Endocrine, *vol. 22, no. 2, 119–126, November 2003* 0969–711X/03/22:119–126/$25.00

Tissue-Specific Regulation by Estrogen of Ezrin and Ezrin/Radixin/Moesin-Binding Protein 50

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The morphology and function of rat GH_3 pituitary cells are profoundly affected by estradiol-17β (E_2), presumably due to changes in the profile of gene expression. We recently reported that a major target of E_2 in these cells is the ezrin gene, which encodes a cytoskeletal linker protein that forms a complex with ezrin/radixin/moesin-binding protein 50 (EBP50) in some cell types. Other studies have shown that EBP50 levels are increased by E_2 in human breast and uterine tissue. Thus, we examined whether ezrin and EBP50 expression is coordinately increased by E_2 in GH_3 cells in vitro and rat pituitary glands in vivo. Ezrin levels are repressed by the steroidal antiestrogen, ICI 182780, and this effect is abrogated by E_2 and the ERα-specific agonist, PPT, in GH_3 cells. In contrast, EBP50 levels remained constant during these treatments. Ezrin and EBP50 did not display extensive colocalization. Moreover, ezrin was not co-immunoprecipitated by an EBP50 antibody in parental GH_3 cells or in GH_3 cells stably overexpressing EBP50, but was co-immunoprecipitated with EBP50 in human breast MCF-7 cells. Disruption of the actin cytoskeleton of GH_3 cells changed the distribution of ezrin within subcellular fractions, but had no effect on EBP50. Finally, in juvenile female rats, E_2 injections increased ezrin expression in the pituitary and uterus, but increased EBP50 expression only in the uterus. These findings demonstrate tissue specificity in the formation of ezrin–EBP50 complexes and in the regulation of EBP50 expression in estrogenresponsive tissues.

Key Words: Ezrin; EBP50; ERM proteins; estrogen; swinholide A.

Introduction

Pituitary lactotropes produce the hormone prolactin (PRL), which is primarily required for mammary gland development and function *(1)*. Estradiol 17-β (E_2) acts on lactotropes, particularly during pregnancy, to induce cellular hypertrophy and hyperplasia, to increase lactotrope survival, and to increase the production and secretion of PRL. E_2 also stimulates the release of autocrine/paracrine factors that further amplify lactotrope growth and PRL production and enhance the blood supply to lactotropes through intrapituitary angiogenesis *(2–7)*. Estrogens induce about a doubling of pituitary volume during pregnancy, which is almost solely due to the enlargement and mitosis of lactotropes, and may lead to problems associated with an increased sellar mass [e.g., visual field problems, headaches, lymphocytic hypophysitis *(8)*]. Moreover, reports have linked estrogen to the progression of prolactinomas, which represent the most common form of pituitary tumor (see ref. *7* and references cited therein). Despite the profound effects of E_2 on lactotrope function, size, and proliferation, the specific proteins and protein complexes that comprise the basis of these effects remain largely unidentified.

The use of cDNA expression array to screen for estrogen-responsive genes in the rat somatolactotropic GH_3 cell line revealed that E_2 markedly stimulates ezrin gene expression *(9)*. E_2 increases ezrin gene transcription, ezrin mRNA levels, and total cellular ezrin protein levels. E_2 also increased the relative amount of ezrin in the detergent insoluble fraction *(9)*, which is indicative of "activated," F-actin-bound ezrin *(10)*. Examination by immunofluorescent scanning laser confocal microscopy demonstrated that E_2 increases ezrin near the "free" or "apical" membrane of cell clusters *(9)*. Microarray cDNA expression analysis and histochemistry also revealed that ezrin expression is upregulated in the epithelial cells of the ventral prostate by testosterone (T) replacement in castrated rats *(11)*. Thus, ezrin may also be regulated through the androgen receptor, or through the aromatization of T to E_2, in the prostate gland.

Ezrin, radixin, moesin (collectively referred to as ERM proteins), and the closely related tumor-suppressor, merlin/schwannomin, comprise a subgroup of the band 4.1 family of proteins. ERM proteins serve as molecular scaffolds

Received June 23, 2003; Revised July 30, 2003; Accepted August 4, 2003.

