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Reciprocal Regulation by Estradiol 17-β of Ezrin and Cadherin–Catenin Complexes in Pituitary GH_3 Cells

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The antiestrogen, ICI 182780, and estradiol-17β (E_2) regulate cadherin-mediated cell adhesion in pituitary GH_3 cells. Using a cDNA expression array to screen for E_2-regulated genes that are associated with the cytoskeleton, we observed that E_2 stimulated ezrin gene expression and confirmed that ezrin gene expression is regulated pretranslationally by ICI 182780 versus E_2. E_2 increased ezrin protein levels in whole-cell lysates and in the cytoskeletal-associated, detergent-insoluble fraction. Confocal microscopy revealed that ezrin was associated with free apical membranes of E_2-treated cells. E_2 decreased N-cadherin and β-catenin levels and induced a redistribution of $p120^{ctn}$ to the cytoplasm. In GH_3 transfectants overexpressing E-cadherin, E_2 had no effect on adhesiveness or on E-cadherin and $p120^{ctn}$ distribution, but increased levels of active ezrin. Ezrin was concentrated at free and apical membranes. These studies provide the first demonstration of the regulation of ezrin by E_2 and show that the ER signaling pathway coordinately regulates two cytoskeletal-associated protein complexes, with mutually exclusive cellular distributions, in a reciprocal manner. These findings indicate that E_2 enriches the cell membrane with ezrin–membrane protein complexes by both increasing ezrin expression and by enlarging the relative area of nonadhesive membrane to which ezrin is targeted.

Key Words: Estrogen; antiestrogen; ezrin; cadherin–catenin complex; cDNA expression array.

Introduction

The primary function of the pituitary lactotrope is to produce and secrete prolactin (PRL). This function is strongly upregulated during pregnancy, primarily in response to estrogens of placental origin. Estrogens have direct effects on lactotropes, as well as indirect effects through the production of autocrine/paracrine signals *(1)*. Estrogens enhance pituitary PRL production in several ways. First, estrogens stimulate PRL gene expression and PRL secretion *(2,3)*. Second, estrogens induce hypertrophy of lactotropes *(4,5)*, which may increase the translational potential of individual lactotropes *(6)*. Estrogens also promote lactotrope proliferation and survival *(7)*. It is notable that the estrogen-induced hypertrophy and hyperplasia of lactotropes cause the human pituitary to increase in size by more than twofold during pregnancy *(5,8)*.

The estrogen-induced changes in the size and proliferation of lactotropes, as well as changes in storage and exocytosis of PRL *(9)*, are likely to require significant rearrangements of the cytoskeleton and of cytoskeletal-associated protein complexes. Antakly et al. *(3)* described the induction of microvilli and blebs in pituitary lactotropes in primary culture. More recently, we reported that estradiol 17β (E_2) decreased cell–cell adhesion and the levels of cytoskeletal-associated proteins, N-cadherin and β-catenin, in GH_3 cells *(10)*. In the present study, we utilized a cDNA expression array approach to search for other cytoskeletal-associated proteins that are regulated by estrogen in GH_3 cells. This screen revealed that E_2 strongly enhances ezrin gene expression.

Ezrin belongs to the ezrin–radixin–moesin (ERM) family of proteins. A more diverged member of the ERM gene family is merlin/schwannomin, which is the tumor-suppressor gene product mutated in neurofibromatosis type 2 *(11)*. ERM proteins collectively function as a noncovalent bridge between the cortical actin cytoskeleton, which binds to ERM proteins near the carboxyl terminus, and integral membrane proteins, which bind near the amino terminus *(12–17)*. ERM proteins also indirectly bridge the actin cytoskeleton to surface proteins via binding to ezrin-binding protein 50 (EBP50; also named NHERF 1) and related proteins *(15–17)*. In the context of our study, it is important to note that EBP50 is induced by estrogen in human mammary epithelial MCF7 cells *(18)*. In addition to their structural role, ERM proteins are involved in several cell signaling pathways that are related to exocytosis *(19)*, cell survival *(20)*, and oncogenic transformation *(21)*. Here, we show that E_2 increases ezrin gene expression and the level of cytoskeletal-associated (detergent-insoluble) ezrin in rat pituitary GH_3 cells. To our knowledge, these findings represent the first demonstration of estrogen regulation of ezrin in any cell or tissue type.

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Results

The ER Signaling Pathway Stimulates Ezrin Gene Expression at a Pretranslational Level

Our previous finding that the ER signaling pathway regulates N-cadherin and β-catenin levels and decreases cell–cell adhesion *(10)* prompted us to examine whether the ER signaling pathway regulates the expression of other genes encoding cytoskeletal-associated proteins. Using a cDNA expression array to screen for E_2-responsive genes, we discovered that ezrin gene expression was strongly increased in E_2-treated GH_3 cells relative to ICI 182780-treated cells (Fig. 1A). As discussed in the Introduction, ezrin acts as an important linker between the cortical actin cytoskeleton and membrane proteins. Therefore, we elected to examine the effects of the ER signaling pathway on ezrin expression more closely. Using RT-PCR with primers designed to amplify gene-specific fragments from the 3' untranslated region (3' UTR) of ERM gene family members, we observed that GH_3 cells express ezrin and radixin, but not moesin (Fig. 1B). The 3' UTR fragments were cloned, confirmed by sequencing, and used as probes in Northern blots. Northern analysis confirmed the results from the cDNA expression array (Fig. 1C), showing that 50 n*M* ICI 182780 significantly decreased ezrin mRNA levels below SFM levels ($p < 0.05$; $n = 4$). Further, the effects of 50 n*M* ICI 182780 were blocked by 10 and 100 n*M* E_2 ($p < 0.05$; 10 n*M* E_2 plus 50 n*M* ICI 182780 vs 50 n*M* ICI 182780 alone). Radixin mRNA levels were not altered by E_2 or ICI 182780 treatments (data not shown). The 3' UTR fragments were also used in nuclear run-on transcription assays (Fig. 1D), which revealed that 10 n*M* E_2 specifically increased ezrin gene transcription about twofold over ICI 182780-treated cells.

The ER Signaling Pathway Regulates Ezrin and Cadherin–Catenin Complexes in a Reciprocal Manner

Immunoblot assay showed that the ER signaling pathway increased ezrin protein levels (Fig. 2A). Statistical analysis using a paired *t*-test showed that E_2 significantly increased cellular ezrin protein levels over ICI 182780-treated cells (3.3 ± 0.8–fold; $p < 0.05$; $n = 4$). As described in the Discussion section, activation of ezrin by posttranslational modification (e.g., phosphorylation) disrupts intramolecular folding *(15)*. This allows the exposed carboxyl terminus to bind to cytoskeletal actin. Binding to F-actin is associated with a reduced ability to extract ezrin by 0.5% Triton X-100 [i.e., an increase in detergent-insoluble ezrin *(22)*]. In GH_3 cells, E_2 increased the amount of ezrin in the detergent-insoluble (i.e., actin-bound) form (Fig. 2B), indicating that E_2 promotes the activation of ezrin. This was corroborated by the specific cortical localization of ezrin in E_2-treated cells (Fig. 3). Confocal imaging of serial sections also revealed that ezrin was associated with free or apical membranes and was largely absent from membranes involved in cell–cell adhesion (Fig. 3 and data not shown).

