Glucocorticoid Down-regulation of Fascin Protein Expression Is Required for the Steroid-induced Formation of Tight Junctions and Cell-Cell Interactions in Rat Mammary Epithelial Tumor Cells*

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Vivian Wong†, Dixie Ching‡, Pierre D. McCrea§, and Gary L. Firestone¶

From the †Department of Molecular and Cell Biology and the Cancer Research Laboratory, University of California, Berkeley, California 94720-3200 and the §Department of Biochemistry and Molecular Biology, University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030-4095

Glucocorticoid hormones, which are physiological regulators of mammary epithelium development, induce the formation of tight junctions in rat Con8 mammary epithelial tumor cells. We have discovered that, as part of this process, the synthetic glucocorticoid dexamethasone strongly and reversibly down-regulated the expression of fascin, an actin-bundling protein that also interacts with the adherens junction component b-catenin. Ectopic constitutive expression of full-length mouse fascin containing a Myc epitope tag (Myc-fascin) in Con8 cells inhibited the dexamethasone stimulation of transepithelial electrical resistance, disrupted the induced localization of the tight junction protein occludin and the adherens junction protein b-catenin to the cell periphery, and prevented the rearrangement of the actin cytoskeleton. Ectopic expression of either the carboxyl-terminal 213 amino acids of fascin, which includes the actin and b-catenin-binding sites, or the amino-terminal 313 amino acids of fascin failed to disrupt the glucocorticoid induction of tight junction formation. Mammary tumor cells expressing the full-length Myc-fascin remained generally glucocorticoid responsive and displayed no changes in the levels or protein-protein interactions of junctional proteins or the amount of cytoskeletal associated actin filaments. However, a cell aggregation assay demonstrated that the expression of Myc-fascin abrogated the dexamethasone induction of cell-cell adhesion. Our results implicate the down-regulation of fascin as a key intermediate step that directly links glucocorticoid receptor signaling to the coordinate control of junctional complex formation and cell-cell interactions in mammary tumor epithelial cells.

The apical junctional complex of epithelial cells is a specialized set of cell-cell contacts consisting of the tight junction and the adherens junction (1). The more apically located tight junction serves as a selective barrier to the paracellular diffusion of solutes and thereby separates the apical and basolateral fluid compartments of the epithelial cell monolayer (2–4). By limiting the lateral movement of plasma membrane lipids and proteins, the tight junction also contributes to the maintenance of cellular polarity, physically defines the border between the apical and basolateral plasma membrane surfaces (2–4), and is essential for the polarized functions of differentiated epithelial cells such as secretion, absorption, and responsiveness to extracellular signals. At the molecular level, the tight junction is formed by the interaction between extracellular domains of the transmembrane protein occludin from neighboring cells (5–8) and an intracellular protein complex that includes occludin and the cytoplasmic proteins ZO-1 (9), ZO-2 (10, 11), cingulin (12, 13), 7H6 antigen (12–15), symplekin (16), and rali3 (17). The adherens junction, which lies immediately below the tight junction toward the basal side of the cell (1), is formed by the calcium-dependent, homophilic binding of E-cadherin extracellular domains from adjacent cells (18, 19). The adherens junction is responsible for intercellular adhesive function (20), is a requirement for the generation of epithelial cell polarity by tight junctions (21–24), and plays a key role in regulating cell-cell interactions involved in tissue morphogenesis and differentiation (25). The E-cadherin-mediated adherens junction formation is performed by the specific association of a number of cytoplasmic proteins with the cytoplasmic tail of E-cadherin (26, 27); these include a-catenin (28, 29), b-catenin (30), plakoglobin (31–33), and p120CTN (34–36). Other cytoplasmic molecular components of the adherens junction include actin, a-actinin (37), vinculin (38), radixin (39), tennin (40), Src (41), and fascin (42).

The tight junction and the adherens junction structures are highly dynamic in that their formation, assembly, and/or disassembly can be controlled by a variety of intracellular signals that ultimately influence cell-cell interactions in a physiologically appropriate manner (43–48). For example, the permeability properties of the tight junction and/or adhesive properties of the adherens junction can be altered by intracellular calcium, protein kinase C activity, and certain lipid-mediated cell signaling cascades (43, 49). The Rac and Rho members of the Ras superfamily of small GTPases appear to provide another common regulatory connection between tight junctions and adherens junctions because these signaling molecules can control intercellular adhesion, permeability, and apical junction assembly (50–53). The actin cytoskeleton, which forms the characteristic perijunctional ring underlying the adherens junction (54) and has been shown to associate with the tight junction (55), represents a structural link between the tight junction and the adherens junction. One potential molecular connection between the actin cytoskeleton and the apical junction may be through the fascin actin-bundling protein (56, 57) which also binds b-catenin to form a biochemically distinct non-cadherin complex (42), although a functional role for fascin in the hormonal control of apical junction dynamics has not been characterized.

Mammary-derived cells represent a biologically useful system to explore hormone-activated signaling pathways that po-
tentially influence and mediate cell-cell interactions. We have established that glucocorticoids can induce tight junction formation in a receptor-dependent manner in both nontumorigenic and tumorigenic rodent mammary epithelial cells (58–62), which implicates this lactogenic steroid (63, 64) as a key physiological signal for the increased junctional complex formation and mammary intercellular contacts that occur during the onset of lactation (65). In the rat Con8 mammary tumor epithelial cell line, which was derived from a 7,12-dimethylbenz(a)anthracene-induced rat mammary adenocarcinoma (66, 67), glucocorticoids induce the remodeling of the apical junction and a polarized phenotype that results in the localization of the ZO-1 tight junction protein from the cytoplasm to the cell periphery at the site of cell-cell contacts. This regulated response induces the barrier function of the tight junction (60, 61), which decreases paracellular permeability and stimulates the transepithelial electrical resistance (TER).1 Given the known transcriptional mechanism of action of glucocorticoid receptors (68–73), we propose that the regulated expression of a specific set of glucocorticoid responsive gene products act as molecular switches and/or structural components that functionally control the formation of intercellular junctions to induce an epithelial-like phenotype. Although, in general, relatively little is known about the regulated expression and/or activity of apical junction-associated structural or regulatory proteins by extracellular stimuli. In this study, we demonstrate that the glucocorticoid down-regulation of fascin protein expression is a critical event for this steroid to induce the organization of the apical junctional complex which provides, for the first time, a direct functional link between a steroid regulated gene and the control of cell-cell interactions.