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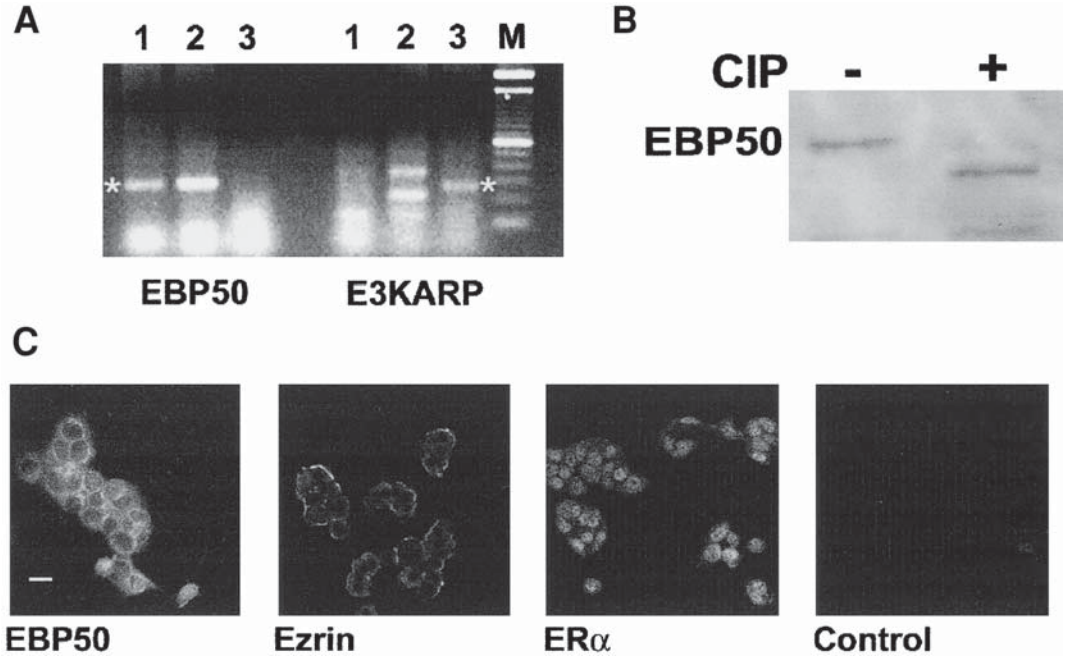

Fig. 1. EBP50 is present in pituitary cells. (**A**) RT-PCR was performed to detect ezrin-binding proteins in RNA extracted from rat pituitary (**lane 1**), GH_3 cells (**lane 2**), and SIGCs (**lane 3**). Primers were made against the 3' UTR or EBP50 and E3KARP. The left asterisk (*) indicates the appropriate size for EBP50. The right asterisk indicates the appropriate size for E3KARP. (**B**) EBP50 detected by Western blot analysis of protein isolated from GH_3 cells, incubated for 1 h at 37°C with or without 25 U calf intestinal phosphatase (CIP). (**C**) Comparison of the subcellular distribution of ezrin, EBP50, and ERα in GH_3 cells in growth medium (GM). Nonimmune rabbit IgG was used as a control. Scale bar = 20 μm.

that link integral membrane proteins to filamentous actin *(12,13)*. ERM proteins bind to membrane proteins, either directly, or indirectly through one of two PDZ-domain proteins, ezrin/radixin/moesin-binding protein 50 (EBP50; also called NHERF-1) or E3KARP (also called NHERF-2). ERM proteins and EBP50 (or E3KARP) form heterotrimeric complexes with membrane proteins, as exemplified by ezrin–EBP50–CFTR complexes in respiratory epithelia *(14,15)*. Owing to the presence of other protein-interactive domains in ERM proteins and EBP50/E3KARP *(16)*, these scaffolding proteins potentially promote the formation of larger protein complexes that integrate structural and signaling information at the cell membrane. ERM proteins are activated by phosphorylation *(17–23)* and/or interaction with polyphosphoinositides *(17,24,25)*, which promote the unfolding of latent ERM proteins and exposure of protein-interactive sites.

EBP50 was recently identified as an estrogen-responsive gene in human MCF-7 breast cancer cells *(26)*. The human EBP50 gene has been shown to harbor multiple half estrogen response elements (EREs) in its promoter region, some of which confer estrogen responsiveness in a heterologous promoter–reporter assay *(27)*. These findings are consistent with the report that EBP50 expression correlates positively with estrogen receptor (ER) status in breast carcinoma specimen *(28)*. Furthermore, EBP50 expression has been shown to be increased in proliferative (i.e., estrogen-stimulated) uterine endometrium over secretory (i.e., estrogen and progesterone-stimulated) endometrium *(28)*.

In order to better understand the role of ezrin induction in the response of lactotropes to E_2, it is important to identify proteins that potentially interact with ezrin and that are coregulated by E_2. The present study focused on whether E_2 coordinately regulates the levels of an ezrin/EBP50 complex in pituitary cells.

Results

EBP-50 Is Present in GH_3 Cells and Rat Pituitary

In order to understand the significance of the E_2-induced levels of ezrin in the pituitary, we extended our studies to ezrin-binding proteins. Here, we focused on EBP50 (NHERF-1) and E3KARP (NHERF-2). Ingraffea et al. *(29)* reported a mutually exclusive pattern of expression for EBP50 and E3KARP in several tissues. RT-PCR analysis indicated that EBP50 mRNA was detectable in GH_3 cells and the male rat pituitary but not in a line of rat ovarian spontaneously immortalized granulosa cells (SIGCs) (Fig. 1A). In contrast, E3KARP mRNA was detected in SIGCs, but specific bands were not seen in pituitary or GH_3 cells (Fig. 1A). Western blot analysis also showed that EBP50 was expressed in GH_3 cells (Fig. 1B). As expected from previous studies in other