E_2 also coordinately decreased cellular levels of N-cadherin (Fig. 2). As expected, N-cadherin (N-cad), β-catenin (β-cat), and $p120^{ctn}$ all localized to adherent membranes in ICI 182780-treated cells (Fig. 4). E_2 treatment resulted in a loss of N-cadherin and β-catenin from the cell membrane. The E_2-induced loss of N-cadherin from the cell membrane was associated with a decrease in the cellular levels of β-catenin (Fig. 2) and a general reduction in β-catenin immunofluorescence (Fig. 4). Nuclear localization of β-catenin was not observed in E_2-treated cells (Fig. 4). Another cadherin-binding catenin, $p120^{ctn}$, was expressed as two forms in GH_3 cells (Fig. 2). In contrast to β-catenin, cellular levels of both forms of $p120^{ctn}$ remained constant (Fig. 2). However, $p120^{ctn}$ immunofluorescence shifted from a predominantly membrane localization in ICI 182780-treated cells to a diffuse, cytoplasmic distribution in E_2-treated cells (Fig. 4).

The Level of Ezrin Expression, but not its Subcellular Distribution, is Independent of Cadherin-mediated Cell–Cell Adhesion

The above findings demonstrate that the ER signaling pathway coordinately regulates two protein complexes that are associated with the cortical actin cytoskeleton. Ezrin is increased, whereas components of the cadherin–catenin complex are downregulated and/or redistributed. Further, ezrin and the cadherin–catenin complex show essentially mutually exclusive subcellular distribution. Given the potential signaling functions of components of the cadherin–catenin complex (see the Discussion section), we examined whether forced expression of cadherin altered the ability of E_2 to increase levels of active ezrin or altered the distribution of ezrin.

GH_3 cells were transfected with an E-cadherin expression construct and subjected to selection. Two G418-resistant phenotypes emerged: one resembling parental GH_3 cells [termed E-cad (–) cells; Fig. 5A, Par] and a highly adherent phenotype [termed E-cad (+) cells; Fig. 5A, Adh]. Western blot analysis in Fig. 5B of GH_3 (lane 1), E-cad (–) (lane 2), and one clonal line of E-cad (+) cells (lane 3) showed that E-cadherin was expressed at a high level only in E-cad (+) cells. This high level of E-cadherin was associated with elevated levels of β-catenin, but essentially no change in endogenous N-cadherin or $p120^{ctn}$.

E-cad (+) cells displayed sensitivity to ER activity, as evidenced by levels of intracellular and secreted PRL in ICI 182780 versus E_2 treated cells (Fig. 6A). Confocal imaging of serial Z-sections showed a greater intensity of PRL immunofluorescence in E_2-treated cells (Fig. 6B). Despite the responsiveness E-cad (+) cells to ER signaling, the forced expression of E-cadherin blocked the ability of E_2 to reduce cell–cell adhesion. Accordingly, confocal microscopy showed that E-cadherin, $p120^{ctn}$, and β-catenin were highly concen-

