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# Estradiol stimulates tyrosine phosphorylation of the insulin-like growth factor-1 receptor and insulin receptor substrate-1 in the uterus

**(epithelium/signal transduction/proliferation)**

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ABSTRACT **The signaling pathways associated with estrogen-induced proliferation of epithelial cells in the reproductive tract have not been defined. To identify receptor tyrosine kinases that are activated *in vivo* by 17β-estradiol (E2), uteri from ovariectomized mice were examined for enhanced tyrosine phosphorylation of various receptors and a receptor substrate following treatment with this hormone. Within 4 hr after hormone exposure, extracts showed increased phosphotyrosine (P-Tyr) immunoreactivity at several bands, including 170- and 180-kDa; these bands were still apparent at 24 hr after E2. Analysis of immunoprecipitates from uterine extracts revealed that E2 enhanced tyrosine phosphorylation of the insulin-like growth factor-1 receptor (IGF-1R) and insulin receptor substrate-1 (IRS-1) by 6 hr. Comparison of supernatants from IRS-1 and control rabbit IgG immunoprecipitates indicated that the 170-kDa P-Tyr band in extracts was equivalent to IRS-1. The receptors for epidermal growth factor, platelet-derived growth factor, and basic fibroblast growth factor did not exhibit an E2-induced increase in P-Tyr content. The nonestrogenic steroid hormones examined did not stimulate the P-Tyr content of IGF-1R or IRS-1. Immunolocalization of P-Tyr and IRS-1 revealed strong reactivity in the epithelial layer of the uterus from E2-treated mice, suggesting that the majority of P-Tyr bands observed in immunoblots originate in the epithelium. Since hormonal activation of IRS-1 is epithelial, estrogen-specific, and initiated before maximal DNA synthesis occurs following treatment with hormone, this protein, as part of the IGF-1R pathway, may be important in mediating estrogen-stimulated proliferation in the uterus.**

One of the major actions of estrogens in target tissues is the stimulation of cellular proliferation. The rodent uterus has served as an experimental model for evaluating steroid hormone action. In the adult ovariectomized rodent, the proliferative response to 17β-estradiol (E2) occurs mostly in the luminal and glandular epithelial cells (1, 2). It is postulated that steroid hormone-enhanced uterine growth is mediated by locally derived polypeptide growth factors (reviewed in refs. 3 and 4). Receptors for various growth factors, including epidermal growth factor (EGF)/transforming growth factor α, platelet-derived growth factor (PDGF), and insulin-like growth factor-1 (IGF-1) are present in various cell types of the uterus, including the epithelium (5–8). A regulatory pathway for cell growth is apparently coupled to some receptors, since growth factors, such as EGF, can exert potent mitogenic effects on uterine epithelial cells, *in vitro* (9) and *in vivo* (10). Previous studies have shown E2-stimulation of various growth factor transcripts or immunoreactivity in the rodent uterus (6, 11–13), which suggests that growth factor bioavailability is a rate-limiting event in hormonal control of uterine growth. It is not known whether enhanced level of transcript and immunoreactivity for a growth factor signifies activation of the corresponding signaling pathway, or, furthermore, whether these findings reflect the requirement for multiple growth factor pathways to mediate estrogen action.

Ligand-induced tyrosine phosphorylation of the cognate receptor is an important event in the regulation of many growth factor signaling pathways. Tyrosine autophosphorylation of some growth factor receptors, such as the EGF receptor (EGFR), occurs mainly at sites located outside the tyrosine kinase domain (14). These sites are important for the selective recruitment of downstream signaling molecules (reviewed in ref. 15). Unlike the EGFR, tyrosine-phosphorylated IGF-1/insulin receptors associate poorly with SH2-containing proteins. Instead, the insulin and IGF-1 receptor kinases phosphorylate the insulin receptor substrate-1 (IRS-1) on multiple tyrosine residues; this protein serves to couple these receptors to SH2-signaling proteins (reviewed in ref. 16).

To identify those growth factor pathways that are actually stimulated in uteri of ovariectomized mice as a result of estrogen action, this study examined various receptors and a receptor substrate for increased phosphotyrosine (P-Tyr) content following hormone exposure. Immunolocalization was used to determine specific uterine cell types that contain components of the activated growth factor receptor pathway. Identification of sex steroid-induced signaling pathways that are associated with proliferation of target tissues may provide insight into biochemical factors that may be implicated in cancer risk and better rationales for interrupting the development or progression of tumors that originate in these tissues.