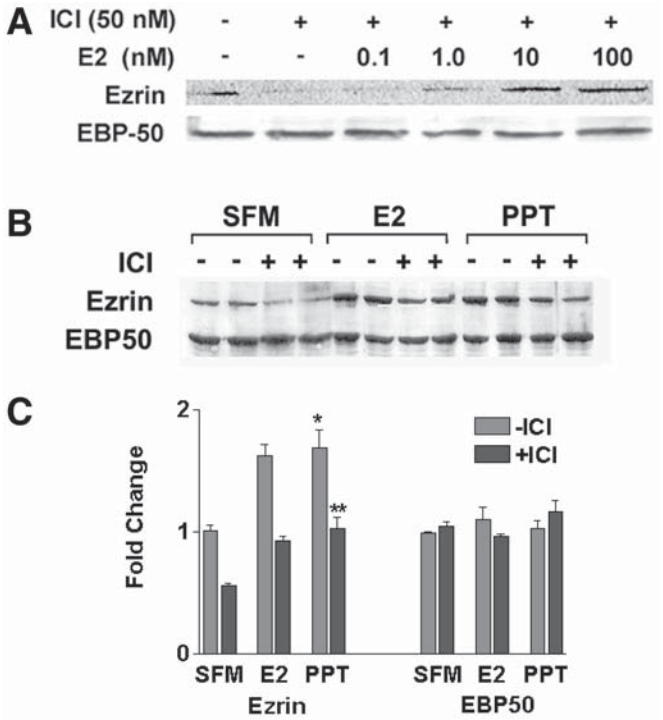

Fig. 2. Effects of ER stimulation on the levels of ezrin and EBP50 in lactotropes. (**A**) GH_3 cells were incubated for 2 d in SFM alone, in the presence of ICI (50 n*M*) alone, and ICI with increasing amounts of E_2. Equal amounts of protein were assayed by Western blot using antibodies against ezrin and EBP50. Results are representative of three separate experiments. (**B**) GH_3 cells were incubated for 2 d in SFM with and without ICI (50 n*M*) in the presence of E_2 (100 n*M*) or PPT (10 n*M*). Equal amounts of protein were analyzed with antibodies against ezrin and EBP50. (**C**) Results were quantified using Scion imaging software (NIH) and graphed (mean ± SEM) with the mean of the control group defined as one. A single asterisk (*) indicates a value significantly different ($p < 0.01$) from SFM control but indistinguishable ($p > 0.05$) from E_2 treatment. A double asterisk (**) indicates a value significantly different ($p < 0.01$) from ICI treatment but indistinguishable ($p > 0.05$) from E_2 + ICI treatment. $n = 3$ from two separate experiments.

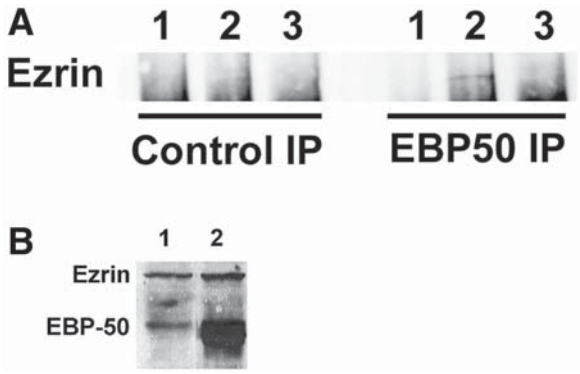

Fig. 3. Ezrin does not co-immunoprecipitate with EBP-50 in GH_3 cells. (**A**) Lysates from GH_3 (**lane 1**), MCF-7 (**lane 2**), and a line of GH_3 cells overexpressing human EBP50 (GH_3–EBP50+, **lane 3**) were incubated with control or anti-EBP50 antibodies, and the precipitated protein was immunoblotted using anti-ezrin antibody. (**B**) Characterization of GH_3–EBP50+ (**lane 2**) cell line compared to parental GH_3 cells (**lane 1**). Equal amounts of protein were analyzed with antibodies against ezrin and EBP50.

cell types *(30)*, treatment of lysates with a phosphatase induced a mobility shift (Fig. 1B), confirming that EBP50 is expressed constitutively as a phosphoprotein in GH_3 cells. Confocal microscopy using a polyclonal antibody against EBP50 showed diffuse cytoplasmic staining of GH_3 cell clusters (Fig. 1C). This subcellular localization of EBP50 is distinctly different from the apical distribution of ezrin (Fig. 1C), calling into question the assumption that EBP50–ezrin complexes are formed in GH_3 cells.