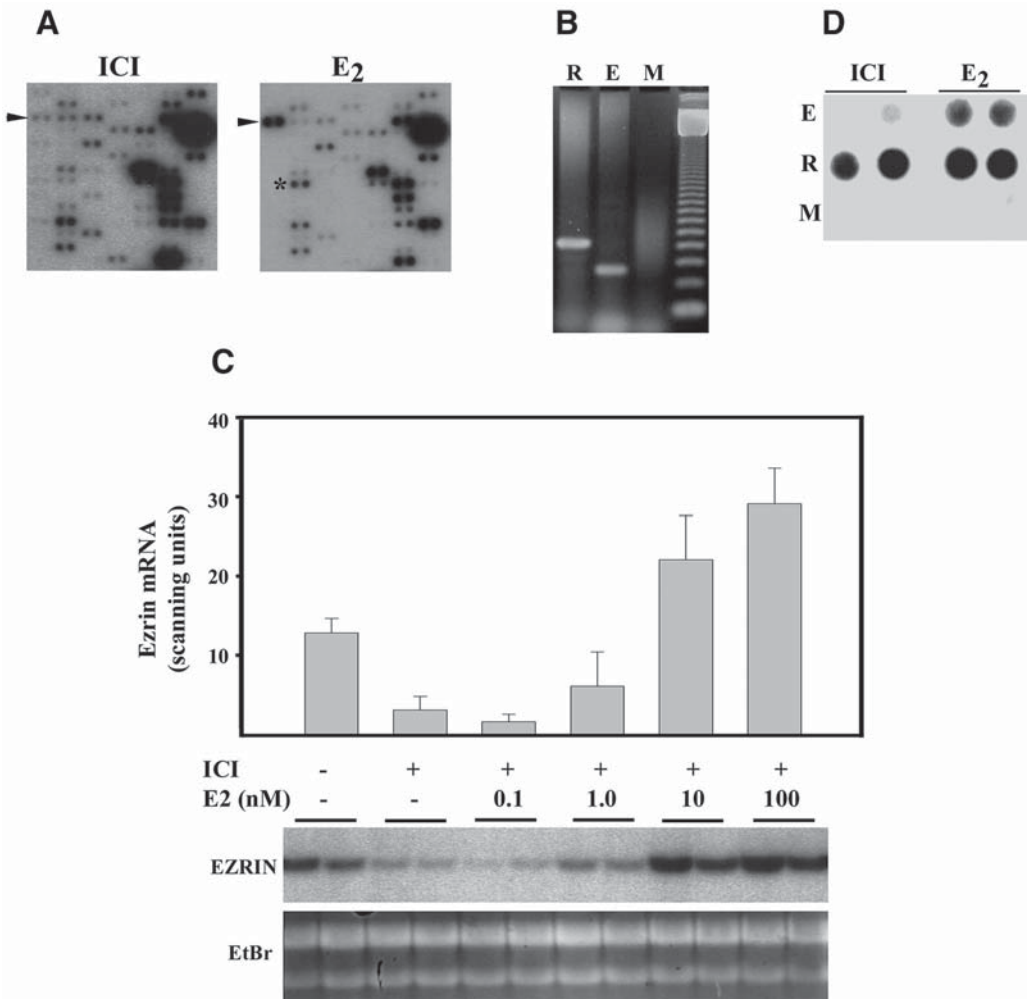

Fig. 1. The ER signaling pathway increases ezrin expression pretranslationally. (**A**) Section A from the Atlas (Clontech) cDNA expression array. Cells were treated with 10 n*M* E_2 or 50 n*M* ICI 182780 in SFM as described in the Materials and Methods section, and gene expression examined by cDNA expression array. A somewhat darker exposure is shown for the ICI 182780 sample. The arrowhead denotes the position of duplicate ezrin dots. The asterisk denotes the position of FRA-2, the one other mRNA in Panel A of the array that is apparently increased by E_2. (**B**) Reverse transcription–polymerase chain reaction (RT-PCR) of GH_3 cell mRNA for ERM gene products. RT-PCR was performed as described in the Materials and Methods section, using primers to amplify the 3' UTR region of radixin (R), ezrin (E), and moesin (M). The right lane shows 123-bp molecular-weight markers (Life Technologies). Radixin and ezrin primers amplified fragments of 513 and 310 bp, respectively. Note that moesin primers correctly amplified a 244 moesin fragment from a different cell line (data not shown). (**C**) Effects of ICI 182780 and E_2 on ezrin mRNA levels as assayed by Northern blot. GH_3 cells cultured in duplicate dishes for 1 d in SFM only or SFM containing 50 n*M* ICI 182780 (ICI) with the indicated amounts of E_2. RNA was assayed for ezrin using the ezrin 3' UTR probe, as described in the Materials and Methods section. The lower panel shows an autoradiogram for ezrin probe (EZRIN) and ethidium bromide (EtBr) staining of RNA on nitrocellulose blot before hybridization from one experiment. The upper panel shows quantification (mean ± SEM) of results from two experiments performed in duplicate (n = 4 for each treatment). Analysis of multiple treatments was performed by one-way analysis of variance, followed by the Student–Newman–Keuls test. (**D**) Effects of ICI 182780 versus E_2 on ezrin transcription rate. ^{32}P-Labeled RNA was generated in vitro from duplicate samples of nuclei from ICI 182780 and E_2 treated cells as described in the Methods and Materials section. Labeled RNA was hybridized to the 3' UTR fragments of ezrin (E), radixin (R), and moesin (M) as described in the Materials and Methods section.

trated at the membrane in both ICI 182780- and E_2-treated cells (Figs. 7 and 9).

The inability of E_2 to decrease the membrane-associated cadherin–catenin complex and to promote redistribution of $p120^{ctn}$ to the cytoplasm did not block E_2 from increasing ezrin levels (Fig. 8). Western blot analysis showed that E_2 increased the levels of total cellular ezrin, as well as the amount in the cytoplasmic, detergent-soluble and detergent-insoluble fractions (Fig. 8). Ezrin and E-cadherin localization was assessed by dual labeling of E-cad (+) cells and confocal imaging of serial sections. This analysis revealed that ezrin distribution was essentially the same in GH_3 and

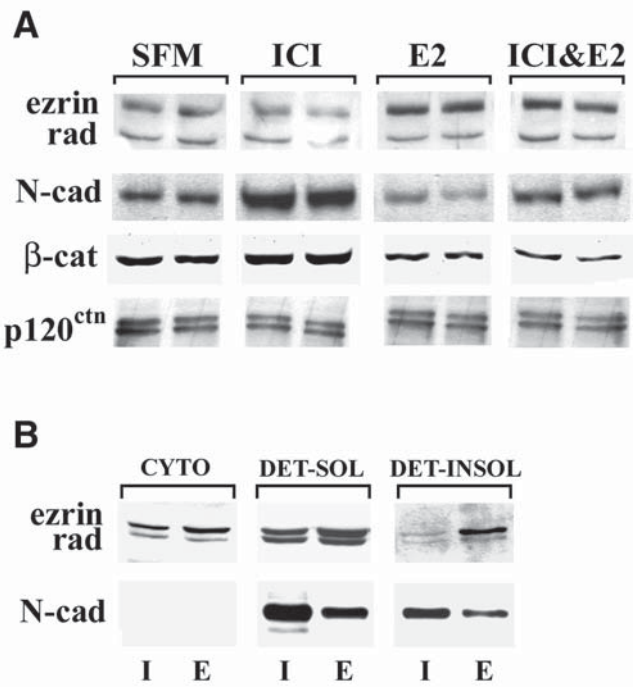

Fig. 2. Effects of ICI 182780 and E_2 on protein levels of ezrin, radixin (rad), N-cadherin (N-cad), β-catenin (β-cat), and $p120^{ctn}$. (**A**) Effects of ICI 182780 and E_2 on cellular protein levels. GH_3 cells were treated as indicated in duplicate cultures for 2 d, processed for whole-cell lysates, and assayed by Western blot. Note that the ezrin antibody used in this figure crossreacts with radixin. These results are representative of four experiments. (**B**) Effects of ICI 182780 and E_2 on ezrin/radixin and N-cadherin levels in cell fractions. GH_3 cells were treated for 2 d with ICI 182780 or E_2, and then processed for sequential fractionation as described in the Materials and Methods section. The three fractions obtained were cytosolic (CYTO), proteins soluble in 0.5% Triton X-100 (DET-SOL) and proteins not soluble in 0.5% Triton X-100 (DET-INSOL). These results are representative of assays on four separate samples.

E-cad (+) cells (compare Fig. 3 to Fig. 9), in that ezrin was concentrated at the cortical regions of free or apical membranes. In contrast to previous studies in other cell types (see the Discussion section), ezrin was largely absent from the cortical regions of E-cadherin-enriched membranes involved in cell–cell adhesion (Fig.9A) and did not coimmunoprecipitate with E-cadherin (Fig. 9B). Thus, cadherin expression does not alter the ability of estrogen to increase levels of activated ezrin, but it does limit the spatial distribution of ezrin.

Discussion

The effects of estrogen on several aspects of cell structure, including shape, size, membrane specializations, and adhesion, have been described for pituitary lactotropes *(3)* as well as several other cell types (e.g., ref. *23*). These observations indicate that estrogen regulates the expression and function of cytoskeletal-associated protein complexes. For example, estrogen regulates cadherins and catenins in several tissues *(24)*. Several cytoskeletal-associated genes have been identified as targets of estrogen regulation through the use of expression screens. These include Arp 3, which was detected by subtractive hybridization *(25)*, EBP 50, which was detected by differential display *(18)*, and ezrin, which was detected using a cDNA expression array (this study). Given the fact that the three types of screens described primarily detect those genes that display striking changes in expression, these findings indicate that cytoskeletal-related genes represent an important subclass of estrogen-regulated genes.

Our study demonstrates that estrogen upregulates ezrin gene expression at a pretranslational level. E_2 could increase ezrin transcription directly or indirectly through the induction of a transcription factor. E_2 regulates c-fos in pituitary lactotropes *(26,27)*, and Fos-induced transformation of cells is associated with a high level of ezrin gene expression *(22, 28)*. It is noteworthy that the results from the array (see asterisk, Fig 1), although not yet confirmed by Northern blot, indicate that estrogen also stimulates expression of the AP-1 family gene, Fra-2, in GH_3 cells. Other transcriptional regulatory factors regulated by E_2 in the pituitary include c-myc *(27,29,30)* and *pttg (29)*. Further work, including the cloning and characterization of the rat ezrin promoter, is needed to sort out the exact mechanism by which activation of the ER signaling pathway leads to changes in ezrin gene expression.

