Insulin-like Growth Factor-1 (IGF-1) Receptor-Insulin Receptor Substrate Complexes in the Uterus

ALTERED SIGNALING RESPONSE TO ESTRADIOL IN THE IGF-1<sup>m/m</sup> MOUSE*

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Some of the actions of estradiol occur through stimulation of growth factor pathways in target organs. Tyrosine-phosphorylated (Tyr(P)) insulin-like growth factor-1 receptor (IGF-1R) and the insulin receptor substrate (IRS)-1 are found in the uterus of mice treated with estradiol. Immunoprecipitates of uterine Tyr(P) IRS-1 contained both p85, the regulatory subunit of phosphatidylinositol (PI) 3-kinase, and PI 3-kinase catalytic activity. Estradiol also stimulated binding of IRS-1 and PI 3-kinase to the IGF-1R. Depletion of IRS-1 from uterine extracts reduced PI 3-kinase associated with the receptor, which suggests that binding of the enzyme to IGF-1R occurs primarily in a complex that also contains IRS-1. Following treatment with estradiol, formation of Tyr(P) IGF-1R, Tyr(P) IRS-1, and the p85 IRS-1 complex was very weak in the uterus of IGF-1<sup>m/m</sup> mice, which are severely deficient in IGF-1. This indicated that most, if not all, of the estradiol-stimulated Tyr phosphorylation of uterine IRS-1 originates from ligand activation of IGF-1R kinase. IRS-2 was also Tyr-phosphorylated in the normal uterus and bound more IGF-1R and p85 in response to estradiol; however, a marked decrease in levels of uterine IRS-2 occurred 12–24 h after treatment with estradiol. Since IRS-2 was present in IGF-1R precipitates and a recombinant form of IGF-1 (long R<sup>1</sup> IGF-1) stimulated formation of Tyr(P) IRS-2, hormonal activation of this docking protein probably occurs through the IGF-1R. In summary, our findings show that estrogen activation of uterine IGF-1R kinase results in enhanced binding of p85 (PI 3-kinase) to IRS-1 and IRS-2. The formation of one or both of these complexes may be important for the potent mitogenic action of this steroid. That estradiol stimulated a decrease of IRS-2, but not of IRS-1, suggests that these docking proteins have different roles in hormone-induced signaling in the uterus.

Estradiol exerts potent effects on gene expression and cell proliferation in the uterus. Several earlier studies indicated that local growth factor pathways may be important for the mitogenic action of estradiol (reviewed in Refs. 1–3). Our laboratory conducted a partial screen of known receptor tyrosine kinases in the mouse uterus for enhanced tyrosine phosphorylation in response to estradiol and found that the insulin-like growth factor-1 receptor (IGF-1R)<sup>1</sup> participates in hormone-induced signaling in this organ (4). This finding is compatible with the estradiol-stimulated increase of insulin-like growth factor-1 (IGF-1) transcripts in the uterus of some mammals (5–7), the presence of the IGF-1R mRNA in various uterine cell types, including the epithelium (8), and the property of the pure estrogen antagonist ICI 182,780 to reduce the level of IGF-1 mRNA in the rodent uterus (9). In addition, estradiol-enhanced uterine epithelial DNA synthesis was significantly decreased in castrated mice overexpressing rat IGF-binding protein-1 in the uterine epithelium, as compared with that for wild-type mice, providing evidence that IGF-1 is an important effector of estradiol-induced DNA synthesis in this tissue (10).

The IGF-1R is a heterotetrameric glycoprotein consisting of two extracellular α-subunits and two transmembrane β-subunits linked by disulfide bonds (reviewed in Ref. 11). Ligand binding to the α-subunit activates the tyrosine kinase of the β-subunit, resulting in autophosphorylation and phosphorylation of cellular substrates. A key component of several signaling intermediates is the Src homology 2 (SH2) domain that binds tyrosine-phosphorylated proteins in a sequence-specific manner (12). In vitro binding studies (13), as well as yeast two-hybrid assays (14–17), demonstrated that the IGF-1R can bind directly to the 85-kDa regulatory subunit (p85) of phosphatidylinositol (PI) 3-kinase, the protein-tyrosine phosphatase Syp, and the adapter protein Grb-10. Also, several of the SH2 domain-containing proteins can associate indirectly with the IGF-1R via cytoplasmic docking proteins (18, 19). One of the first docking molecules described was the insulin receptor substrate (IRS)-1 (20), a major substrate of the insulin and IGF-1 receptors (18–20) as well as receptors for growth hormone, several interleukins (IL-2, IL-4, IL-7, IL-9, IL-13, and IL-15), and interferons (αβ and γ) (21–25). IRS-1 belongs to a family of docking proteins that includes IRS-2 (26, 27), IRS-3 (28, 29), IRS-4 (30), and Gab-1 (31), which have binding modules and functional properties similar to those of IRS-1. Tyrosine phosphorylation sites in the IRS proteins and Gab-1 provide binding motifs for several distinct SH2 domain-containing proteins, including p85, Syp, and Grb-2 (28–32). Shc is another substrate of the IGF-1R tyrosine kinase (33). When tyrosine-phosphorylated, Shc can bind to the SH2 domain of Grb-2 (34), which, in turn, complexes with the GTP exchange protein Sos to activate Ras and the downstream mitogen-activated protein kinase cascade (35, 36).