## EXPERIMENTAL PROCEDURES

**Materials.** Estradiol, progesterone, dexamethasone, and 5α-dihydrotestosterone were from Sigma. EGF was purchased from Collaborative Biomedical Products (Bedford, MA). Protein A-Sepharose was from Pharmacia. The Xcell II mini-cell for SDS/PAGE and the blot module for Western transfer were from NOVEX (San Diego). Prestained $M_r$ markers were from Bio-Rad, and the polyvinylidene fluoride membrane was from

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Abbreviations: E2, 17β-estradiol; EGF, epidermal growth factor; IGF-1, insulin-like growth factor-1; PDGF, platelet-derived growth factor; EGFR, EGF receptor; IRS-1, insulin receptor substrate-1; P-Tyr, phosphotyrosine; IGF-1R, IGF-1 receptor; PDGFR, PDGF receptor.

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NOVEX or Millipore (Immobilon-P). Enhanced chemiluminescence reagents were obtained from Amersham.

**Antibodies.** Horseradish peroxidase-conjugated anti-phosphotyrosine monoclonal antibody was obtained from ICN (PY20). A polyclonal antibody against P-Tyr-containing proteins was purchased from Transduction Laboratories (Lexington, KY). A rabbit polyclonal antiserum was raised against a peptide corresponding to the extreme C-terminal region (residues 1157–1186) of the rat EGFR (17), as described (18), and was affinity-purified. Polyclonal antibodies against the human IGF-1 receptor (IGF-1R, $\beta$-subunit) and the human fibroblast growth factor type 2 receptor were purchased from Santa Cruz Biotechnology. Antisera against the $\alpha$-subunit of the human PDGF receptor (PDGFR), the murine PDGFR $\alpha$-subunit and the human PDGFR $\beta$-subunit were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-rat IRS-1 antibody was purchased from Upstate Biotechnology. Horseradish peroxidase-conjugated donkey anti-rabbit IgG was from Amersham; rabbit IgG was obtained from Vector Laboratories.

**Animals, Treatments, and Preparation of Tissue Extracts.** Procedures with experimental animals followed the guidelines of the National Institute of Environmental Health Sciences Animal Care and Use Committee. CD-1 mice (Charles River Laboratories) were ovariectomized at 76–82 days of age and were treated at 12–48 days after castration. Each mouse was injected s.c. with 1 $\mu$g E2 in 0.1 ml PBS/1% ethanol, and uterine or liver tissue collected at various times after treatment; controls received only the vehicle. For hormone specificity experiments, CD-1 castrates were injected s.c. with 1 $\mu$g E2, 10 $\mu$g dexamethasone, 10 $\mu$g 5$\alpha$-dihydrotestosterone, 100 $\mu$g progesterone, or vehicle (50 $\mu$l DMSO/4% ethanol); uteri were collected at 6 hr following treatment with hormones. Tissue extracts were prepared as described (19) with the following modifications: tissues were disrupted at 4°C in buffer A [1% Triton X-100/2 mM EDTA/2 mM EGTA/1 mM $Na_3VO_4$/20 mM NaF/50 $\mu$M $Na_2MoO_4$/20 $\mu$g/ml aprotinin/20 $\mu$g/ml leupeptin/15 $\mu$M (4-amidinophenyl)-methanesulfonyl fluoride in 20 mM Hepes, pH 7.4] with three 10-sec bursts of a Brinkmann polytron at the highest setting. A constant volume (600 $\mu$l) of buffer A was used to homogenize each uterus. A section of liver was disrupted in buffer A on a 7.5% wet weight/volume basis. After centrifugation at 21,000 $\times$ *g* for 1 min, aliquots of the uterine (100–250 $\mu$g protein) and liver (300 $\mu$g protein) extracts were boiled in Laemmli sample buffer for 5 min. An equivalent volume (15 $\mu$l) of each extract was evaluated by Western blot analysis as detailed below. Protein concentrations were determined by the Pierce BCA Protein Assay.

**Immunoprecipitation and Western Blot Analysis.** Additional aliquots of the uterine (300–600 $\mu$g protein) and liver (750 $\mu$g protein) tissue extracts were added to buffer B (150 mM NaCl/5 mM EDTA/0.5% Triton X-100 in 50 mM Tris, pH 8.5) and incubated with specific antibody, antiserum or control rabbit IgG followed by the addition of protein A-Sepharose. Immunoprecipitates were washed three times with buffer B and, along with corresponding supernatants, were boiled in Laemmli sample buffer for 5 min. Proteins were resolved by SDS/8.0% PAGE and transferred to polyvinylidene fluoride membrane. The membrane was blocked with TBS/0.1% Tween 20/5% bovine albumin and probed with the appropriate antibodies. Immunoreactive proteins were detected using enhanced chemiluminescence.