**EXPERIMENTAL PROCEDURES**

**Cell Culture. Glucocorticoid Treatment, and Measurement of Transepithelial Electrical Resistance—Con8 rat mammary epithelial cells** (61) were grown on standard tissue culture plates or Anopore membrane filters (0.2 μm, Nalgene Nunc International, Naperville, IL) in Dulbecco’s modified Eagle’s medium/F-12 supplemented with 10% calf serum and penicillin/streptomycin (BioWhittaker, Walkersville, MD), Dulbecco’s modified Eagle’s medium/F-12 supplemented with 10% calf serum and penicillin/streptomycin (BioWhittaker, Walkersville, MD), or RPMI-1640 medium (2X) (Life Technologies Inc., Gaithersburg, MD) containing 5% dialyzed fetal bovine serum (Atlanta Biologicals Inc., Atlanta, GA). The media, each cell pellet was detergent extracted by suspension in 1 ml of lysis buffer (0.5% Triton X-100, 20 mM PIPES, pH 6.8, 2 mM EDTA, 15% sucrose, 2% SDS, and 50 mM dithiothreitol) containing protease inhibitors (5 mM phenylmethylsulfonyl fluoride, 5 μg/ml pepstatin, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 50 μg/ml antipain, 2 μM benzamidine, 50 μg/ml soybean trypsin inhibitor, and 2.5 mM iodoacetamide). Samples were boiled for 15 min and cooled to room temperature before the addition of 1 mM iodoacetamide and 125 mM SDS. SDS-PAGE was performed using the Mini-protein II cell electrophoresis unit (Bio-Rad) according to the manufacturer’s guidelines. After electrophoresis, proteins were electrophoretically transferred to nitrocellulose membranes (Micron Separations Inc., Westboro, MA) using the Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) according to manufacturer’s guidelines. The blots were incubated with blotting buffer (5% non-fat dry milk, 0.15 M NaCl, 0.2% Triton X-100) before probing with a 1:100 dilution of primary antibodies (anti-occludin, anti-β-catenin, anti-E-cadherin, anti-α-catenin, anti-fascin, or anti-e-Myc epitope). The mouse monoclonal anti-fascin antibody (gift of DAKO Corp., Carpenteria, CA) was obtained as a crude supernatant of hybridoma media and was not diluted prior to use. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies (Bio-Rad) were used as secondary probes, and the blots were developed by an ECL kit (Amersham Life Sciences, Inc.).

**Immunoprecipitation of Junctional Complex Proteins**—For the examination of co-immunoprecipitated proteins, each 100-mm2 plate of confluent Con8 cells was extracted in 5 ml of 1% Tween 20 buffer (1% Tween 20, 0.15 M NaCl, 5 mM EDTA, 20 mM HEPES, pH 7.4) in the presence of protease inhibitors (5 mM phenylmethylsulfonyl fluoride, 5 μg/ml pepstatin, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 50 μg/ml antipain, 2 μM benzamidine, 50 μg/ml soybean trypsin inhibitor, and 2.5 mM iodoacetamide). Extraction was carried out on ice for 30 min and all samples were preclarified by centrifugation at 12,000 × g for 15 min before immunoprecipitation. Co-immunoprecipitation was performed with Protein A-Sepharose CL-4B (Pharmacia Biotech Inc.) in the presence of rabbit anti-β-catenin or preimmune serum at 4 °C for 4 h. Immunoprecipitates were washed five times in the same immunoprecipitation buffer, followed by a final wash with phosphate-buffered saline. Co-immunoprecipitated proteins were solubilized immediately in SDS-PAGE sample buffer and prepared for SDS-PAGE as described above for Western blotting.

**Assay for Cytoskeletal-associated Actin Filaments**—The Triton X-100 extraction method (74) was used to determine the levels of F-actin and the amount of cytoskeleton-associated fascin and β-catenin. The mammary tumor cells were grown to confluency and treated with or without 1 μM dexamethasone for 96 h. The cells were then trypsinized into a single-cell suspension, counted using a hemacytometer, and then 2 million cells collected into a pellet by centrifugation. After aspirating the media, each cell pellet was detergent extracted by suspension in 1 ml of 1% Triton X-100, 20 mM HEPES, 50 mM MgCl2, 50 mM KCl, 5 mM EDTA, 5 mM dithiothreitol, 1 mM ATP, and 1 mM MgCl2 each of leupeptin, pepstatin, and aprotonin) and then immediately centrifuged at 8,700 × g in a microcentrifuge for 3 min. The resulting pellets (containing the Triton X-100-resistant proteins) were suspended in 100 μl of 20 mM phosphate buffer (14.5 mM NaH2PO4 and 5.2 mM KH2PO4, pH 7.0, and 1 μM each of leupeptin, pepstatin, and aprotonin). A parallel set of cells was centrifuged and then directly suspended in 100 μl of 20 mM phosphate buffer and represents the total cell extracts. For the Western blots, the Triton X-100-resistant proteins and total cell extracts were dissolved in an equal volume of 2 × SDS-PAGE sample buffer (described above) and the proteins electrophoretically fractionated by SDS-PAGE.