To identify signaling intermediates immediately down-

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‡ The abbreviations used are: IGF-1R, insulin-like growth factor-1 receptor; IGF-1, insulin-like growth factor-1; SH2, Src homology 2; PI, phosphatidylinositol; IRS, insulin receptor substrate; IL, interleukin; Gab-1, Grb-2-associated binder-1; Tyr(P), phosphotyrosine; PAGE, polyacrylamide gel electrophoresis.

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stream of the ligand-activated IGF-1R tyrosine kinase following estrogen exposure in the uterus, tissues from castrated mice were examined for the hormone-induced binding of IRS-1 and SH2 domain-containing proteins to the IGF-1R. We utilized IGF-1-/-mice, which are profoundly growth-retarded due to low IGF-1 synthesis (37), to evaluate the relative importance of the IGF-1R ligand in mediating the estradiol-stimulated tyrosine phosphorylation of uterine IRS-1. We also examined the tyrosine-phosphorylated IGF-1R following hormone treatment for enhanced binding of PI 3-kinase (p85), Grb-2, and Syp and determined the importance of IRS-1 in the estradiol-stimulated association of p85 with the IGF-1R. Finally, changes in tyrosine phosphorylation, IGF-1R interaction, and SH2-containing protein association with IRS-2 following estradiol treatment were compared with those of IRS-1.

**EXPERIMENTAL PROCEDURES**

**Materials**—17β-Estradiol, l-α-PI, t-α-PI-4-monomophosphate, and ATP were purchased from Sigma. Long R3-IGF-1 was obtained from Diag-nostic Systems Laboratories, Inc. (Webster, TX). Protein A-Sepharose was purchased from Amersham Pharmacia Biotech. The precast minigels for electrophoresis were from NOVEX (San Diego, CA) or Bio-Rad. Prestained molecular weight standards were purchased from Bio-Rad and NOVEX, and the polyvinylidene fluoride membrane (Immobilon-P) was from Millipore Corp. (Bedford, MA). Reagents for enhanced chemilumi-nescence, Hyperfilm-ECL and [32P]PiATP were from Amersham Pharmacia Biotech. Silica gel (LHPKDF 60–A) TLC plates were from Whatman (Maidstone, United Kingdom).

**Antibodies**—Polyclonal antibodies against the extreme C terminus (amino acids 1222–1235) of rat IRS-1 (number 06–248), a glutathione S-transferase fusion protein containing residues 6–125 of rat IRS-1 pleckstrin homology domain (number 06–524), a glutathione S-transferase fusion protein containing residues 976–1094 of mouse IRS-2, the human Shc proteins, and the rat p85 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-serum raised against a glutathione S-transferase fusion protein containing amino acids 618–747 of mouse IRS-2 was a gift from Morris White (Joslin Diabetes Center, Boston, MA). Polyclonal antibodies against human IGF-1R (β-subunit), human SHP-2 or Syp, human Grb-2, and mouse Grb-10 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxi-dase-conjugated anti-phosphotyrosine (Tyr(P)) monoclonal antibody (PY20) was obtained from ICN (Costa Mesa, CA). A goat polyclonal anti-anti-Tyr(P) monoclonal antibody was from Calbiochem. Horseradish peroxidase-conjugated donkey anti-rabbit IgG was from Amersham Pharmacia Biotech; rabbit IgG was obtained from Vector Laboratories (Burlingame, CA).