Abundance of EBP50 Is Not Regulated by Estrogen in GH_3 Cells

Having established that EBP50 is expressed in GH_3 cells and pituitary, we next examined whether EBP50 and ezrin are coordinately regulated by E_2 in GH_3 cells. As shown previously *(9)*, levels of ezrin protein were depressed by the steroidal antiestrogen, ICI 182780, and this effect could be reversed by increasing amounts of E_2 (Fig. 2A). In contrast to ezrin, EBP50 protein levels remained constant (Fig. 2A).

GH_3 cells express multiple complete and truncated forms of the estrogen receptor *(31)*, including intracellular and membrane forms of the ERα *(32)*. Under our culture conditions, GH_3 cells uniformly express high levels of nuclear ERα (Fig. 1C). In order to examine whether the observed effects of estrogen on ezrin levels could be attributed to the actions of ERα, GH_3 cells were treated for 2 d in SFM alone or with ICI 182780 in the presence or absence of E_2 or PPT, an ERα-specific agonist. Both E_2 and PPT effectively antagonized the effects of the antiestrogen, ICI 182780, on the levels of ezrin (Fig. 2B,C). In contrast, EBP50 levels were not affected by any of the three ERα ligands (Fig. 2B,C).

EBP50 Does Not Strongly Interact with Ezrin in GH_3 Cells

EBP50 co-immunoprecipitates with ezrin in other cell types *(30)*. Attempts to co-immunoprecipitate endogenous ezrin with EBP50 in GH_3 cells failed, even though these co-immunoprecipitates are observed in MCF-7 cells (Fig. 3A). In order to test the presence of ezrin–EBP50 complexes in GH_3 cells more rigorously, we established a GH_3 cell line stably expressing an HA-tagged human EBP50 (GH_3–EBP50+ cells) at a level several-fold higher than the endogenous rat form (Fig. 3B). Even using GH_3–EBP50 cell lysates, we did not observe EBP50 antibodies to immunoprecitate ezrin (Fig. 3A). These findings do not support the presence of significant quantities of ezrin–EBP50 complexes in GH_3 cells.

We also used a biochemical approach to test for the presence of ezrin–EBP50 complexes that was based on the fact