Activation of dormant ezrin allows it to form a molecular bridge between the actin cytoskeleton and membrane proteins *(14–17)* and is associated with a shift of ezrin into the detergent-insoluble fraction *(22)*. Immunoblot assays showed that total cellular ezrin levels were increased about threefold in E_2-treated versus ICI 182780-treated GH_3 cells, and cell fractionation revealed an E_2-induced increase in the level of ezrin protein in the detergent-insoluble fraction of both GH_3 and E-cad (+) cells. These results were corroborated by confocal microscopy of E_2-treated GH_3 and E-cad (+) cells, which show that ezrin becomes concentrated near the cell membrane. Because activation of ezrin occurs primarily through phosphorylation of its carboxyl terminus *(14–17)*, these findings provide indirect evidence that estrogen regulates ezrin posttranslationally. We are currently examining whether E_2 induces phosphorylation of ezrin through either nuclear (i.e., transcriptional) actions of the ER or by rapid, membrane-initiated signaling from the ER.

Previous studies have reported that ERM proteins localize to sites of cell–cell contacts *(17)* and references therein) and that ablation of ezrin expression through antisense oligodeoxynucleotides leads to reduced cell–cell adhesion *(31,32)*. Hiscox and Jiang *(32)* also provided biochemical evidence for an interaction between ezrin and E-cadherin–catenin complexes using coimmunoprecipitation assays. However, ezrin is localized to apical, free membranes in many cell

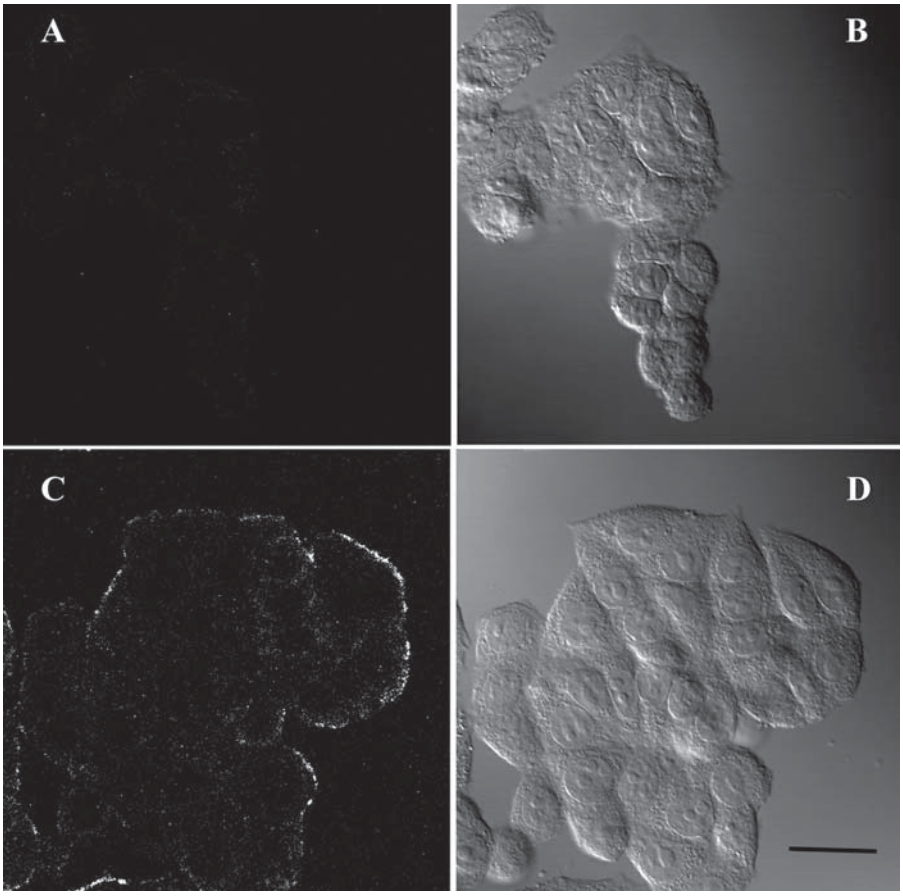

Fig. 3. Effects of ICI 182780 (**A, B**) and E_2 (**C, D**) on the level and subcellular distribution of ezrin. GH_3 cells were treated with ICI 182780 or E_2 for 2 d as described in Fig. 1 and then processed for ezrin immunofluorescent confocal microscopy (left) and Nomarski differential interference microscopy (right). Scale bar = 20 μm.

types (e.g., ref. *33*). In GH_3 cells, E_2 induces the concomitant increase in active ezrin and downregulation of cadherin–catenin complexes. Further, ezrin and N-cadherin display a largely mutually exclusive distribution, and ezrin does not coimmunoprecipitate with N-cadherin or β-catenin in GH_3 cells (our unpublished observations). Similarly, ezrin and E-cadherin do not colocalize or coimmunoprecipitate in E-cad (+) cells (Fig. 9). It thus appears that the subcellular distribution of ezrin is cell-specific, probably dictated by differences in the relative abundance, binding affinity, and/or distribution of ezrin-binding membrane proteins and EBP50-related proteins. Cell-specific proteins that link ezrin to cadherins may also exist. In any case, our findings indicate that ezrin is not physically involved in cell–cell adhesion in GH_3 cells.

Despite the spatial separation of ezrin and cadherin–catenin complexes in GH_3 cells, it is possible that a change in the level of cadherin–catenin complexes alters signaling pathways that upregulates ezrin, or vice versa. The former possibility is based primarily on evidence from other cell types that upon release from cadherin–catenin complexes, catenins gain potent signaling functions. Translocation of β-catenin to the nucleus allows it to heterodimerize with Tcf/LEF transcription factors and regulate gene expression *(34)*. Although nuclear localization of β-catenin has been described in pituitary adenomas *(35)*, the E_2-induced decrease in N-cadherin did not result in an apparent translocation of nuclear β-catenin GH_3 cells after 2 d. However, we could not discount the possibility that β-catenin accumulates in the nucleus and alters gene expression at an earlier time-point after E_2 treatment or that related catenins (plakoglobin) are involved in signaling. Specific splice variants of $p120^{ctn}$ have also been shown to translocate to the nucleus *(36)*, and $p120^{ctn}$ was shown to interact with the transcription factor, Kaiso *(37)*. Like β-catenin, $p120^{ctn}$ does not appear to translocate to the nucleus in GH_3 cells. Rather, $p120^{ctn}$ accumulates in the cytoplasmic E_2-treated cells. Recent studies have shown that "free" cytoplasmic $p120^{ctn}$ decreases Rho activity and/or increases Rac and Cdc42 activity *(38–40)*. In these studies, cadherin overexpression was used to reduce levels of "free" cytoplasmic $p120^{ctn}$, which blocked the effects of $p120^{ctn}$ on Rho-related GTPases. In the current study, we utilized