**Immunohistochemical Localization.** Ovariectomized mice were injected s.c. with E2 (1 $\mu$g) or vehicle (0.1 ml PBS/1% ethanol). At 6 hr following treatments, uteri were perfusion-fixed with freshly prepared 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) (20). Immunolocalization of P-Tyr and IRS-1 was determined on trypsin-treated paraffin sections by the double peroxidase–antiperoxidase technique (21). The anti-P-Tyr antibody was from Transduction Laboratories (catalog no. P11230). Substitution of rabbit IgG for the primary antibodies served as the control.

## RESULTS

**Estradiol Stimulates Tyrosine Phosphorylation of Uterine and Liver Proteins.** To evaluate estrogen-induced phosphorylation of uterine and liver proteins, tissue extracts prepared at various times after treatment with E2 were evaluated by immunoblotting with anti-P-Tyr antibody. Within 4 hr, uterine extracts from hormone-treated animals revealed an increase in P-Tyr immunoreactivity at 76-, 170-, and 180-kDa; these bands were still apparent at 24 hr after E2 treatment (Fig. 1). A cluster of phosphorylated bands at 120–130 kDa that was observed in untreated animals intensified as a result of hormone treatment (Fig. 1). Immunoblots of extracts from livers of these animals showed an increase in P-Tyr immunoreactivity at 76 kDa by 4 hr after E2 exposure; this band was not apparent at 24 hr after hormone exposure (data not shown).

**Tyrosine Phosphorylation of Uterine IGF-1R.** To identify one or more of the uterine proteins that exhibited an increase in tyrosine phosphorylation as a result of treatment with E2, immunoprecipitates of various known receptor tyrosine kinases were evaluated with anti-P-Tyr antibody. Analysis of immunoprecipitates of the IGF-1R ($\beta$-subunit) revealed enhanced phosphorylation at 110-kDa in uterine extracts obtained 6 hr after E2 treatment (Fig. 2*A*). In contrast to the IGF-1R, the EGFR detected in uterine extracts did not exhibit a hormone-dependent increase in P-Tyr content at 6 hr following estrogen exposure (Fig. 2*B*). The immunoblots probed with receptor-specific antibodies revealed that receptor protein levels were not significantly altered following treatment with E2 within the time period studied (Fig. 2 *A* and *B*). Evaluation of the immunoprecipitation supernatants showed that the quantitative removal of each receptor prior to immunoblotting with anti-P-Tyr antibody did not reduce the levels of the E2-stimulated P-Tyr bands at 120 to 130-, 170-, and 180-kDa (Fig. 2 *A* and *B*). Incubating the extract from estrogen- or vehicle-treated (6 hr) mice with antiserum or antibody specific to either the PDGFR ($\alpha$- and $\beta$- subunits) or fibroblast growth factor type 2 receptor also resulted in precipitates that did not show a change in P-Tyr content (data not shown). Further examination of EGFR, PDGFR, and fibroblast growth factor type 2 receptor immunoprecipitates obtained from uterine extracts at 2 and 12 hr after treatment with E2 also showed no change in phosphorylation (data not shown).

To confirm that our methods can detect modulation of receptor phosphorylation in uteri of mature mice, we tested the capacity for EGF to stimulate tyrosine phosphorylation of uterine EGFR *in vivo*. Immunoblots of the EGFR immuno-

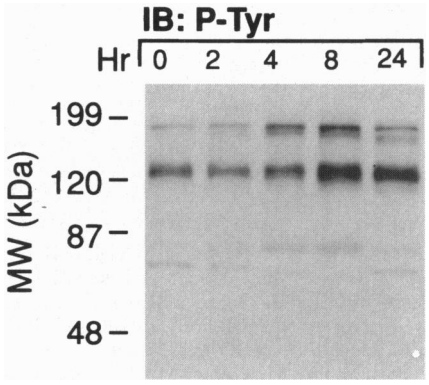


FIG. 1. Tyrosine phosphorylation in uterine extracts from ovariectomized CD-1 mice at various times after treatment with 1 $\mu$g E2. Immunoblots (IB) were evaluated with antibody to phosphotyrosine (P-Tyr). MW, molecular weight markers.