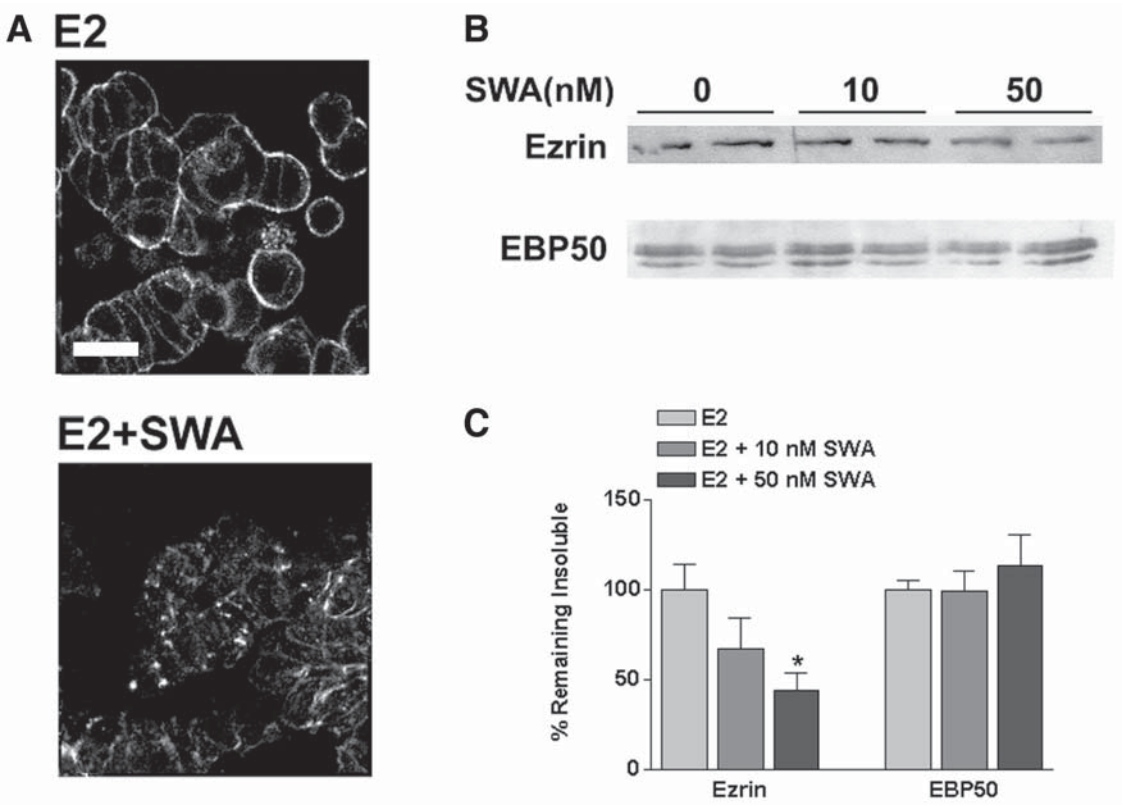

Fig. 4. Swinholide A (SWA), an actin-severing agent, decreases levels of ezrin but not EBP50 in the detergent-insoluble fraction. (**A**) Changes in the actin cytoskeleton in GH_3 cells treated with SWA. Cells grown in SFM plus E_2 for 24 h were incubated in the absence or presence of 30 n*M* SWA. The actin cytoskeleton was visualized using BODIPY-FL phallacidin. Scale bar = 20 μm. (**B**) Detergent-insoluble fractions were isolated as described from cells treated with no. 0, 10 n*M*, or 50 n*M* SWA. (**C**) Densitometric quantification of ezrin and EBP50 levels. The mean values for ezrin and EBP50 in cells treated with E_2 alone were designated as 100%. An asterisk indicates a significant change from cells treated with E_2 alone ($p < 0.05$, $n = 4$ from two separate experiments.)

that such complexes are linked to the cortical actin cytoskeleton. As discussed in the Introduction, the activated, F-actin-bound form of ezrin is not extractable by 0.5% Triton X-100 *(9,10)*. About 10% of total ezrin and EBP50 is found in the 0.5% Triton X-100-insoluble fraction in E_2-treated GH_3 cells (data not shown). However, this "detergent-insoluble fraction" (DI fraction) is comprised of other insoluble structures, such as chromatin, intermediate filaments, etc., and thus, does not exclusively contain F-actin-binding proteins. Therefore, we tested whether ezrin and/or EBP50 were linked to the actin cytoskeleton in GH_3 cells by examining whether the actin-severing drug swinholide A (SWA) reduced the level of either protein in the DI fraction. GH_3 cells were treated in SFM for 24 h with E_2 followed by a second 24 h period in E_2 with 0, 10, and 50 n*M* SWA. At 30 n*M*, SWA partially disrupted the actin cytoskeleton as detected by the appearance of specks with phalloidin staining (Fig. 4A). Disruption of the actin cytoskeleton by SWA decreased the amount of ezrin residing in the DI fraction by approx 50%. In contrast, levels of EBP50 in the DI fraction remained constant (Fig. 4B,C). These data indicate that ezrin is, in fact, linked to F-actin, but probably not as an ezrin–EBP50 complex, in the DI fraction of GH_3 cells.

E_2 Regulation of Ezrin and EBP50 In Vivo: Tissue-Specific Responses by EBP50

The absence of regulation of EBP50 by E_2 in the GH_3 cell pituitary tumor cell line was somewhat surprising in light of studies using other cell types *(26,27)*. Therefore, we examined whether E_2 regulates ezrin and EBP50 in vivo in the pituitary gland and in another well-established E_2-target tissue, the uterus. Sexually immature 21-d-old female rats, which have low endogenous levels of gonadal steroids, were used for these experiments. Three daily injections of E_2 significantly ($p < 0.05$) increased total pituitary ezrin levels over sham-injected controls (Fig. 5A,C). In contrast to ezrin, E_2 and ICI 182780 did not significantly alter EBP50 levels from sham-injected controls. As expected, E_2 significantly ($p < 0.05$) increased uterine weights. In contrast to its effects in the pituitary, E_2 increased the levels of both ezrin and EBP50 in the uterus (Fig. 5B,D).

Discussion

The application of cDNA expression array to screen for estrogen-responsive genes identified the ezrin gene as a notably estrogen-inducible target in GH_3 cells *(9)*. This find-