**Stable Con8 Cell Lines Expressing Full-length, NH2-terminal Portion and COOH-terminal Portion Myc-Fascin**—The full-length mouse fascin containing a terminal fragment of mouse fascin containing amino acids 281 to 493 was tagged with a 6X c-Myc epitope at its COOH terminus and the amino terminus of fascin containing amino acids 1–213 was tagged with a 6X c-Myc epitope at its NH2 terminus were cloned into the pCMV-1-tk mammalian expression vector. The fascin sequences in this expression vector are driven by the simian CMV IE94 promoter and the vector backbone of pCS2+ is pBluescript KS+ (75, 76). Plasmid DNA was expanded in XL-1 Escherichia coli cells and purified with a plasmid purification kit (Qiagen, Inc., Chatsworth, CA).

1 The abbreviations used are: TER, transepithelial electrical resistance; PAGE, polyacrylamide gel electrophoresis; SGK, serum and glucocorticoid inducible protein kinase; TLCK, Np-tosyl-l-lysine chloromethyl ketone; PIPES, 1,4-piperazinediethanesulfonic acid; CMV, cytomegalovirus.

Stained cells were mounted with SlowFadeLight Antifade reagent (Molecular Probes, Inc.) and stored at 4 °C before visualization.
The pcDNA3-fascin, pCS2 + MT NH2-terminal fascin, and the pCS2 + MT COOH-terminal fascin DNA were introduced into Con8 cells at 24 h after plating of cells at 30% confluency. One set of cells was also transfected with the pCS2 + MT vector alone. In transfections with the pCS2 + MT based vectors, the cells were co-transfected with the neomycin resistance gene driven by the CMV promoter. Transfection of cells was performed using LipofectAMINE reagent according to the manufacturer’s protocol (Life Technologies, Inc., Gaithersburg, MD). Potential expressing vectors were selected at 600 μg/ml G418 (Life Technologies, Inc.), which was cytotoxic to ~99% of parental Con8 cells. Single clones were isolated, expanded, and Myc-fascin expression was assessed by antigen blotting with either the anti-fascin or anti-Myc epitope antibodies. Multiple fascin expressing clonal cell lines derived from the parental Con8 cells were analyzed in parallel to control for clonal variation. The mammary tumor cells expressing high levels of full-length Myc-fascin are FN12 and FN36, and of the COOH-terminal portion of Myc-fascin are FC10 and FC12. The FN3, FN3.3, and FN3.2 do not express Myc-fascin although the original transfected contained the full-length Myc-fascin gene. The C1 cell lines were transfected with the pCS2 + MT expression vector without any fascin sequences.

**RESULTS**

**Time Course and Dose Response of the Glucocorticoid Down-regulation of Fascin Protein in Rat Mammary Epithelial Tumor Cells**—We have previously established that glucocorticoids stimulate tight junction formation in Con8 mammary epithelial tumor cells (61). Given the well characterized effects of glucocorticoids on gene expression (68–73), it seemed likely that this steroid induces the formation of tight junctions by altering the expression of a select subset of proteins that are associated with and that may regulate the overall junctional complex. Furthermore, the formation of intercellular junctions is intimately linked to the cytoskeleton, and we have observed that dexamethasone causes a rearrangement of Con8 mammary tumor cells from round-shaped cells that grow on top of each other to cuboidal shapes characteristic of an epithelial monolayer (60, 61). Thus, potential targets of glucocorticoid signaling may include proteins that interact with both the tight junction and the adherens junction as well as the cytoskeleton. Recently, fascin, an actin-bundling protein (56, 57), was found to interact with the adherens junction protein β-catenin (42), and therefore provided a candidate regulatory protein involved in the glucocorticoid control of junctional complex formation.

To explore this possibility, the level of fascin protein produced was compared with the kinetics of tight junction formation in Con8 mammary tumor cells treated with the synthetic glucocorticoid dexamethasone over a 4-day time course. At the indicated times in dexamethasone, the generation of TER in cell monolayers grown on Anopore membrane filters was used to monitor tight junction formation, and a parallel set of Western blots was probed for fascin protein or for the tight junction protein occludin. As shown in Fig. 1a, dexamethasone induced a significant increase in the TER that continued to rise throughout the time course. In contrast, the TER of cells not treated with glucocorticoids remained essentially unchanged. Western blots showed that total cellular fascin protein began to decrease at approximately 48 h and was markedly reduced (>80%) by 72 h of dexamethasone treatment (Fig. 1a). During this time period in which fascin levels are reduced, occludin protein levels remained unchanged. The overall time frame of induced TER and fascin protein down-regulation is consistent with a functional relationship between these processes.

As a complementary approach to confirm the correlation between the glucocorticoid down-regulation of fascin with the formation of tight junctions, the production of fascin and the induction of TER was analyzed in mammary tumor cells treated with various doses of steroid for 4 days. As shown in Fig. 1b, half-maximal down-regulation of fascin protein and the half-maximal stimulation of TER was observed at approximately 10−8 M dexamethasone, which correlates with the Kd of glucocorticoid-receptor occupancy for these cells (67). The maximal effect of dexamethasone on both fascin down-regulation and the stimulation of TER was observed at 10−7 M (Fig. 1b), with higher concentrations of dexamethasone having no additional effects (data not shown). A similar dose-dependent re-
sponse on fascin down-regulation and stimulation of TER was observed in mammary tumor cells treated for shorter durations with dexamethasone. Thus, the down-regulation of the 45-kDa fascin expressed in mammary tumor cells correlated with the formation of tight junctions in a dose- and time-dependent manner that is consistent with a glucocorticoid receptor-dependent process.