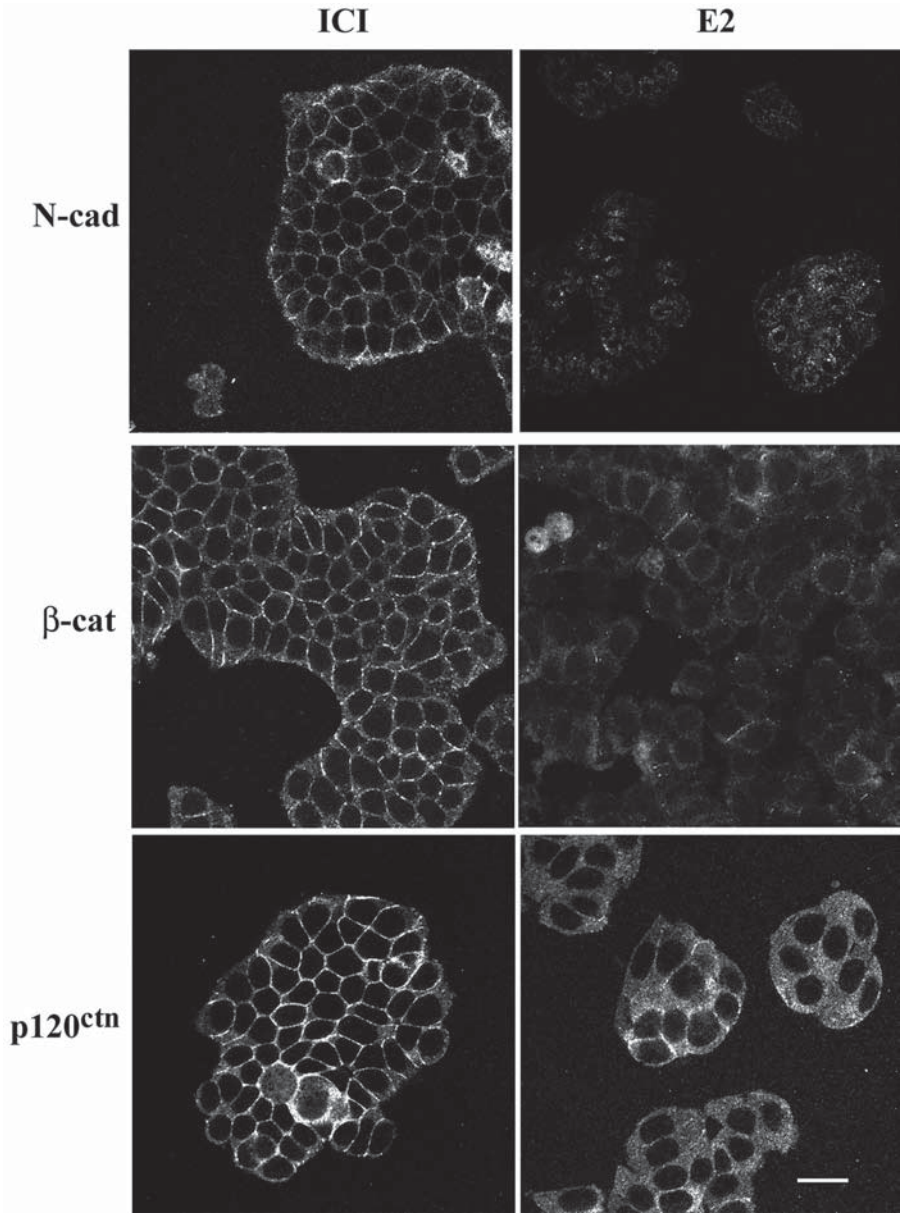

Fig. 4. Effects of 50 n*M* ICI 182780 (ICI) and 10 n*M* E_2 (E_2) on the levels and subcellular distribution of N-cad, β-cat, and $p120^{ctn}$. GH_3 cells were treated as described in Fig. 1 and then processed for immunofluorescent staining and confocal microscopy. Scale bar = 10 μm. Note that the apparent nuclear staining of N-cad that is visible in the E_2-treated cells in this figure is spurious and has been observed in ICI 182780-treated cells and negative controls.

E-cadherin overexpression as a means to recruit and retain catenins within cadherin–catenin complexes at the cell membrane and to maintain cadherin-mediated cell–cell adhesion in E_2-treated cells. As expected, E-cadherin overexpression blocked the ability of E_2 to promote a less adhesive phenotype or to alter E-cadherin, β-catenin, and $p120^{ctn}$ localization. Nevertheless, E-cad (+) cells remained estrogen responsive and displayed an E_2-induced increase in total and detergent-insoluble levels of ezrin. Thus, the E_2-induced increase in ezrin expression and activation occurs independently of the concomitant decrease in cadherin–catenin complexes. However, examination of ezrin versus cadherin distribution, particularly in E-cad (+) cells, reveals that cadherin-mediated cell–cell adhesion does have an effect on the spatial distribution of ezrin. In both GH_3 and E-cad (+) cells, ezrin is largely excluded from membranes associated with cell–cell contact. Thus, the degree of cadherin-mediated cell–cell adhesion limits the area of membrane with which ezrin can interact and, therefore, may limit ezrin-dependent function(s). Although E_2 injections significantly increase total pituitary ezrin levels in immature female rats (our unpublished observations), more work is required to determined how E_2 influences cadherin-mediated cell–cell adhesion and ezrin localization within lactotropes in vivo.

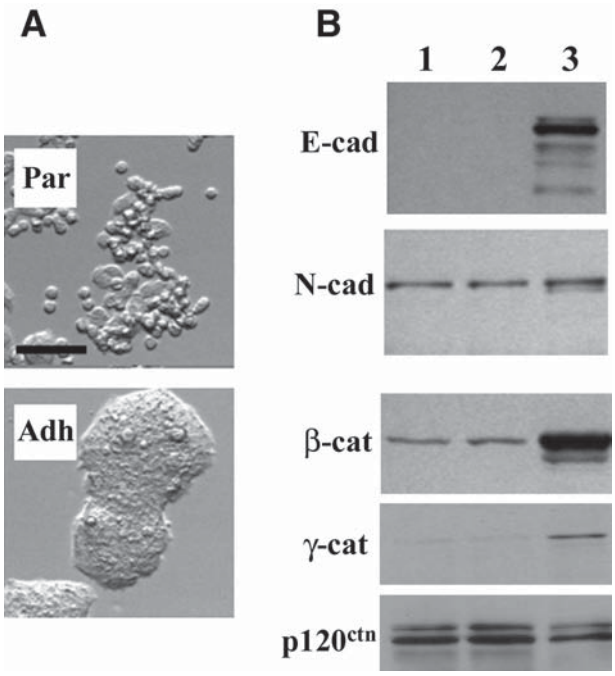

Fig. 5. Characterization of stable transfectants overexpressing E-cadherin. (**A**) Phenotype of cells as observed by Hoffman modular contrast optics. Panel A: G418-resistant cells that display the parental GH_3 cell phenotype [E-cad (–) cells]; Panel B: a tightly adherent colony of E-cad (+) cells, in which it is difficult to discern individual cell boundaries. Scale bar = 50 μm. (**B**) Cellular levels of E-cad, N-cad, β-cat, γ-catenin (γ-cat; also termed plakoglobin), and $p120^{ctn}$ in parental GH_3 cells (lane 1), E-cad (–) cells (lane 2), and E-cad (+) cells (lane 3).