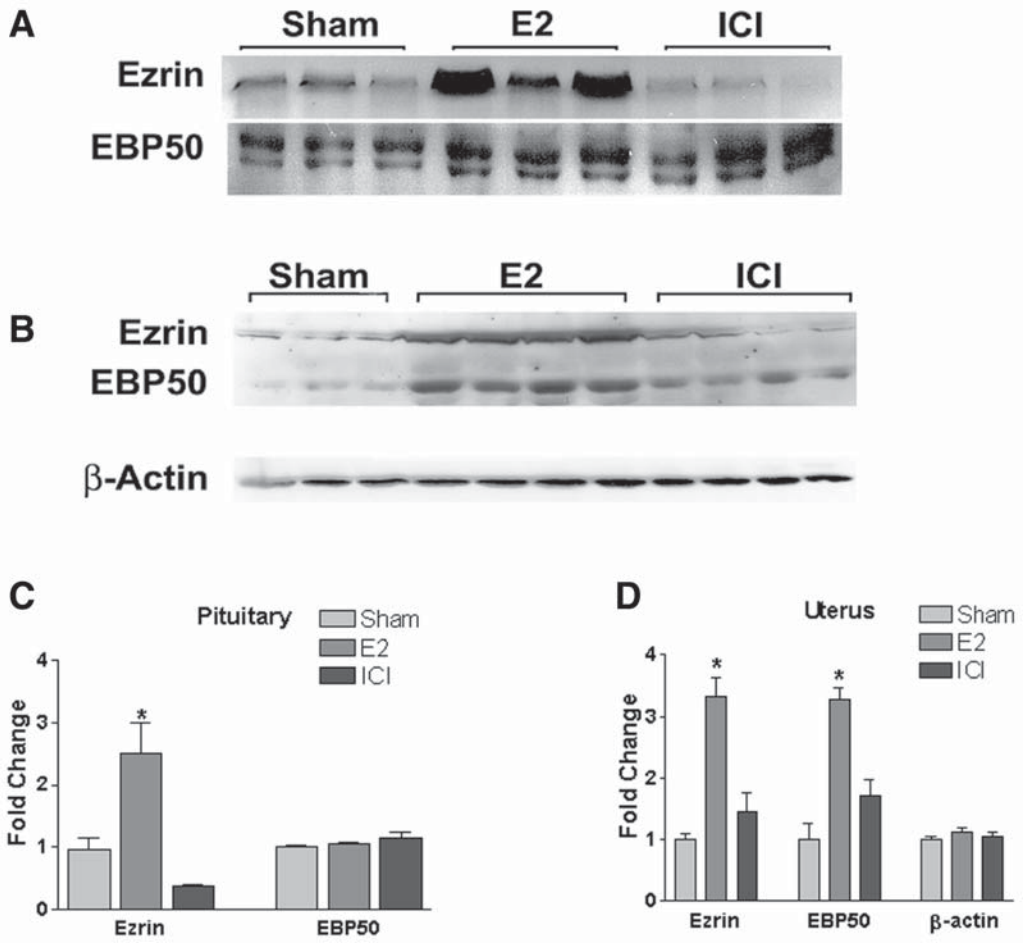

Fig. 5. In vivo regulation of Ezrin and EBP50 by E_2 is tissue-specific. 21-d-old sexually immature female Wistar rats were given three daily injections of oil vehicle, E_2 (5 μg), or ICI (50 μg). (**A,B**) Equal amounts of pituitary and uterine proteins were immunoblotted for ezrin and EBP50. Uterine proteins were additionally stained for β-actin. (**C,D**) Densitometric quantification of proteins in pituitary and uterine samples. The mean values for ezrin and EBP50 in rats treated with vehicle injections was designated as unity. An asterisk indicates a significant change from control ($p < 0.05$, $n = 3$ or 4). Results are representative of three separate experiments.

ing was then confirmed by nuclear run-on transcription assay, Northern blot hybridization, immunoblot, and confocal microscopy *(9)*. The present study extends these findings by showing that ezrin expression is enhanced by both E_2 and the ERα-specific analog, PPT, in GH_3 cells. The present study also shows that E_2 significantly induces ezrin in the pituitary and uterus in vivo. Ezrin has also been identified as an androgen- (but possibly an aromatized-androgen) inducible gene in the rat prostate *(11)*. Given the magnitude of these responses, and their occurrence in at least two estrogen-target tissues in vivo, it is likely that an elevated ezrin level plays an important role in overall cellular response to estrogen.

Ezrin is involved in a broad range of cellular processes, often in a cell-specific nature (see below). One approach to elucidating the function of ezrin in the lactotrope in the context of estrogenic stimulation is the identification of proteins that interact with ezrin, and which may also be upregulated by E_2. A fundamental distinction to be made during this approach is the determination of whether ezrin primarily binds directly to the cytoplasmic domain of an integral membrane protein(s) in lactotropes, or primarily interacts with membrane proteins indirectly through the formation of a complex with PDZ-containing proteins such as EBP50. The present study focused on EBP50 because it had been identified as an estrogen-responsive gene in human MCF7 breast ductal carcinoma cells *(26)*, is expressed in ER-positive breast cancer cells, and is elevated in proliferative versus secretory uterine endometrium *(28)*. Also, the promoter region of the human EBP50 gene harbors multiple ERE half-sites, some which bind to the ER in electrophoretic mobility shift assays and confer estrogen-responsiveness to heterologous promoter–reporter constructs in transient transfection assays *(27)*.

EBP50 expression was observed in both GH_3 cells and the rat pituitary. In contrast to previous studies on breast and uterine tissue *(26,28)*, EBP50 levels were unaffected by ICI 182780 or E_2 treatment in GH_3 cells, and E_2 injections had no effect on total pituitary EBP50 levels in immature female rats. Notably, E_2 increased pituitary ezrin levels, and

co-stimulated ezrin and EBP50 expression in the uterus. These findings show that, although E_2 increases EBP50 expression in the rat, as has been shown in the human, this regulation is cell-specific. Cell-specific responses have been reported for E_2 regulation of other genes. For example, E_2 increases expression of the high-density lipoprotein receptor, SR-BI, in steroidogenic tissues, but represses SR-BI in the liver *(33)*. A molecular basis for the cell-specific response of specific genes to E_2 may include differences in the E_2 signaling pathway itself [e.g., membrane-initiated versus nuclear ER signaling *(32)*, or ERα versus ERβ *(31)*], the presence of other signaling pathways that modulate the ER [e.g., EGF *(34)*, BMPs *(35)*], different levels of and competition for co-activator proteins *(36)*, and/or the interaction of cell-specific transcription factors with the ER, particularly in the context of half-ERE sites [e.g., Pit-1/ER interaction in lactotrope-specific expression of the PRL gene *(37)*]. An understanding of the cell-specific regulation of EBP50 awaits the characterization of positive E_2 regulation in the uterus and breast.