Glucocorticoid Withdrawal Reverses the Rearrangement of the Apical Junction and the Down-regulation of Fascin Protein—To test whether glucocorticoids are required for the maintenance of the organized apical junction and for the continued down-regulation of fascin, we examined the effects of dexamethasone withdrawal on the overall rearrangement of junctional proteins and on fascin protein production. Con8 mammary tumor cells were treated with dexamethasone for 6 days, at which time the TER is strongly induced. Subsequently, the culture medium was replaced with fresh medium without added glucocorticoids for 4 days and the TER returned to basal levels. The subcellular localization of the tight junction protein, occludin, and the adherens junction protein, β-catenin, were examined by indirect immunofluorescence microscopy. Dexamethasone withdrawal effectively reversed the glucocorticoid-induced rearrangement of both occludin and β-catenin (Fig. 2a, panels ± versus +). Both occludin and β-catenin redistributed from the junctional complex at the cell periphery back to an unorganized pattern indistinguishable from the untreated cells upon withdrawal of dexamethasone (Fig. 2a, panels ± versus −).

A parallel set of Con8 mammary tumor cells were examined for the protein expression of fascin, α-catenin, β-catenin, and cingulin. As shown in Fig. 2b, dexamethasone withdrawal (lower panel, lane ±) reversed the dexamethasone-mediated down-regulation of fascin protein under conditions in which the expression of tight junction and adherens junction proteins remained constant. Fascin expression was almost restored to pretreatment levels by glucocorticoid withdrawal. It is worth mentioning that the lane for ± was slightly under loaded which is noticeable on the β-catenin blot. Therefore, it appears that the glucocorticoid regulation of junctions assembly and disassembly correlated closely with the expression levels of fascin protein. In addition, the reversibility of the glucocorticoid-induced junctional proteins formation implicates this steroid as a maintenance factor rather than a terminal differentiation signal.

Ectopic Expression of Full-length Fascin Prevents the Glucocorticoid Induction of Transepithelial Electrical Resistance in Mammary Tumor Cell Monolayers—Conceivably, the down-regulation of fascin is a critical functional event in the glucocorticoid signaling pathway in that fascin may inhibit key cellular events that lead to the formation of tight junctions. To directly investigate this possibility, we tested whether the ectopic constitutive expression of fascin would prevent the dexamethasone induction of tight junction formation. Con8 mammary tumor cells were stably transfected with cDNA of full-length mouse fascin containing a Myc epitope tag at the NH² terminus (77). This fascin mammalian expression vector contains the neomycin resistance gene (Fig. 3a) and stable clonal cell lines that express different levels of Myc-fascin were obtained by selection using a lethal dosage of G418. Based on the open reading frame, the Myc-fascin cDNA is expected to encode an approximate 65-kDa protein, taking into account the 10-kDa increase in molecular weight due to the Myc epitope. However, during our initial screening for expressing clones, we consistently observed that the Con8 mammary tumor cells expressed Myc-fascin as an 80-kDa protein species. Fig. 3a shows a Western blot characterizing the ectopically expressed Myc-fascin from one of the transfected cell lines (f3.1). Cell extracts from f3.1 cells were electrophoretically fractionated and Western blots were probed with c-Myc monoclonal antibodies, fascin polyclonal antibodies, or fascin monoclonal antibodies. As a control, a Western blot of a cell extract isolated from non-transfected Con8 cells was also incubated with c-Myc monoclonal antibodies. As shown in Fig. 3a, the c-Myc antibody recognized an 80-kDa protein that is not present in the non-transfected Con8 cells. Similarly migrating forms of Myc-fascin were detected in the f3.1 cells using the polyclonal or monoclonal fascin antibodies (Fig. 3a). Endogenous fascin is not apparent in the blots because of the short time of exposure to the chemiluminescence reagents before development of the x-ray film. Taken together, these results verify that the 80-kDa protein observed in transfected Con8 cells is the ectopically expressed, Myc-tagged mouse fascin. In this regard, transfection of this same Myc-fascin expression vector, or a fascin expression vector with a COOH-terminal Myc epitope, also produced an 80-kDa protein species in a human tumor cell line.2

Several individual single cell-derived subclones of transfected Con8 cells were screened for expression of Myc-fascin and for the ability of glucocorticoids to induce tight junction formation as assessed by the development of TER. In the three clonal transfected cell lines that express high levels of Myc-fascin (f3.1, f3.8, and f3.11), dexamethasone failed to stimulate the monolayer TER under conditions in which the level of ectopically expressed fascin remained constant (Fig. 3b, upper panel versus middle panel). In contrast, in three recovered transfected cell lines that express little or no Myc-fascin (f3.4, f3.3, and f3.2), the TER was glucocorticoid inducible indicat-

2 P. McCrea, unpublished observation.
amino-terminal Myc epitope tag linked to the pcDNA3.1 expression vector. Western blots of electrophoretically fractionated cell extracts from nontransfected Con8 cells or a Myc-fascin transfected Con8 subclone denoted f/3.1 were probed with anti-Myc primary antibodies (myc). Parallel Western blots of the f/3.1 cell extracts were also probed with polyclonal (poly) or monoclonal (mono) antibodies to mouse fascin. b, individual subclones of transfected Con8 mammary tumor cells were grown on Anopore membrane filter supports and treated with or without 1 μM dexamethasone for 4 days. The f/3.1, f/3.8, and f/3.11 subclones produce varying levels of constitutively expressed Myc-fascin, whereas, the f/3.4, f/3.3, and f/3.2 subclones do not produce Myc-fascin and represent transfection controls. The monolayer TER was monitored in each cell culture and Western blots of parallel sets of cells were probed using Myc epitope or SGK-specific primary antibodies.