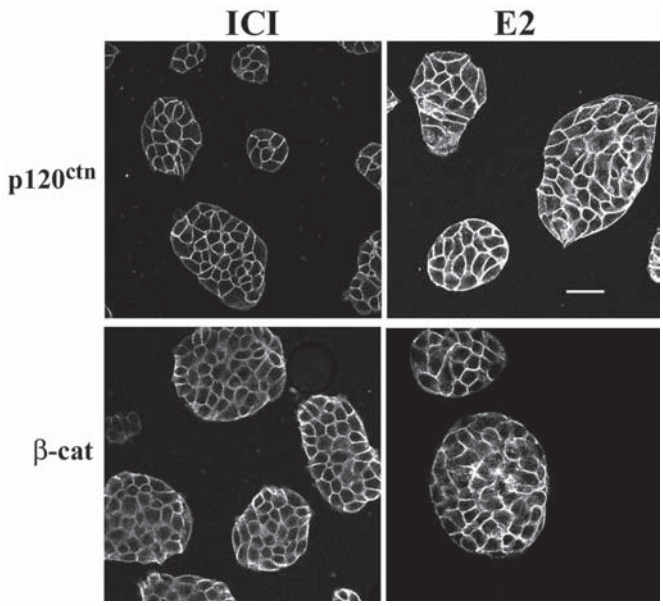

Fig. 7. Absence of effect of ICI 182780 versus E_2 on the subcellular distribution of $p120^{ctn}$ and β-catenin (β-cat) in E-cad (+) cells. Cells were treated as described in Fig. 1 for 2 d and processed for $p120^{ctn}$ and β-catenin immunofluorescence and confocal microscopy.

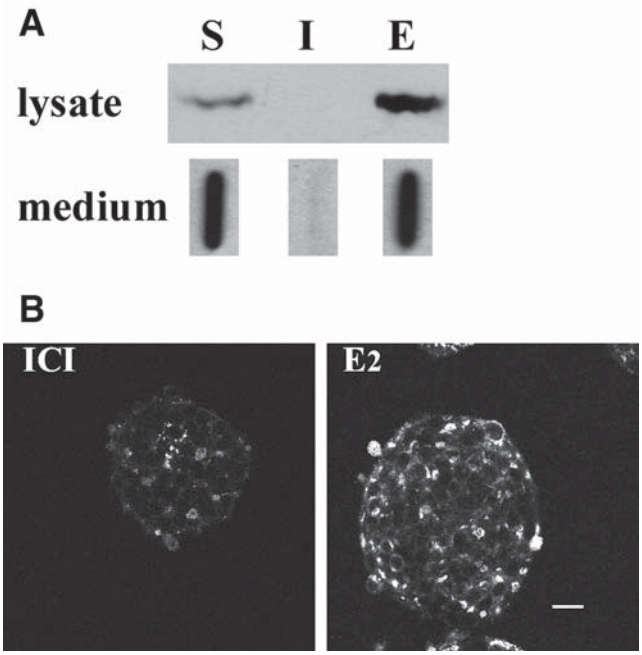

Fig. 6. E-cad (+) cells are responsive to E_2. (**A**) Cellular and secreted levels of PRL in E-cad (+) cells cultured for 2 d in SFM alone (S) or with 50 n*M* ICI 182780 (I) or 10 n*M* E_2 (E). Upper and lower panels show PRL levels as determined by immunoblot of cell lysates or by slot blot of culture media, respectively. (**B**) PRL immunofluorescence in E-cadherin cells treated for 1 d with 50 n*M* ICI 182780 (ICI) or 10 n*M* E_2, as detected by confocal microscopy. Scale bar = 50 μm.

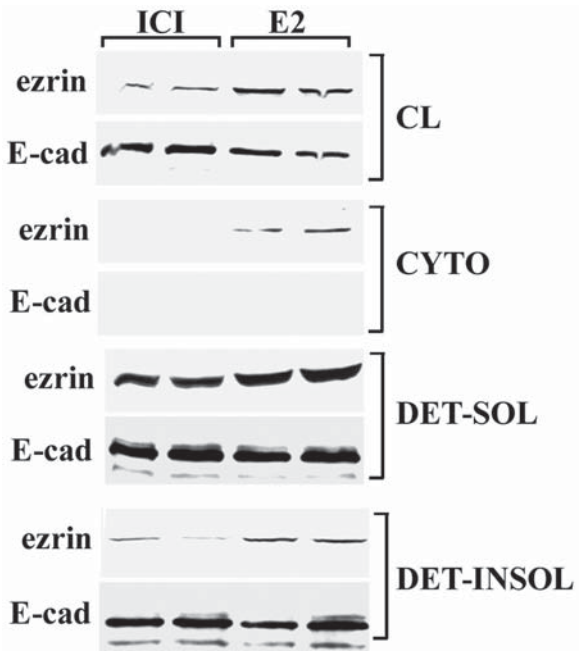

Fig. 8. Effects of ICI 182780 and E_2 on ezrin and E-cadherin levels in E-cad (+) cells. Two sets of duplicate cultures of cells were treated for 2 d with ICI 182780 or E_2 as described in Fig. 1. One set was processed for whole-cell lysate (CL), and the second set was serially extracted to generate cytoplasmic (CYTO), detergent-soluble (DET-SOL), and detergent insoluble (DET-INSOL) fractions as described in the Materials and Methods section. The immunoblot was serially stained for ezrin and E-cadherin.