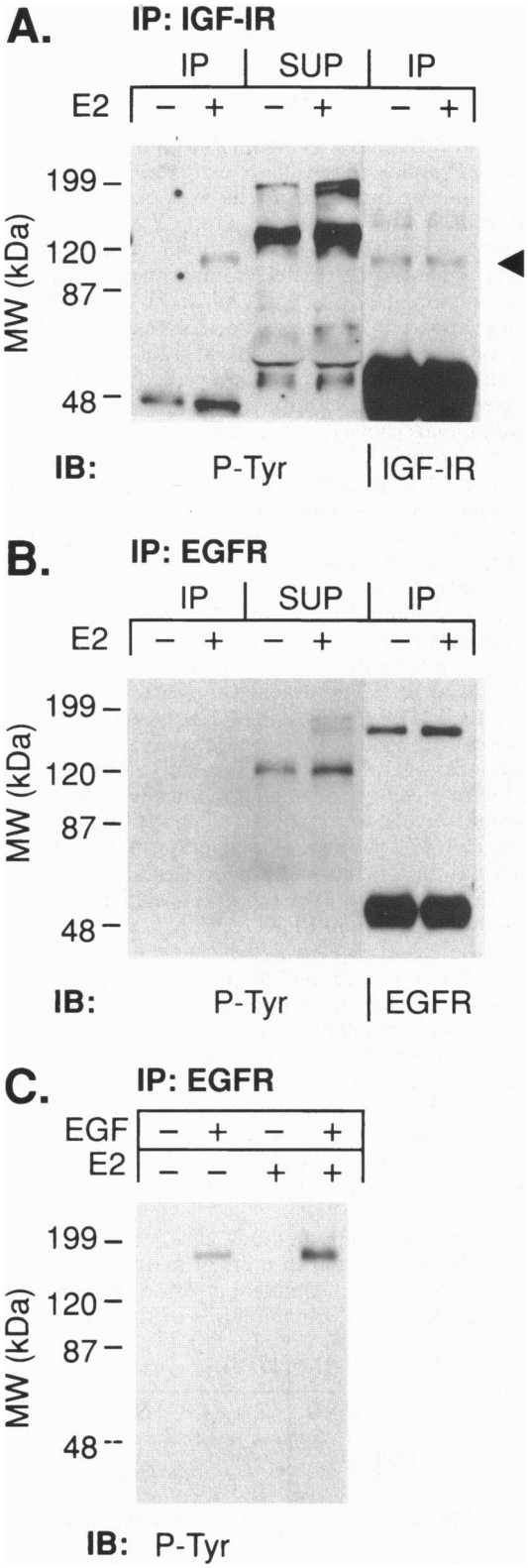


FIG. 2. Estradiol stimulates tyrosine phosphorylation of uterine IGF-1R. Uterine extracts were collected from CD-1 castrates 6 hr following exposure of 1 μg E2 (+) or vehicle (−). Aliquots of extracts were incubated with specific antibody to the (*A*) IGF-1R or (*B*) EGFR. Immunoprecipitates (IP) and IP supernatants (SUP) were evaluated by immunoblots (IB) with specific antibody to phosphotyrosine (P-Tyr) or to each receptor. The arrow denotes the β-subunit of the IGF-1R (110 kDa). (*C*) Six hours following treatment with E2 or PBS vehicle, mice were injected with EGF (100 μg) or vehicle (100 μl PBS) and uteri were collected 5 min later. EGFR IP were evaluated with P-Tyr antibody. MW, molecular weight markers.

precipitates showed increased P-Tyr content at a 170-kDa band as a result of treatment with EGF in the absence of E2 exposure (Fig. 2*C*). Estradiol apparently enhanced EGF-induced phosphorylation of the EGFR (Fig. 2*C*). Though uterine EGFR protein levels in the immature rat (22) and ovariectomized, mature mouse (this study, data not shown) were elevated at 12 hr after E2 exposure, the increase in EGFR levels at 6 hr after treatment with E2 was negligible compared with that of controls, indicating that differences in receptor content at 6 hr after hormone exposure do not account for the enhanced phosphorylation of the EGFR following treatment with EGF. This effect may be due to increased uterine vascular permeability following treatment with E2, which allows for greater influx of plasma proteins, including exogenously administered EGF, into the uterine tissue.

**Estradiol Enhances the P-Tyr Content of Uterine IRS-1.** Activation of the IGF-1R causes rapid stimulation of tyrosine phosphorylation of the 165- to 180-kDa IRS-1 (23). Since phosphorylation of the uterine IGF-1R β-subunit was induced by E2, we examined whether phosphorylation of IRS-1 was also estrogen-dependent. IRS-1 immunoprecipitates from uterine extracts revealed an increase in tyrosine phosphorylation of this protein in E2-treated mice when compared with that from controls (Fig. 3). This hormone-induced increase in phosphorylation was associated with a slightly decreased mobility on SDS/PAGE as compared with IRS-1 in controls (Fig. 3). To determine whether the 170- or 180-kDa P-Tyr band in uterine extracts has IRS-1 immunoreactivity, the extracts from hormone- or vehicle-treated animals were incubated with anti-IRS-1 antibody or control rabbit IgG. After immunoprecipitation with anti-IRS-1 antibody, examination of the clarified supernatants showed a negligible increase in the P-Tyr immunoreactivity of the 170-kDa band; the P-Tyr level of this band was markedly increased in supernatants collected after immunoprecipitation with rabbit IgG (Fig. 3).