For the remainder of the experiments, the f/3.1 clone was utilized because this cell line produced the highest constitutive level of Myc-fascin. The f/3.2 clone expressed an insignificant level of Myc-fascin and therefore was employed as one of the transfection control cells for comparison to the f/3.1 clone. As discussed below, the C1 cell clone represents the other transfection control cell line used in certain experiments. As shown in Fig. 4, ectopic expression of Myc-fascin in the f/3.1 clone inhibited the dexamethasone-stimulated TER over a 4-day time course, whereas the TER was rapidly stimulated in f/3.2 cells in a manner similar to that of parental Con8 mammary tumor cells (see Fig. 1a). Indirect immunofluorescence microscopy using Myc antibodies showed the localization of Myc-fascin throughout the cells in both 4-day dexamethasone-treated and untreated f/3.1 cells (Fig. 4). In contrast, Myc-fascin was not detected by immunofluorescence staining in either untreated (Fig. 4) or dexamethasone-treated (data not shown) f/3.2 cells. Taken together, these results demonstrated that ectopic expression of the full-length fascin gene inhibited the glucocorticoid-stimulated formation of tight junctions and further implicates the down-regulation of fascin as an essential intermediate step in the glucocorticoid signaling pathway that controls the junctional complex in mammary tumor cells.

**Ectopic Expression of Amino-terminal or Carboxyl-terminal Fragments of Fascin Fail to Prevent the Glucocorticoid Induction of Transepithelial Electrical Resistance**—Previous studies have shown that the COOH-terminal 216 amino acids of fascin (residues 277 to 493) can bind *in vitro* to actin (56, 57, 77), whereas, analysis by the yeast two-hybrid assay revealed that the fascin coding region corresponding to amino acids 324–453 functionally interact with β-catenin (42). Although the precise structural boundaries involved in each interaction have not been defined, the fascin molecule can be approximately dissected into an NH2-terminal fragment that does not bind either actin or β-catenin and a COOH-terminal fragment that binds to both actin and β-catenin. Therefore, Con8 mammary tumor cells were transfected with expression vectors that encode either the amino-terminal 313 amino acids (FN construct) or the carboxyl-terminal 213 amino acids (FC construct) of the fascin coding region that contains both the actin and the β-catenin-binding sites. These constructs overlap by 32 amino acids between residues 281 and 313 and each contain six copies of the Myc epitope tag at the amino terminus (Fig. 5a). The Con8 cells were stably transfected with each construct as well as with an empty pSC2+MT expression vector and individual cell colonies isolated from each population of G418-resistant cells. Two single cell-derived subclones of cells transfected with either the NH2-terminal fascin fragment (FN12 and FN36 cells) or the COOH-terminal fascin fragment (FC10 and FC12 cells) as well as one of the vector-transfected cell lines (C1 cells) were examined for expression of the Myc-tagged fascin domains by Western blots using the Myc antibodies and for the glucocorticoid stimulation of TER. As shown in Fig. 5a, the transfected FC10 and FC12 cell lines produced a 34-kDa Myc-tagged COOH-terminal fascin protein and the transfected FN12 and FN36 cell lines produced a 45-kDa Myc-tagged NH2-terminal fascin protein, compared with the 80-kDa full-length Myc-fascin expressed in the f/3.1 cells. The ectopically expressed COOH-terminal and NH2-terminal fascin proteins correspond to the predicted sizes based on the fascin amino acid sequence in each construct and the 11-kDa size of the Myc epitope tag.
expected, the vector-transfected control cells, C1, do not express any Myc epitope-tagged fascin proteins.

The ability of glucocorticoids to stimulate the TER was monitored in each of these transfected mammary tumor cells after 96 h treatment with or without 1 μM dexamethasone (Dex), and the monolayer TER was measured at the indicated days in culture. Lower panel, the localization of Myc epitope-fascin protein was examined in f/3.1 and f/3.2 cells treated with or without 1 μM dexamethasone for 4 days by indirect immunofluorescence microscopy using Myc epitope antibodies. The scale bar represents 25 μm.

**Fig. 5.** Stable ectopic expression of amino-terminal and carboxyl-terminal Myc-fascin fragments and effects on the transepithelial electrical resistance. a, the FN expression construct encodes the NH₂-terminal 313 amino acids of fascin with a 6X Myc-epitope tag on the amino terminus and driven by the CMV promoter. The FC expression construct encodes the COOH-terminal 213 amino acids of fascin with a 6X Myc epitope tag on the amino terminus and driven by the CMV promoter. Con8 mammary tumor cells were co-transfected with a CMV driven neomycin resistance gene together with the FN construct, FC construct, or a pCS2+ MT empty vector and individual single cell-derived colonies were collected. FC10 and FC12 cells express COOH-terminal Myc-fascin, FN12, and FN36 express NH₂-terminal Myc-fascin, f/3.1 cells produce full-length Myc-fascin (see Fig. 3), and the C1 cells are a vector-transfected control cell line. Western blots of electrophoretically fractionated cell extracts from each of these transfected cells were probed with anti-Myc primary antibodies. b, individual subclones of transfected Con8 cells were grown on Anopore membrane filter supports and treated with or without 1 μM dexamethasone (DEX) for 4 days and the monolayer TER was monitored in each cell culture as described under "Experimental Procedures."

in Fig. 5b, in contrast to inhibitory effects of full-length Myc-fascin (Fig. 3), the ectopic expression of either the COOH-terminal or NH₂-terminal fragments of fascin, in two different transfected cell lines, failed to disrupt the dexamethasone stimulation of TER (Fig. 5b). The overall range of the glucocorticoid-
induced TERs in the transfected cells approximated that observed in the vector control C1 cells (Fig. 5b) and other stable transfected control cell lines (Fig. 3). Thus, the ability of constitutively expressed fascin to inhibit the glucocorticoid regulation of tight junction function does not simply require the portion of the protein containing both the actin and β-catenin-binding sites.

**Ectopic Fascin Expression Prevents the Glucocorticoid-induced Rearrangement of β-Catenin and Occludin to the Cell Periphery and Disrupts the Regulated Reorganization of the Actin Cytoskeleton**—To determine whether the ectopic expression of fascin disrupts the formation of both tight junctions and adherens junctions, the glucocorticoid-regulated rearrangement of the adherens junction protein β-catenin and the tight junction protein occludin was examined in the parental and transfected mammary tumor cell clones. Myc-fascin expressing f/3.1 cells, control f/3.2 cells, and untransfected Con8 cells were treated with 1 μM dexamethasone for the indicated times and the localization of β-catenin was examined by indirect immunofluorescence microscopy. The scale bar represents 50 μm.