**Animals**—Procedures with experimental animals followed the guide-lines of the NIEHS, National Institutes of Health, Animal Care and Use Committee. C57BL/6 mice (Charles River Laboratories) were ovariecto-mized at 76–82 days of age and were treated at 13–48 days after castration. Mice homozygous (IGF-1-/-) for a site-specific insertional mutation of the ifg1 allele were obtained from Lyn Powell-Braxton (Genentech, Inc.; San Francisco, CA). These mice are 36% smaller than the wild-type counterparts (37). In contrast to the ifg1 null mutants (38–40), the IGF-1-/-mice are viable and fertile, and they produce a small amount of wild-type IGF-1 mRNA and protein (37). Serum IGF-1 levels in IGF-1-/-mice are 30% of wild-type values (37). The IGF-1-/-mice and wild-type mice were ovarioctomized at 100–135 days of age and were treated at 14–47 days after castration. Each CD-1 mouse was treated subcutaneously with either a single injection of 1 μg of estradiol in 0.1 ml of PBS, 1% ethanol or three successive injections, at 5-min intervals, of 200 μg of long R3 IGF-1 in 0.1 ml of PBS, 0.2% bovine albumin. Each IGF-1-/-mice and wild-type mouse was injected subcutane-ously with estradiol (20 μg/kg) in 0.1 ml of PBS, 1% ethanol. Uterine tissues from all mice were collected at various times after treatment; controls received vehicle only. Tissue was homogenized at 4 °C as de-scribed previously (4). After centrifugation of uterine homogenates at 21,000 × g for 4 min, aliquots of the resulting supernatants (2–4 μg of protein) were applied in Laemmli sample buffer for 5 min, and an equivalent volume (10 μl) of each was evaluated by Western blot anal-ysis as detailed below. Protein concentrations were determined by the Pierce BCA protein assay.

**Immunoprecipitation and Western Blot Analysis**—Immunoprecipi-tates were obtained from additional aliquots of uterine supernatants (400–800 μg of protein), as previously reported (4), using specific anti-body (5 μg), antisera (1:80), or nonspecific rabbit IgG (5 μg). The immunoprecipitates were resolved by SDS-PAGE and transferred to polyvinylidene fluoride membrane. The membrane was blocked with either Tris-buffered saline, 0.1% Tween 20, 5% bovine albumin or PBS, 3% nonfat dry milk and probed with the appropriate antibodies. Immun-ohistochemical procedures were described previously.

**Sequential Immunoprecipitations**—Supernatants of uterine homogene-sates from estradiol- or vehicle-treated mice were incubated with anti-IRS-1 (C terminus) antibody or nonspecific IgG. Following adsorption of immune complexes to protein A-Sepharose, immunoprecipitation sup-ernatants were precipitated again with anti-IRS-1 antibody or non-specific IgG. After the second round of anti-IRS-1 antibody or nonspe-cific IgG immunoprecipitation, resulting supernatants were precipitated with anti-IGF-1R antibody. All immunoprecipitates were subjected to Western blot analysis, and additional IGF-1R precipitates were evaluated for PI 3-kinase activity as described below.

**PI 3-Kinase Activity**—Immunoprecipitates were subjected to an in vitro PI 3-kinase assay as described previously (19). The precipitates obtained 6 h after treatment of mice with estradiol or vehicle were washed successively with 1% Triton X-100, 100 μM Na3VO4 in PBS (three times); 500 mM LiCl, 100 μM Na3VO4 in 100 mM Tris-HCl, pH 7.5 (three times); and 100 mM NaCl, 1 mM EDTA, 100 μM Na3VO4 in 10 mM Tris-HCl, pH 7.5 (two times). The pellets were resuspended in 50 μl of 100 mM NaCl, 1 mM EDTA in 10 mM Tris-HCl, pH 7.5, and combined with 50 μl of 10 mM MgCl2, 100 μM ATP containing 300 μCi of [γ-32P]ATP. After 10 min at 22 °C with constant shaking on a Eppendorf thermomixer, the reaction was stopped with 20 μl of 8% HCl and 160 μl of CHCl3/methanol (1:1). After centrifugation, the lower organic phase was recovered and, along with the PI-4-monophosphate standard, applied to a silica gel TLC plate. TLC plates were developed in CHCl3/CH3OH/H2O/ONH4OH (99:78:18:5.3), dried, and visualized by autoradiography. [32P] incorporation into PI-3-monophosphate was quantified using a PhosphorImager (Molecular Dynamics, Inc.).