**Time-Dependent, Uterine-, and Hormone-Specific Stimulation of Tyrosine Phosphorylation of the IGF-1R and IRS-1 by E2.** A coordinated increase in tyrosine phosphorylation of the IGF-1R and IRS-1 in the uterus was observed by 6 hr and was still apparent at 30 hr after E2 administration (Fig. 4 *A* and *B*). Estradiol did not increase tyrosine phosphorylation of liver

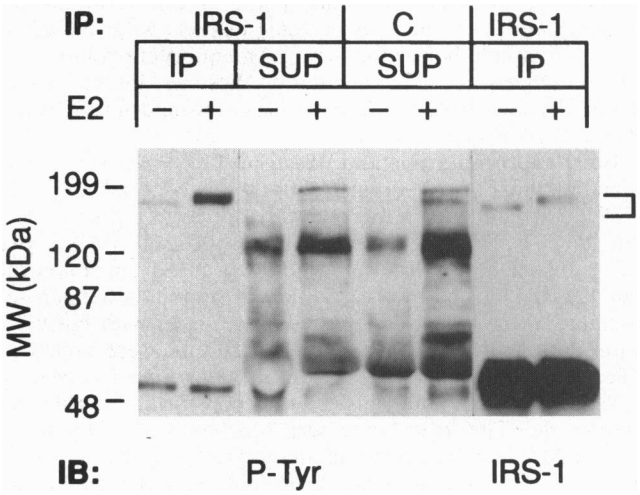


FIG. 3. Tyrosine phosphorylation of uterine IRS-1 is stimulated by E2. Uterine extracts were obtained from ovariectomized mice 6 hr after treatment with 1 μg E2 (+) or vehicle (−). Aliquots of extracts were incubated with anti-IRS-1 antibody or control rabbit IgG (C). Immunoprecipitates (IP) and IP supernatants (SUP) were examined by immunoblots (IB) with antibody to phosphotyrosine (P-Tyr) or IRS-1. The bracket denotes IRS-1, which decreases in mobility (160–170 kDa) on SDS/PAGE after treatment with E2. MW, molecular weight markers.

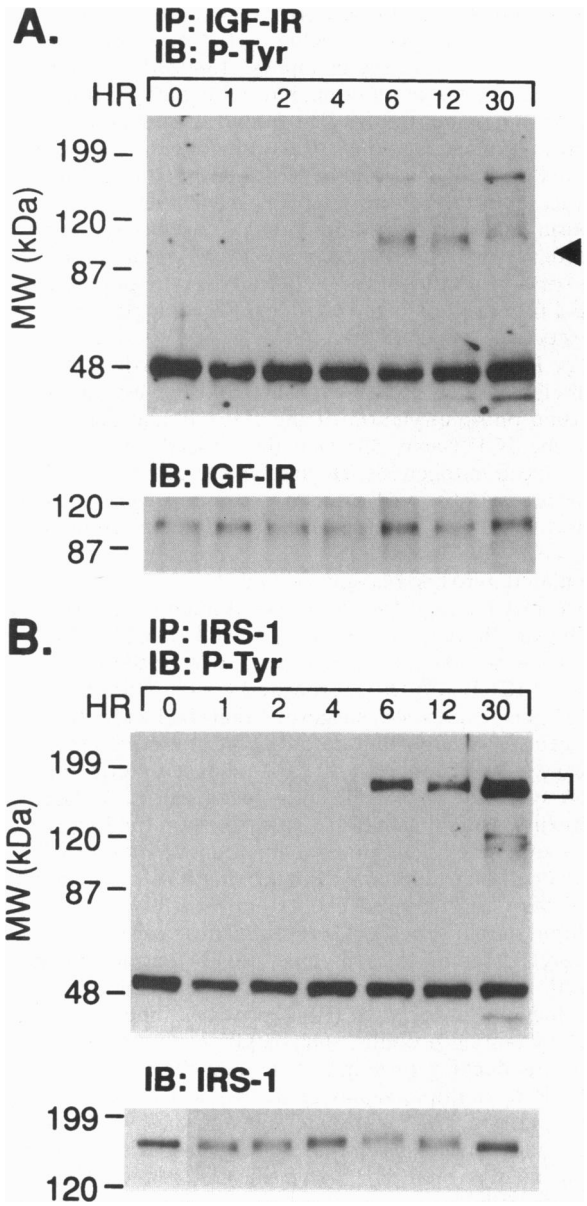


FIG. 4. Time-dependent stimulation of tyrosine phosphorylation of (*A*) IGF-1R and (*B*) IRS-1 by E2. At various times following exposure to E2 (1 μg), uterine extracts were obtained from ovariectomized mice. Immunoblots (IB) of the IGF-1R (β-subunit) or IRS-1 immunoprecipitates (IP) were evaluated with antibody to phosphotyrosine (P-Tyr), IGF-1R (β-subunit), or IRS-1. The arrow denotes the β-subunit of the IGF-1R (110 kDa). The bracket identifies the range of IRS-1 mobility (160–170 kDa) over time. The 160-kDa P-Tyr band that apparently coimmunoprecipitated with IGF-1R β-subunit has not been identified and is not immunoreactive with anti-IRS-1 antibody. MW, molecular weight markers.