![Image](image_url)

**Fig. 6.** Ectopic expression of fascin prevents the glucocorticoid-induced rearrangement of β-catenin to the junctional complex. Transfected f/3.1 high Myc-fascin expressing (b-b’’) or f/3.2 transfection control (c-c’’) as well as the parental Con8 mammary tumor cells (a-a’’) were treated with 1 μM dexamethasone for the indicated times and the localization of β-catenin was examined by indirect immunofluorescence microscopy. The scale bar represents 50 μm.

in f/3.2 cells was inhibited in f/3.1 cells which produce high levels of Myc-fascin (Fig. 7, panels b and b’). Taken together, these results demonstrated that ectopic expression of fascin interfered with the ability of glucocorticoids to induce a global rearrangement of the tight and adherens junction proteins, and hence the formation of the junctional complex.

The effect of ectopic fascin expression on the organization of the actin cytoskeleton was examined by indirect immunofluorescence staining for actin. As shown in Fig. 7 (panels c versus d), constitutive expression of Myc-fascin did not alter the actin cytoskeleton staining pattern in cells that were not treated with glucocorticoids. However, in f/3.1 cells (Fig. 7, panel d’), the expression of Myc-fascin prevented the dexamethasone-induced rearrangement of the actin cytoskeleton that was observed in both parental and f/3.2 cells (Fig. 7, panels c’ and e’). A similar result was obtained with phallolidin staining which confirms that the observed differences are due to polymerized actin (data not shown). Thus, ectopic expression of fascin did not change the basal actin cytoskeleton organization, but rather it disrupted the glucocorticoid-induced signal that controls the membrane rearrangement that leads to junctional complex formation.

Because fascin binds and bundles actin filaments as well as binds to β-catenin in a non-E-cadherin complex (56, 57, 77), the level of cytoskeletal-associated F-actin, fascin, and β-catenin were biochemically examined in the Myc-fascin transfected f/3.1 cells compared with the C1 vector-transfected control cells. Cells were treated with or without dexamethasone for 4 days and level of total Myc-fascin, actin, or β-catenin in the whole cell extracts were compared with the level of each protein detected in a Triton X-100-resistant cell pellet, which represents the cytoskeletal associated actin filaments (74). As shown
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Fig. 8. Characterization of the cytoskeletal-associated actin filaments and association of β-catenin with the cytoskeleton in Myc-fascin-expressing and control transfected cells. The f/3.1 cells expressing Myc-fascin and the vector control C1 mammary tumor cells were grown to confluency and treated with or without 1 μM dexamethasone (DEX) for 96 h. After trypsinization into single-cell suspensions, 2 million cells per condition were collected by centrifugation, extracted with a Triton X-100 containing buffer, and the detergent-resistant cell pellet (Triton X-100 resistant) fractionated by SDS-polyacrylamide gel electrophoresis. A second 2 million cell pellet collected from each condition was electrophoretically fractionated without detergent extraction (total cell extracts). The Western blots were probed for Myc-fascin (using Myc antibodies), actin, and β-catenin using the appropriate primary antibodies.

Fig. 9. Ectopic expression of fascin does not alter the expression levels and complex formation of junctional proteins. a, transfected f/3.1 Myc-fascin expressing and f/3.2 transfection control mammary tumor cells were treated with or without 1 μM dexamethasone for 0, 3, 5, and 7 days. Parallel sets of Western blots of total cell extracts were probed for the production of the Myc epitope, occludin, E-cadherin, β-catenin, actin, and SGK. b, the transfected f/3.1 and f/3.2 mammary tumor cells were treated with or without 1 μM dexamethasone for 4 days and β-catenin immunoprecipitates isolated from the corresponding cell extracts. The Western blots of electrophoretically fractionated immunoprecipitated protein were probed for E-cadherin, α-catenin, β-catenin, and the Myc epitope.

in Fig. 8, most of the actin expressed in the presence or absence of glucocorticoids can be detected in the Triton X-100-resistant cell pellet and the ectopic expression of Myc-fascin did not alter the actin content of this fraction. Also, the same amount of β-catenin was detected in the Triton X-100-resistant fraction regardless of the level of Myc-fascin or the steroid treatment (Fig. 8). Approximately 50% of the expressed Myc-fascin fractionated with the detergent-resistant cell pellets which likely results from its binding to actin. These results suggest that the constitutive expression of fascin did not disrupt the apical junctions or the glucocorticoids regulated organization of the actin cytoskeleton by an alteration in the cytoskeletal associated actin filaments or the interaction of β-catenin with the cytoskeleton.

