell junction, with less obvious differences in E-cadherin and β-catenin distribution. The SCp2-Id-S cells are capable of forming three-dimensional alveolar structures in response to EHS-ECM and lactogenic hormones, but these alveoli appeared to lose their adhesive property and became dispersed after longer periods (19). We utilized dexamethasone, a potent glucocorticoid, to induce tight junction formation on cells cultured on filter inserts, whereas hydrocortisone, insulin, prolactin, and EHS-ECM were required to induce the alveolar structures in the three-dimensional cell cultures, which may attenuate the stability of the cell-cell interactions in this cell system. In addition, SCp2 cells that overexpressed Id-1 secreted a 120-kDa gelatinase that contributed to the instability of its three-dimensional structure and invasive behavior in the surrounding matrix (71). In this regard, a connection between ECM signaling through integrins and the formation of cell-cell junctions has been established in mammary epithelial cells (72). The nature of the ECM also reconciles the opposing effects of the Rac GTPase, determining whether it would promote E-cadherin-mediated cell-cell adhesion or induce migration in epithelial cells (73). Our data demonstrate that E-cadherin accumulated in response to dexamethasone in the SCp2 and SCp2-Id-S cells. E-cadherin plays a key role in the initial formation of cell-cell contacts and linkage of E-cadherin to the cortical actin cytoskeleton. It is likely that higher levels of E-cadherin are required to maintain more stable adhesive contacts, since glucocorticoids also induced the assembly of the cortical actin belt in the control SCp2 and SCp2-Id-S cells.

Taken together, our results implicate the transcriptional regulator Id-1 as an important element in the glucocorticoid-activated cell signaling pathway that controls tight junction dynamics in mammary epithelial cells. At an early stage in this program, Id-1 establishes a competent state for mammary epithelial cells to form normal cell-cell contacts, presumably by altering the expression of a specific set of gene products involved in the later steps in the steroid-induced cascade. In addition, the cellular context and the status of junctional maturation may determine the precise role of Id-1 in controlling mammary cell-cell interaction as well as affecting the growth and differentiated properties. Further characterization of the downstream targets of Id-1 may provide important insights into the hormonal control of the epithelial junctional complexity.

Acknowledgments—We thank Anita Maiyar and Vivian Wong for helpful suggestions during the course of this work. We also thank Minnie Wu and Hal Light for technical assistance.

REFERENCES

1. Matter, K., and Balda, M. S. (1999) Int. Rev. Cytol. 186, 117–146
2. Stevenson, B. R., and Keon, B. H. (1998) Ann. Rev. Cell Dev. Biol. 14, 89–109
3. Mito, L. L., and Anderson, J. M. (1998) Annu. Rev. Physiol. 60, 143–159
4. Madara, J. L. (1998) Annu. Rev. Physiol. 60, 143–159
5. Gumbiner, B. M. (1996) Cell 84, 345–357
6. Ozawa, M., Baribault, H., and Kemler, R. (1989) EMBO J. 8, 1711–1717
7. Fanning, A. S., Jameson, B. J., Jessazia, L. A., and Anderson, J. M. (1998)