IGF-1R or IRS-1 within the time period (0–12 hr) examined (data not shown). The hormone-induced decrease in the gel mobility of IRS-1 from uterine extracts, as demonstrated in Fig. 3, was time-dependent. When compared with IRS-1 from extracts collected at 0 hr (≈160 kDa), the mobility of this protein by 6 hr after E2 was slightly decreased (≈170 kDa); by 30 hr, the mobility of IRS-1 was similar to that observed in control (0 hr) samples (Fig. 4*B*). Estrogen-dependent changes in the gel mobility of liver IRS-1 were not detected (data not shown). At each period examined, IGF-1R and IRS-1 protein levels in uterine (Fig. 4 *A* and *B*) and liver (data not shown) extracts were not significantly altered by treatment with E2. In contrast to estrogen treatment, the P-Tyr content of the uterine IGF-1R and IRS-1 was not enhanced by progesterone, dexamethasone, or 5α-dihydrotestosterone within the time period examined (data not shown).

**Immunolocalization of P-Tyr and IRS-1 in Uterine Epithelial Cells.** Immunoreactive P-Tyr and IRS-1 were examined in sections of uteri from CD-1 castrates following treatment with E2 or vehicle to identify cell populations that contain components of the estrogen-activated IGF-1R pathway. Prior to hormone exposure, immunolocalization with anti-P-Tyr antibody revealed weak, variable staining among luminal and glandular epithelial cells; stromal and myometrial cells were negative (Fig. 5*A*). By contrast, sections from hormone-treated mice exhibited intense staining in most of the luminal and glandular epithelial cells; the stromal cells showed weak to moderate staining (Fig. 5*B*). Though IRS-1 immunoreactivity was weakly detectable in the epithelium of control mice (data not shown), the cytoplasm of nearly all luminal (Fig. 5*C*) and glandular (data not shown) epithelial cells exhibited strong IRS-1 immunoreactivity at 6 hr following treatment with E2. Stromal staining was not apparent in the estrogen-treated group (Fig. 5*C*). Staining was negligible in the epithelium and stroma of the uteri from mice treated with E2 or vehicle when control rabbit IgG was substituted for primary antibody (data not shown).

## DISCUSSION

In the reproductive tract, the interaction between the stroma and epithelium is crucial in mediating the biological response to steroid hormones (reviewed in ref. 24). On this basis, we felt it was critical in this study to use the whole animal to accurately assess what receptor tyrosine kinases are activated by estrogens. We evaluated the E2-dependent activation of various receptor tyrosine kinases in the adult mouse uterus to elucidate growth factor signaling pathways associated with the uterotropic response to estrogens. The IGF-1R pathway was activated by E2 in the mouse uterus as indicated by hormone-induced tyrosine phosphorylation of the IGF-1R and IRS-1. The IRS-1 was apparently equivalent to the 170-kDa P-Tyr band in the extract from hormone-treated mice. The uterine extracts contained additional E2-enhanced, P-Tyr bands (p180, p120-130, and p76), suggesting that other receptor tyrosine kinases and/or substrates are activated following estrogen exposure. As with p170 (IRS-1), one or more of these proteins may be part of the IGF-1R signaling cascade. Other receptor systems, including the receptors for growth hormone and various cytokines, can also engage IRS-1 (16) implying that hormonal stimulation of uterine IRS-1 P-Tyr content may be mediated, at least in part, independently of the IGF-1R. However, in this study IGF-1R and IRS-1 demonstrate coordinated increases in phosphorylation following E2 treatment, suggesting that the uterine IGF-1R kinase phosphorylates IRS-1.

The immunoreactive EGFR, PDGFR (α- and β-subunits), and fibroblast growth factor type 2 receptor that were identified in uterine extracts did not exhibit an increase in P-Tyr content by 12 hr following treatment with E2, indicating that these receptor tyrosine kinases are apparently not activated upon estrogen exposure within the time period investigated. Stimulation of tyrosine phosphorylation of the EGFR after EGF exposure was detected in the absence or presence of estrogen, demonstrating that an activated receptor tyrosine kinase can be recovered given the present experimental conditions. Because E2 stimulates the uterine synthesis of EGF (11), transforming growth factor α (12), and PDGF (6), these growth factor pathways may play roles distinct from IGF-1 in normal uterine physiology that is not manifested by the present experimental model, such as the implantation of the blastocyst and proliferation of stromal cells during decidualization (25, 26).