The Levels and Complex Formation of Junctional Proteins Are Not Altered by the Ectopic Expression of Fascin—The expression levels and complex formation of junctional proteins were examined in the transfected mammary tumor cells. Cells that express high (f/3.1) or insignificant (f/3.2) levels of ectopic Myc-fascin were treated with dexamethasone over a 7-day time course and the total cellular proteins were analyzed by Western blotting. As shown in Fig. 9a (top panel, anti-Myc blot), Myc-fascin levels in f/3.1 cells remained constant at a high level throughout the 7-day dexamethasone treatment, while f/3.2 cells expressed virtually no Myc-fascin. During the same 7-day time course, the expression levels of occludin, E-cadherin, β-catenin, and actin remained unchanged in both f/3.1 and f/3.2 cell clones. Under these conditions, the cells maintained their glucocorticoid responsiveness as indicated by the strong induction of SGK protein expression (Fig. 9a, bottom panel). The formation of β-catenin protein complexes with E-cadherin, α-catenin, and fascin protein was examined in the transfected cell clones by Western blot analysis of coimmunoprecipitated proteins. Cell extracts of dexamethasone-treated and untreated cells were first immunoprecipitated with β-catenin antibodies in 1% Tween 20 to preserve the protein-protein interactions of β-catenin. Western blot analysis of the electrophoretically fractionated immunoprecipitates revealed that ectopic expression of Myc-fascin did not alter the ability of β-catenin to complex with E-cadherin or α-catenin (Fig. 9b, upper three panels). Similar amounts of E-cadherin and α-catenin were observed to co-immunoprecipitate with β-catenin in both dexamethasone-treated and untreated f/3.1 and f/3.2 cells. Furthermore, anti-Myc blots showed that the Myc-fascin protein co-immunoprecipitated with β-catenin from f/3.1 cells, but not from f/3.2 cells (Fig. 9b, bottom panel). These results demonstrate that the constitutive expression of Myc-fascin does not alter the expression levels or complex formation of the adherens junction proteins.

The Glucocorticoid Induction of Intercellular Adhesion Is Inhibited by the Ectopic Expression of Fascin—To test whether the ectopic expression of Myc-fascin alters intercellular adhesion through the adherens junction, a cell aggregation assay was performed on parental and transfected mammary tumor cells. Dexamethasone-treated (1 μM for 4 days) or untreated cells were collected by trypsinization in the presence of calcium, which preserves E-cadherin function, and subjected to shear force by gently pipetting through a round-tip pipette until only small cell aggregates remained. As shown in Fig. 10, in the absence of dexamethasone, each of the three tested cell lines formed small aggregates of 50 cells or less. The cell aggregates were loosely attached to each other, the cell borders were rounded and intercellular space was prominently seen as bright lines under the dark field microscope setting. In dexamethasone-treated cultures, parental cells and the f/3.2 transfection control cells formed large compact aggregates (200–1000 cells) in which the cell borders were not readily observed even at the periphery of the cell mass. There were no observable differences in cell aggregate size and characteristics between the parental and f/3.2 cells (Fig. 10, panels a’ and c’). In addition, when the parental and f/3.2 cells were allowed to further aggregate for 15 min after the initial cell adhesion assay, cell aggregates consisting of thousands of cells were formed (data not shown). In contrast, glucocorticoids failed to
stimulate intercellular adhesion in f/3.1 cells, which constitutively produce Myc-fascin (Fig. 10, panel b’). Dexamethasone-treated f/3.1 cells remained small, loosely attached aggregates that were indistinguishable from cell aggregates of f/3.1 cells not treated with steroid and thereby demonstrates that the ectopic expression of Myc-fascin disrupted the glucocorticoid activation of cell-cell adhesion.

**DISCUSSION**

The regulation of cell-cell interactions and junctional complex dynamics is essential for epithelia to reversibly adapt to proliferative signals and to respond to physiological controls during tissue growth and differentiation. In this study, we have discovered that glucocorticoids, a key hormonal regulator of mammary gland differentiation, down-regulated the production of fascin protein, an actin-bundling protein that interacts with β-catenin, concomitant with the induction of tight junction and cell-cell adhesion in Con8 rat mammary epithelial tumor cells. The constitutive expression of fascin disrupted the ability of glucocorticoids to trigger the formation of the apical junctional complex and induce intercellular adhesion. These results implicate the down-regulation of fascin as a key intermediate step in the glucocorticoid signaling pathway that controls mammary cell-cell interactions and provides, for the first time, a direct functional link between a steroid-regulated gene and the control of the apical junctions.

We propose that the selective regulation of fascin production in mammary tumor cells is necessary for the coordinate regulation of tight and adherens junctions formation by glucocorticoids. It is tempting to speculate that fascin acts as an inhibitor of junctional complex formation, and thus the glucocorticoid down-regulation of fascin eliminates an inhibitory signal. The glucocorticoid-mediated decrease in total cellular fascin protein levels temporally and dose-dependently correlated with the generation of functional tight junctions as indicated by the development of transepithelial electrical resistance, as well as with the localization of tight junction protein occludin and the adherens junction protein β-catenin to the cell periphery. Under these conditions, glucocorticoids did not significantly alter the total cellular protein levels of tight junction and adherens junction proteins such as occludin, cingulin, ZO-1 (61), β-catenin, and E-cadherin. Thus, glucocorticoids appear to induce the assembly of both the tight and adherens junctions from pre-existing junctional components. The role of fascin down-regulation in the glucocorticoid-regulated formation of epithelial junctions was directly tested by the ectopic expression of full-length mouse fascin in Con8 rat mammary tumor cells. Characterization of stable clonal cell lines that produce various levels of ectopically expressed full-length Myc-fascin showed a direct correlation between the expression level and the degree of inhibition of glucocorticoid-induced transepithelial electrical resistance. These results show that the inhibition of junctional complex formation is specific to fascin expression and is not due to random clonal variation. The constitutive production of exogenous fascin was sufficient to prevent the glucocorticoid-induced development of transepithelial electrical resistance and the reorganization of occludin and β-catenin to the tight and adherens junctions, respectively. In transfected cells that produce a high level of ectopic fascin, expression of junctional proteins and formation of the E-cadherin-α-catenin/β-catenin complex was unaltered. Therefore, the ectopic expression of fascin appears to specifically disrupt the overall assembly process of the junctional complex that is usually triggered by glucocorticoids and suggests that fascin acts as a negative regulator of the glucocorticoid-mediated formation of epithelial junctions.