In addition to the absence of EBP50 regulation by E_2 in the pituitary and GH_3 cells, several attempts by us failed to detect significant levels of ezrin–EBP50 complexes. First of all, ezrin shows a strict submembrane localization to the "free" surfaces of E_2-treated GH_3 cells, whereas a considerable amount of endogenous EBP50 showed a diffuse intracellular fluorescence. Immunoprecipitation using several different EBP50 antibodies failed to co-immunoprecipitate ezrin in GH_3 cells. However, EBP50 antibodies readily pulled down ezrin from MCF7 lysate. This prompted us to generate the GH_3–EBP50+ cell line expressing EBP50 at elevated levels. Even in these overexpressing cells, the ezrin antibody immunoprecipitated ezrin, but failed to pull down EBP50, as detected either by an EBP50 antibody or by an antibody to the HA-tag epitope (data not shown), and EBP50 antibodies failed to co-immunoprecipitate ezrin.

As negative co-immunoprecipitation data, even when contrasted to MCF7 lysate as a positive control, offer weakly convincing evidence of the absence of a specific complex, we also employed a biochemical approach to the examination of the presence of significant quantities of actin–ezrin–EBP50 complexes in the DI fraction of GH_3 cells. SWA is a cell-permeable, actin-severing macrolide from a marine sponge *(38)*, which can be used to "release" F-actin-binding proteins in vivo or in vitro *(39)*. We reasoned that if the DI fraction-associated EBP50 is specifically bound to the FERM domain of F-actin-bound ezrin, it would be solubilized along with ezrin upon SWA-induced fragmentation of the actin cytoskeleton. Consistent with reports for other cell types *(38)*, SWA disrupted the actin cytoskeleton in GH_3 cells as evidenced by the generation of cytoplasmic specks with phalloidin staining. This was accompanied by a decrease in the relative amount of ezrin and actin in the DI fraction. In contrast, the level of EBP50 was unaltered by SWA treatment, indicating that EBP50 is either nonspecifically sticking to components of the DI fraction, or specifically binding to a component other than F-actin. Collectively, the somewhat diffuse subcellular distribution of EBP50, the inability to coimmunoprecipitate EBP50 with an ezrin antibody, and the inability of reduce EBP50 levels with SWA in the DI fraction argue that GH_3 cells do not contain significant levels of ezrin–EBP50 complexes. It is worth noting that ezrin–EBP50 complexes have been detected exclusively in well-polarized epithelia *(40)*. Similarly, E_2 regulation of EBP50 was observed in polarized exocrine epithelia (i.e., mammary ductal cells and uterine epithelium). In contrast, the endocrine parenchyma of the pituitary have a reduced (if any) degree of polarization *(41)*, which may be related to a different role of EBP50 in lactotropes.

The findings of the present study indicate that ezrin may function in lactotropes primarily through direct interactions with integral membrane proteins, as opposed to indirect binding through EBP50. Examples of direct ezrin-binding membrane proteins include a subset that are involved in cell adhesion (e.g., ICAMs, CD44, CD43, and syndecan 2) and cell size (e.g., NHE1) *(42–45)*. As E_2 regulates both cell–cell adhesion in GH_3 cells *(46)*, and cell size in GH_3 cells (our unpublished data) and pituitary lactotropes *(3)*, ezrin may subserve the control of these states by E_2. However, it should be noted that ERM proteins are required for a diverse and increasingly large number of functions and responses, including maintenance of cell shape *(10)*, cell motility *(22)*, cell survival *(47)* and death *(48)*, cell adhesion *(49)*, growth-factor exocytosis *(50)*, receptor endocytosis and recycling *(51)*, and the maintenance of epithelial phenotype *(52)*. From a pathophysiological standpoint, specific ERM proteins are upregulated in several types of metastatic cancers *(53)*, may be required for tumor invasiveness *(54)*, and serve as a prognostic indicator in some cancers *(55)*. Thus, ezrin may subserve multiple E_2-regulated events, including PRL and growth factor exocytosis, lactotrope hypertrophy, lactotrope survival/apoptosis and prolactinoma progression, in the pituitary gland.

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 F-12 HAM (DMEM; Sigma Chemical Co., St. Louis, MO) supplemented with penicillin/streptomycin (2000 U/mL), 5% horse serum, and 1.25% fetal bovine serum (GIBCO, Grand Island, NY). Cells were grown until a density of >50% confluence was reached. At the time of an experiment, cells were washed with E_2-free, phenol red-free, serum-free DMEM:F12 (SFM; Sigma Chemical Co., St Louis, MO) and treated for 48 h. Water-soluble estradiol 17β (E_2) was obtained from Sigma, the antiestrogen

ICI 182780 and the ERα specific agonist propyl pyrazole triol (PPT) from Tocris Cookson Inc (Ballwin, MO), and swinholide A (SWA) from Calbiochem (La Jolla, CA).