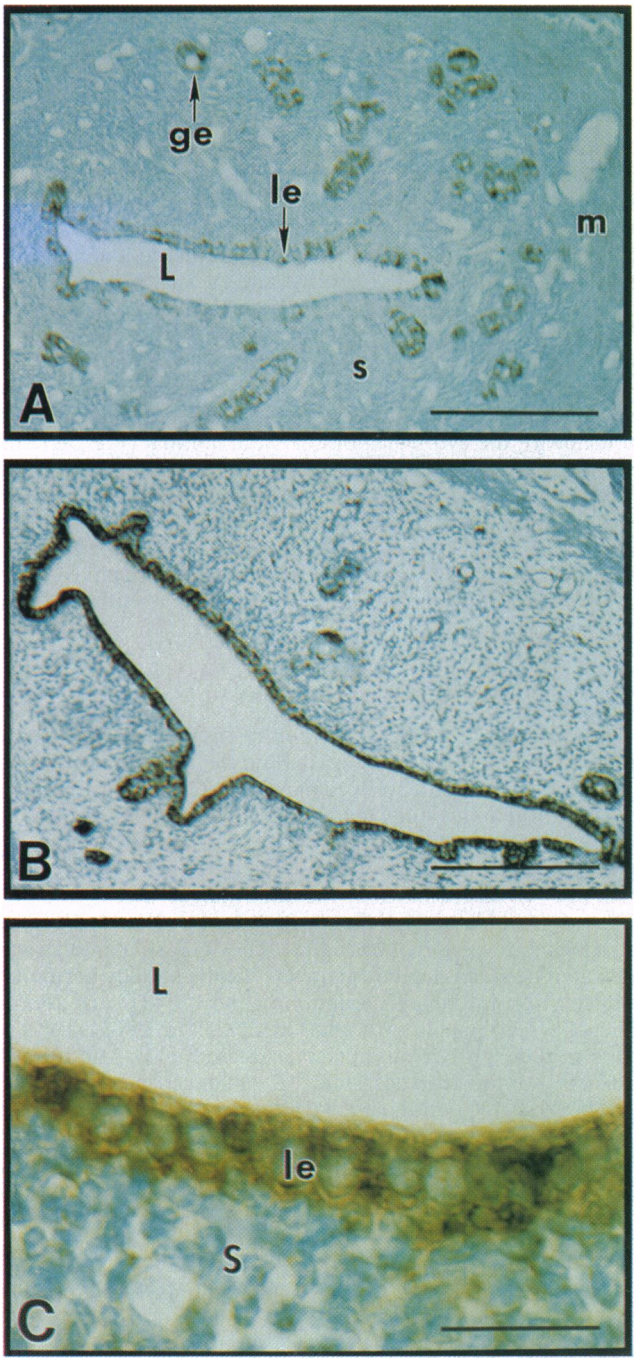


FIG. 5. Uterine epithelial cells contain immunoreactive P-Tyr and IRS-1. Estradiol (1 μg) or vehicle was administered to ovariectomized mice. After 6 hr, immunolocalization of P-Tyr or IRS-1 was determined on trypsinized paraffin sections of perfusion-fixed uteri by the double peroxidase-antiperoxidase technique. (*A*) In control animals, P-Tyr immunostaining was weak and variable among the luminal (le) and glandular (ge) epithelial cells; stromal (s) and myometrial (m) cells were negative. L, lumen. (Bar = 100 μm.) (*B*) Estrogen-treated mice exhibited intense P-Tyr immunoreactivity in virtually all luminal epithelial cells and most glandular epithelial cells; the majority of stromal cells showed weak to moderate staining. (Bar = 100 μm.) (*C*) Following treatment with E2, IRS-1 immunoreactivity was prominent in the cytoplasm of nearly all luminal epithelial cells; stromal staining was not evident. (Bar = 20 μm.) IRS-1 immunoreactivity appeared weaker in epithelial cells of control mice (data not shown) than in hormone-treated mice. This was not due to changes in protein level *per se*, since E2 did not apparently increase uterine IRS-1 levels as observed with immunoblots (see Fig. 4*B*).