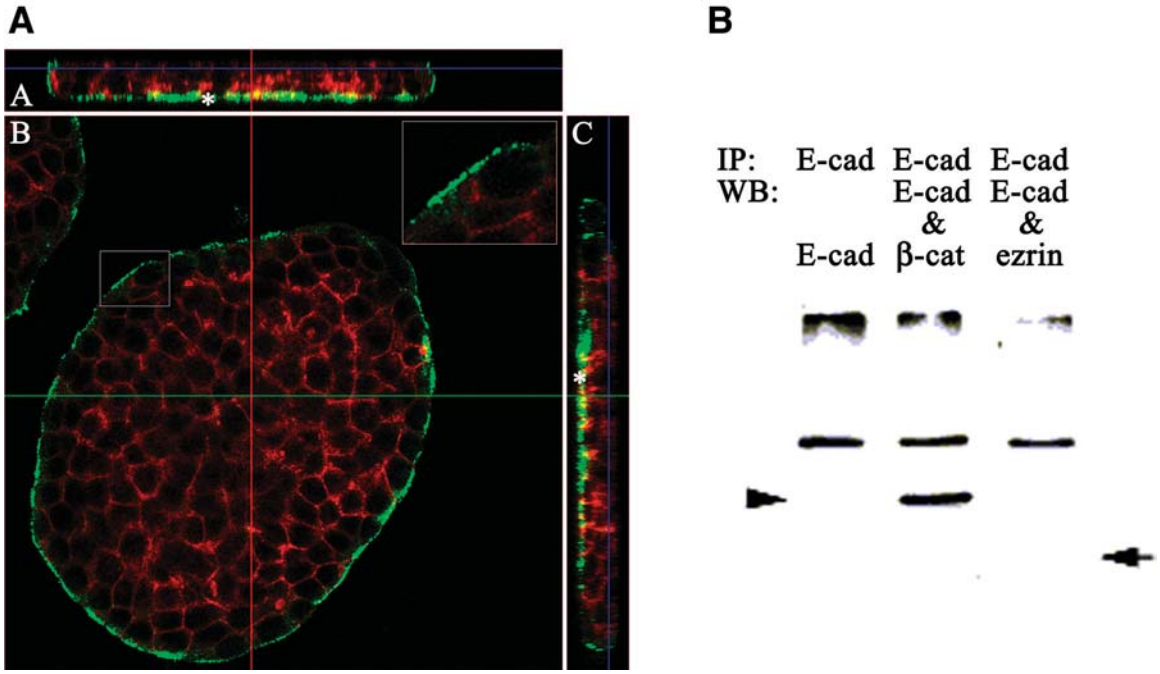

Fig. 9. Ezrin fails to colocalize or coimmunoprecipitate with E-cadherin in E-cad (+) cells. (**A**) Ezrin (green) and E-cadherin (red) immunofluorescence in E_2-treated E-cad (+) cells Serial Z-sections were collected using confocal microscopy and analyzed with Imaris software (Bitplane, Zurich, Switzerland). A middle Z-section (Panel B) and two orthogonal side views (Panels A and C) generated from multiple Z-sections are shown. The asterisk denotes apical surface of side views. The inset shows a higher magnification of boxed region in Panel B. (**B)** E-cadherin immunoprecipitations from E_2-treated E-cad (+) cells. One E-cadherin immunoprecipitation was split into three lanes and first immunoblotted for E-cadherin (upper band in all lanes). One strip was subsequently stained for β-catenin (E-cad & β-cat) as a positive control for coimmunoprecipitation. Position of β-catenin is denoted by the arrowhead. A second strip was subsequently stained for ezrin (the predicted position denoted by an arrow). In a second experiment, ezrin stained a total lysate sample on the same blot and immediately adjacent to an E-cadherin immunoprecipitate, which was negative for ezrin (not shown).

In summary, the ER signaling pathway increases ezrin expression and activation in pituitary GH_3 cells. Estrogen is likely to maximize the function of ezrin by expanding its membrane-associated domain through the downregulation of cadherin–catenin complexes. It is likely that ezrin plays a role in the responses of lactotropes to estrogen, which include PRL and growth factor secretion, cellular hypertrophy, and cellular proliferation and survival. Ezrin has been implicated in growth factor production *(41)*, survival *(20)*, and proliferation *(42)* in other cell types. More work is required to identify the membrane protein(s) that interact with ezrin in lactotropes and GH_3 cells and the specific estrogen-dependent function(s) of ezrin in these cells.

Materials and Methods

Cell Culture

GH_3 cells (ATCC, Rockville, MD) were maintained in growth medium (GM), composed of Dulbecco's modified Eagle's medium (DMEM) F-12 (Sigma Chemical Co., St. Louis, MO) supplemented with antibiotics, 5% horse serum, and 1.25 % fetal bovine serum (Life Technologies, Gaithersburg, MD). Ham's F-12 nutrient mixture without phenol red (E_2-free SFM; Life Technologies) was supplemented with antibiotics. Water-soluble estradiol-17β (E_2) was obtained from Sigma, and the antiestrogen ICI 182780 was obtained from Tocris Cookson, Inc. (Ballwin, MO).

Experiments were performed by plating cells into tissue culture dishes and culturing in GM until a density of >50% confluence was reached. The GM was removed, and the cells washed with E_2-free SFM and cultured in E_2-free SFM containing 50 n*M* ICI 182780 and/or specified amounts of E_2 for 1–2 d.

cDNA Expression Array

GH_3 cells were treated for 2 d in SFM containing either 50 n*M* ICI 182780 or 10 n*M* E_2. RNA was isolated using the Atlas Pure Total RNA Labeling System (Clontech, Palo Alto, CA) and treated with DNase according to the supplier's instructions. An aliquot (10 μg) of each sample was assayed by Northern blot hybridization for PRL mRNA levels to ensure the integrity of the RNA and responsiveness of the cells to treatment. Gene expression was examined using the Rat Atlas cDNA Expression Array (Clontech), with strict adherence to the supplier's protocols, starting with 26 μg of total RNA from each sample. Multiple exposures were obtained of the membranes, using Kodak Biomax MS film and screen (Sigma).

RT-PCR and "T/A" Cloning

Primer pairs (Life Technologies) were designed to amplify the 3' UTRs of (1) rat ezrin (sense: 5'-GCACCTCACAGC AGGCAGGTGTCAC-3'; antisense: 5'-CTAGAGAAAG TATGGCACCCGTGTG), (2) mouse radixin (sense: 5'-CTT GTGATCCATGTCCTTCATAAGC-3'; antisense: 5'-CAGT AAGTGATCCGTTGTGACACC), and (3) rat moesin (sense: 5'-AGTGGGCGCGCAGCCGTTAGGGAC-3'; antisense: GCTATGTTGAATGAGTGTGACAAAG-3').

The total RNA was isolated using Ultraspec reagent (Biotecx Inc, Houston, TX). RNA (5 µg) was reversed transcribed and amplified by 30 cycles of PCR using standard procedures. The ezrin, radixin, and moesin 3' UTR fragments were inserted into the vector pCR3.1 by "T/A-cloning" (Invitrogen, Carlsbad, CA) and their identity confirmed by sequencing by the Molecular Core Facility at UCHC.