The glucocorticoid induction of junctions assembly is accompanied by an activation of cell-cell adhesion that resulted in the formation of compact cell aggregates. In contrast, the Myc-fascin expressing cells remained loosely packed in the presence of dexamethasone. Thus, the constitutive expression of fascin protein not only blocks the glucocorticoid-induced formation of junctional complexes, but also interferes with the glucocorticoid activation of cell-cell adhesion. Fascin might regulate intercellular adhesion directly by modulating the function of E-cadherin or indirectly via its effect on actin which has been implicated to contribute to the adhesive strength of E-cadherin (80, 81). Fascin, which has been shown to bundle actin filaments (56, 82, 83), could potentially influence cell-cell adhesion and the assembly of intercellular junctions by modulating the actin cytoskeleton, a key player in the generation of epithelial cellular architecture and polarity (24). However, this possibility seems unlikely because the ectopic expression of fascin did not disrupt the cytoskeletal-associated actin filaments or the interaction of β-catenin with the cytoskeleton. Although the immediate mechanism of action of fascin is not clear, the discovery of a role for fascin in the regulation of cell-cell adhesion and intercellular junctions formation provides both structural and functional links between the actin cytoskeleton and the control of intercellular junctions.

Different molecular weight forms of fascin have been re-
ported in a variety of rodent and human cells, although to date, most of the characterized cell types express fascin with molecular masses in the range of 54–58 kDa (56, 82, 84). We have observed that the rat ConA mammary tumor epithelial cell line expressed a fascin-like protein at an apparent molecular mass of 45 kDa, which we have shown to be antigenically related to mouse fascin by competition binding to anti-mouse fascin antibodies. The polyclonal anti-fascin antibody used in our experiments was raised against a thioredoxin fusion protein that contains full-length mouse fascin (42, 77) and the primary antibody binding to the 45-kDa fascin protein produced in the mammary tumor cells was completely competed off by the addition of 100 μg of the fusion protein (data not shown). Conceivably, the processing of fascin protein into a smaller form may be important in the regulation of cell-cell adhesion and junctional complex formation. In this regard, it has been shown that fascin can be proteolytically cleaved to a 30-kDa form that binds actin filaments in vitro (56). We have also observed that ectopic expression of the full-length mouse fascin (tagged with the Myc epitope) yielded a form of mouse fascin that electrophoretically migrated as a 80-kDa protein. Although an unusually high molecular weight, this 80-kDa Myc-fascin species is recognized by both polyclonal and monoclonal anti-fascin antibodies. Moreover, our preliminary evidence suggests that expression of this full-length Myc-fascin cDNA, as well as a fascin cDNA with the Myc epitope tag on the COOH terminus, in human epithelial tumor cells also generates a similar 80-kDa species.2 The mechanism by which this high molecular weight form of Myc-fascin is produced is unknown, although stable protein-protein interactions may potentially account for the unexpected size. A dimeric form of the tryptic fragments of purified recombinant fascin has been shown to migrate as a stable complex in SDS-PAGE, suggesting that fascin indeed forms stable dimers under denaturing conditions (83).

It is tempting to consider that the interaction of fascin with components of the apical junction, such as β-catenin, represents a potential regulatory mechanism by which the expression of high levels of exogenous fascin might inhibit glucocorticoid-induced cell-cell adhesion and intercellular junctions formation. Several studies have shown that the regulation of adherens junction assembly correlated with a reduced binding of β-catenin to E-cadherin (44–46). However, our results showed that under conditions in which the ectopic expression of full-length Myc-fascin disrupted the glucocorticoid induction of cell-cell adhesion and intercellular junctions formation, the same level of β-catenin co-precipitated with the E-cadherin immune complex. A small fraction of Myc-fascin coprecipitates with E-cadherin, likely through its interaction with β-catenin. It is not clear whether Myc-fascin and E-cadherin bind to β-catenin in the same complex or in mutually exclusive complexes, although it has been shown that fascin competes with E-cadherin for β-catenin binding in an in vitro assay (42). The ectopic expression of the carboxyl-terminal 213 amino acids of fascin, which includes the actin and β-catenin-binding sites, failed to disrupt the glucocorticoid induction of tight junction formation, which suggests that the ability of fascin to disrupt the glucocorticoid-induced cell-cell interactions involves potentially complicated structure/function relationships within the full-length fascin protein. Conceivably, the inhibition of cell-cell adhesion by exogenous fascin could result from an effect of fascin on the E-cadherin–β-catenin protein complex, such as to induce conformational changes that attenuate the adhesive function of E-cadherin. Alternatively, fascin might indirectly regulate the function of E-cadherin via the modulation of the various functions of β-catenin or of the cortical actin cytoskeleton. Since β-catenin is known to interact with the LEF-1 transcription factor (85) and the adenomatous polyposis coli protein (86), the binding of exogenous fascin to endogenous β-catenin, and perhaps other cellular components, may potentially affect the normal activities of β-catenin in the regulation of its interacting proteins, which might in turn affect intercellular adhesion and junctions formation.

The coordinate induction of tight junction formation and cell-cell adhesion through fascin down-regulation might reflect an important biological switch in mammary cell differentiation. Our results, in which the ectopic expression of fascin disrupted the glucocorticoid stimulation of tight junctions and adherens junctions, implicate fascin as a negative regulator of cell-cell interactions. Thus, the glucocorticoid down-regulation of endogenous fascin expression is necessary for the induced formation of the apical junctional complex in rodent mammary tumor cells. Glucocorticoid receptors can stimulate or inhibit gene transcription by their selective DNA binding to glucocorticoid response elements (68–70), and by their ability to directly bind to and attenuate the function of certain transcription factors (68, 71–73). If the fascin gene is a direct target of glucocorticoid signaling, it is likely that the glucocorticoid receptor inhibits fascin gene transcription by interfering with specific transcription factors that act on the fascin gene promoter or by inducing the expression of transcriptional inhibitors that target the fascin gene. Unraveling the signaling pathway by which glucocorticoid down-regulates fascin protein expression would be an important step toward the understanding of glucocorticoid regulation of epithelial junctions formation, and perhaps the role of intercellular junctions in the regulation of cell growth and differentiation.

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