Stimulation of tyrosine phosphorylation of the uterine IGF-1R and IRS-1 following hormone exposure is compatible with E2-enhanced transcription of the IGF-1 gene in the uterus of several species, including the rat (13), mouse (27), and monkey (28). In the hypophysectomized, ovariectomized rat, it was shown that uterine, but not hepatic or renal, IGF-1 mRNA was increased by 3 hr after E2 treatment (13). Our findings in mice extend these data by demonstrating that E2 stimulates the P-Tyr content of the IGF-1R and IRS-1 in the uterus, but not in the liver, by 6 hr following hormone exposure. The rapid stimulation of uterine IGF-1 mRNA abundance by E2 (13) coincides with the observations that *de novo* protein synthesis is not required for estrogen-induction of IGF-1 transcription in the rat uterus (29) or in primary cultures of osteoblastic cells (30).

The immunolocalization of P-Tyr and IRS-1 in the uterine epithelium following E2 exposure and the estrogen-specific tyrosine phosphorylation of the IGF-1R and IRS-1 suggest that the IGF-1-activated signaling cascade plays a role in E2-induced mitogenesis. Hormonal activation of the IGF-1R pathway was observed prior to the initial increase of DNA synthesis (≈8 hr) observed in mouse luminal epithelial cells following E2 exposure (1). The propagation of hormone-stimulated, tyrosine phosphorylated IGF-1R and IRS-1 in the uterus may be important for the continued progression of the epithelial cells into the S phase of the cell cycle (31). Reduction of uterine weight following treatment with the estrogen antagonist ICI 182,780 was associated with inhibition of uterine IGF-1 gene expression, suggesting that the local expression of this gene is an important determinant of uterine growth (32). Recently, it was reported that E2-enhanced uterine epithelial DNA synthesis was significantly decreased in castrated mice overexpressing rat insulin-like growth factor binding protein-1 in the uterine epithelium, as compared with that for wild-type mice, providing additional evidence that IGF-1 is an important mediator of E2-induced proliferation in this tissue (33). In addition to mitogenesis, IGF-1 stimulates progesterone receptor levels in rat uterine cells in culture (34), suggesting that this growth factor mediates multiple activities in the uterus. Since tyrosine phosphorylated IRS-1 provides binding motifs for SH2-containing proteins, such as Grb2 (35, 36) and PTP1D (37), this docking protein may couple the IGF-1-stimulated IGF-1R to multiple signaling pathways, including the mitogenic pathway (38). Alternate signaling pathways regulating IGF-1-induced cellular proliferation may exist, such as the engagement of the IGF-1R with Shc (38, 39). Identification of the intracellular proteins that are recruited to the uterine IGF-1R/IRS-1 following E2 stimulation will be crucial in defining the hormone-induced signaling pathways that elicit proliferation in this target tissue.

In this study, P-Tyr and a component of the IGF-1R pathway were observed in the uterine epithelium following treatment with E2, suggesting that the tyrosine phosphorylations observed in response to estrogen are reflecting, for the most part, hormonal action on the epithelium. Hormone-induced DNA synthesis in the estrogen-receptor devoid epithelium of the neonatal mouse uterus (40) and reexpression of estrogen-dependent proliferation by mouse uterine epithelial cells when recombined with homologous stroma and transplanted *in vivo* (41) suggest that the proliferative response of the epithelium is mediated by factors produced by the estrogen receptor-positive stroma in response to estrogens. These data and previous studies (3) suggest that IGF-1 may be one of these paracrine factors that mediates E2-induced uterine epithelial proliferation through activation of the IGF-1R and IRS-1 in the epithelium. Tissue recombination experiments with uteri from mature, estrogen receptor null mice (42) and wild-type littermates provide further evidence that the estrogen receptor in the stroma, but not in the epithelium, is required for hormone-stimulated epithelial proliferation (G. Cunha, personal communication).

In summary, we show for the first time, to the best of our knowledge, that E2 stimulates tyrosine phosphorylation of the IGF-1R and IRS-1 in the mouse uterus. Hormonal activation of IRS-1 is epithelial, estrogen-specific, and is initiated well before maximal epithelial DNA synthesis (≈16 hr) occurs following treatment with hormone, indicating that this protein probably has a pivotal role in the transduction of the hormonal signal for mitogenesis in the mouse uterus. We propose that IRS-1 becomes engaged in the proliferative pathway of the uterine epithelium after estrogen stimulation of IGF-1 synthesis in proximal stromal cells, which, in a paracrine manner, activates the IGF-1 receptor kinase in epithelial cells, leading to IRS-1 phosphorylation and subsequent signal coupling. Experiments with intact mice carrying null mutations of the genes encoding IGF-1 (43–45) or IRS-1 (46, 47) should be informative in relating the IGF-1/IGF-1R pathway to E2-induced proliferation and determining the importance of IGF-1 in stimulating other unknown receptor tyrosine kinases or downstream targets following hormone exposure.

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