Protein Analysis From In Vivo and In Vitro Studies

Juvenile female Wistar rates (Charles River) were maintained with food and water ad libetum. Rats received 200 µL sc injections of either vegetable oil vehicle, vehicle containing 5 µg E_2, or vehicle containing 50 µg ICI 182780 for 3 d and were killed on the fourth day, according to a protocol approved by the Committee for Laboratory Animal Care at UCHC. Pituitaries and uteri were dissected out, and homogenized in 100 and 200 µL, respectively, of ice-cold lysis buffer [10 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 5 m*M* EGTA, 1 m*M* $MgCl_2$, 2.5% Triton X-100, supplemented with 1% each of protease inhibitor cocktail and phosphatase inhibitor cocktails I and II (Sigma, St. Louis, MO)]. For in vitro studies, GH_3 cells were collected with a cell scraper in 1 mL of ice-cold lysis buffer. Lysates were centrifuged for 5 min at 16,000*g*, and protein concentrations determined by BCA assay (Pierce, Rockford, IL). Western blots of cell lysates were performed and nitrocellulose filters were probed with anti-ezrin (UBI, Lake Placid, NY), monoclonal anti-EBP50 (AbCam, Cambridge, England), polyclonal anti-EBP50 *(14)*, and anti-β-actin (Sigma, St. Louis, MO).

Immunoprecipitations

Culture dishes of cells were rinsed with PBS and lysed in 500 µL of Immunoprecipitation Buffer (IP buffer: 1% Triton X-100, 150 m*M* NaCl, 10 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA, 1 m*M* EGTA pH 8.0, 0.5% NP-40) supplemented with 1% protease and phosphatase inhibitor cocktails (Sigma, St. Louis, MO) under constant agitation for 30 min at 4°C. Cells were scraped from the dish and passed several times through a 25-gauge syringe to disperse large aggregates. Lysates were clarified by centrifugation of 12,000*g* for 5 min at 4°C. An aliquot of 100 µL of lysate was incubated with 1–5 µg of antibody under agitation for 1 h at 4°C. After adding 20 µL of protein A agarose (Upstate, Lake Placid, NY), samples were incubated for an additional 30 min. Mixtures were then centrifuged at 12,000*g* at 4°C for 1 min and the supernatant removed by aspiration. Pellets were washed three times in IP buffer, resuspended in 20 µL SDS-PAGE sample buffer, boiled for 5 min, and centrifuged again at 12,000*g* for 1 min at 4°C. Supernatant was then analyzed by Western blot.

Immunoflourescence Studies

Cells cultured on glass cover slips were fixed in 4% formaldehyde in PBS for 15 min, washed in PBS for 5 min, permeabilized with 0.5% Triton X-100 in PBS for 5 min, blocked with 3% BSA in PBS for 15 min, and washed again in PBS. Fixed cells were incubated with primary antibody, as described above, or nonimmune control IgG in 0.1% BSA in PBS for 30 min. Cover slips were washed three times in PBS, incubated in secondary antibody [Alexa 488 chicken anti-mouse or anti-rabbit (Molecular Probes, Eugene, OR) or rhodamine F(ab)2 goat anti-mouse (Organon-Technika, Malvern, PA)] for 30 min, washed three times in PBS, and mounted in Slo-Fade mounting reagent (Molecular Probes). Microscopy was done on a Zeiss LSM510 confocal laser scanning microscope using a 40× 1.2 NA c-apochromat lens.

Generation of Stably Transfected Cell Lines

GH_3 cells were cotransfected with 10 µg of an HA-tagged human EBP50expression construct (kindly provided by Dr. M. von Zastrow, UCSF) and 2 µg of pC-E-cad (kindly provided by Dr. M. Ozawa, Kagoshima University, Japan), by electroporation and incubated in GM for 2 d. Medium was changed to GM without penicillin/streptomycin containing 300 µg/mL of Geneticin (Life Technologies). After 3 wk, individual colonies were selected for microdissection based on increased cell–cell adhesion. Individual colonies underwent a second round of selection. EBP50 expression in parental GH_3 cells and GH_3–EBP50+ cell lines was assessed by Western blot.

Acknowledgments

Supported by a grant from The Robert Leet and Clara Guthrie Patterson Foundation (BW, AC) and from the State of Connecticut Department of Public Health (Breast Cancer Research and Education Income Tax Checkoff Fund) to BW, and HL 63755 from the NIH (SLM). Fluorescence microscopy was performed using the facilitites of the Center for Biomedical Imaging Technology at UCHC, which is supported in part by PHS RR13186.

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