Northern Blot Hybridization

RNA (10 µg) was resolved on 1% agarose, 6% formaldehyde gels and assayed by Northern blot hybridization using standard procedures. DNA probes corresponding to the 3' UTR fragments of ezrin, radixin, and moesin mRNAs were generated by PCR amplification of cloned fragments (see above). Size markers used on Northern gels were the 0.25 to 9.4-kb RNA ladder from Life Technologies.

Nuclear Run-on Transcription Assays

GH_3 cells (four 100-mm dishes/sample) were treated with 50 n*M* ICI or IDnME_2 for 1 d, washed in cold PBS, resuspended in 4.5 mL of lysis buffer (10 m*M* Tris-HCl, pH 7.4, 10 m*M* NaCl, 3 m*M* $MgCl_2$), and lysed by the addition of 0.5 mL of 5% NP-40 and incubation on ice for 2 min. Nuclei were centrifuged at 1000*g* for 5 min at 4ºC and resuspended in 100 µL of freshly prepared reaction buffer *(43)*. After the addition of 10 µL of ^{32}P-UTP (100 µCi, 3000 Ci/mmol; Amersham Pharmacia Biotech, Piscataway, NJ), nuclei were incubated at 30ºC for 30 min with shaking, followed by incubation with 2 µL of RQ1 DNase (Promega, Madison, WI) at 37ºC for 10 min. RNA was extracted with Ultraspec reagent. The RNA pellet was resuspended in 50 µL of TES solution (10 m*M* Tris-Cl, pH 7.4, 10 m*M* EDTA, 0.2% sodium dodecyl sulfate [SDS]) and a 2-µL aliquot used to determine incorporated ^{32}P-UTP.

Ezrin, radixin, and moesin 3' UTR fragments were generated by PCR as described above. Samples were adjusted to approximately 5 µg in 50 µL, denatured by addition of 0.1 *M* NaOH and incubation at 94ºC for 10 min, 200 µL of 20X SSC added, and the samples dotted onto Nitroplus nitrocellulose membrane (Osmonics, Westborough, MA) using a vacuum manifold. After ultraviolet (UV) crosslinking, the DNA dots were placed in a scintillation vial containing 1 mL TES, 1 mL TESN (TES plus 0.6 *M* NaCl), 200 µL 50X Denhardt's solution, and 10 µL of 10 mg/mL salmon sperm DNA (Life Technologies) and prehybridized for 6 h at 65ºC. Approximately 50 µL of each RNA sample, adjusted to equal counts per minute was denatured at 65ºC for 10 min, immediately added to the prehybridization vials, and hybridzed at 60ºC overnight. Filters were washed in 2X SSC, 0.1% SDS at room temperature for 15 min, followed by one wash in prewarmed 2X SSC, 0.1% SDS at 60ºC for 45 min, and exposed to BioMax MS film.

Western Blot and Slot Blot Procedures

Western blots of cell lysates were performed essentially as described *(10)*. Primary antibodies were obtained from the following: ezrin (that crossreacts with radixin and moesin) from Santa Cruz Biotechnology Inc (Santa Cruz, CA) ezrin (specific) from Upstate Biotechnology Inc. (Lake Placid, NY), pan-cadherin (used to assess N-cadherin) from Sigma Chemical Co.; β-catenin, $p120^{ctn}$, and E-cadherin from Transduction Laboratories (Lexington, KY), and PRL from the National Hormone and Peptide Program (http://www.humc.edu/hormones).

For cytoskeletal extraction experiments, cells were treated with 50 n*M* ICI 182780 or 10 n*M* E_2 for 24 h in SFM in a 100-mm dish. Medium was decanted and cells collected in 0.5 mL of cytoskeletal stabilization buffer (CSB; *44*) and 5 µL each of PIC, phosphatase inhibitor cocktail 1, and phosphatase inhibitor cocktail 2 (Sigma). Cells were lysed by 10 strokes in a Dounce homogenizer using a B pestle on ice, and then centrifuged at 12,000*g* at 4ºC for 15 min. The supernatant ("cytosolic" fraction) was saved, and the pellet resuspended in 200 µL of CSB, 0.5% Triton X-100, and 2 µL each of the protease and phosphatase inhibitors. After incubation on ice for 10 min, the samples were centrifuged at 12,000*g* at 4ºC and the supernatant ("detergent-soluble" fraction) saved. The detergent-insoluble pellet was then resuspended in 200 µL of 2.5% Triton-X 100 lysis buffer plus 2 µL each of protease and phosphatase inhibitors, incubated on ice for 10 min, and cleared by centrifugation at 12,000*g* for 5 min at 4ºC. The supernatant represents the "detergent-insoluble" fraction (in that "detergent" specifically refers to 0.5% Triton-X 100).

Secreted PRL in conditioned medium was assayed by a slot blot procedure. Equivalent numbers of cells were aliquoted into 60-mm dishes and incubated for 1 d in GM. Cells were then washed and treated for 1 d in 5 mL of SFM in 60-mm culture dishes. The medium was discarded, the cells cultured in 3 ml of fresh SFM, and the medium collected after 1d. An aliquot of 25 µL from each medium sample was added to 175 µL of PBS, applied to nitrocellulose using a slot blot manifold, and washed with 200 µL PBS. After UV crosslinking, blots were processed for PRL staining exactly as described for Western blots.

Immunofluoresecence Studies

Cell cultured on glass cover slips were fixed in 4% formaldehyde in PBS for 15 min, washed with PBS, blocked with 3% BSA in PBS for 30 min, and incubated for 30 min with primary antibody diluted in 0.1% BSA in PBS. Controls

received the appropriate normal IgG for 30 min. Cover slips were washed three times in PBS, incubated for 30 min in secondary antibody, washed in PBS, and mounted in Slo-Fade mounting reagent (Molecular Probes). Microscopy was performed on a Zeiss LSM510 confocal laser scanning microscopy using either a 63X 1.4 NA planapochromat or 40X 1.2 NA c-apochromat lens. Primary antibodies were as described above, with the specific ezrin antibody (UBI) used for immunofluorescence studies. Secondary antibodies used were FITC donkey anti-goat IgG (#sc2024, Santa Cruz Biotechnology), Alexa 488 chcken anti-rabbit (A21441) or anti-mouse (A21200 Molecular Probes), or rhodamine $F(ab)_2$ goat anti-mouse IgG (Organon-Technika, Malvern, PA).

Generation of Stably Transfected Cell Lines

The E-cadherin expression constructs, pC-E-cad, were generously provided by Dr. M. Ozawa (Kagoshima University, Japan *(45)*. GH_3 cells were transfected by electroporation, plated into 100-mm culture dishes, and incubated in GM for 2 d. Medium was then changed to GM without penicillin/streptomycin, containing 300 µg/mL of Geneticin (Life Technologies). After 3 wk, individual colonies were microdissected by suction into a glass micropipet, trypsinized and, plated in tissue culture dishes in GM plus Geneticin. Individual colonies underwent a second round of microdissection and then cultured as cell lines.

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