**RESULTS**

**Formation of an IGF-1R-IRS-1 Complex in the Uterus following Estrogen Exposure**—IGF-1R is a major substrate of the IGF-1R tyrosine kinase (19) and binds to a juxtamembrane motif (NPEY) of the IGF-1R as a result of ligand-induced phos-phorylation of the receptor (41). Since estradiol induces coordi-nated phosphorylation of the IGF-1R β-subunit and IRS-1 in the uterus (4), we examined whether this steroid hormone stimulates the formation of a complex containing both the IGF-1R and IRS-1. Immunoblot analysis of IGF-1R (β-subunit) precipitates from uterine extracts obtained 6 h after estradiol treatment showed increased tyrosine phosphorylation at 110 and 170 kDa; a 185-kDa Tyr(P) protein was evident by 12 h after hormone treatment (Fig. 1A). The tyrosine-phosphorylated 110-kDa protein was also present at 12 h after estradiol treatment, whereas phosphorylation of the 170- and 185-kDa proteins was still evident at 72 h after estrogen exposure (Fig. 1A). Immunoblot analysis of the IGF-1R (β-subunit) precipitates revealed that the 110- and 170-kDa Tyr(P) proteins correspond to the IGF-1R (β-subunit) and IRS-1, respectively (Fig. 1A). The identity of the 185-kDa Tyr(P) protein that coprecipitated with the IGF-1R β-subunit has not been determined. At each time period examined, there was no apparent change in IGF-1R protein levels following treatment with estradiol (Fig. 1A).

As previously reported (4), tyrosine phosphorylation of uterine IRS-1 increased by 6 h following estradiol treatment (Fig. 1B). When compared with IRS-1 from extracts of control mice (0 h), the Tyr(P) content of this docking protein was enhanced at 72 h after estrogen exposure (Fig. 1B). IRS-1 from extracts of control mice displayed a mobility of 160 kDa but shifted to 170 kDa by 6 h after estradiol treatment; by 48 h, the migration of IRS-1 was similar to that observed in control (0 h) samples (Fig. 1B). IRS-1 protein levels were comparable throughout the time period investigated (Fig. 1B).

** Estradiol-stimulated Tyrosine Phosphorylation of IGF-1R and IRS-1 is Impaired in the IGF-1R-/- Mouse**—The apparent
formation of an IGF-1R-IRS-1 complex suggests that IRS-1 is a substrate for this receptor; however, since various receptors have the potential to tyrosine-phosphorylate IRS-1 (21–25), we wanted to determine whether the observed estradiol-stimulated tyrosine phosphorylation of uterine IRS-1 is catalyzed predominantly by the IGF-1R kinase. Therefore, the hormone-induced change in Tyr(P) content of IGF-1R and IRS-1 in uterine extracts from IGF-1m/m mice was compared with that of wild-type mice. Following estrogen exposure, the increase in tyrosine phosphorylation of the uterine IGF-1R (Fig. 2A) and IRS-1 (Fig. 2B) of IGF-1m/m mice was much less than that of wild-type mice. Although estradiol-induced tyrosine phosphorylation of IRS-1 was negligible in the IGF-1m/m mutants, the IRS-1 immunoreactive band exhibited the same hormone-dependent decrease in gel mobility observed with that in wild types (Fig. 2B). The IGF-1R (Fig. 2A) and IRS-1 (Fig. 2B) protein levels in uteri from either vehicle- or estradiol-treated animals were comparable in mutant and wild-type mice. The uterine weights of ovariectomized wild-type and mutant mice both increased about 2-fold 6 h after treatment with hormone. When uterine sections from both groups were evaluated by light microscopy, no gross difference in histology was observed.

Estradiol Enhances the Interaction of p85 with the IGF-1R and IRS-1—To identify SH2 domain-containing proteins that associate with the IGF-1R and IRS-1 as a result of estrogen action, uterine extracts were examined by immunoprecipitation for possible interactions between either the IGF-1R or IRS-1 and various SH2-containing proteins. IGF-1R- and IRS-1-associated p85 was detected in control (0 h) samples, and binding of p85 to both the IGF-1R (Fig. 3A) and IRS-1 (Fig. 3B) protein levels in uteri from either vehicle- or estradiol-treated animals were comparable in mutant and wild-type mice. The uterine weights of ovariectomized wild-type and mutant mice both increased about 2-fold 6 h after treatment with hormone. When uterine sections from both groups were evaluated by light microscopy, no gross difference in histology was observed.

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of Grb-2 and Syp with IRS-1 were confirmed with anti-IRS-1 antibodies derived against two different regions of the molecule, the extreme C terminus region (Figs. 3, A and B, and 4A) and an N terminus region containing the pleckstrin homology domain (data not shown).

Immunoprecipitates of Grb-2 and Syp were also subjected to immunoblot analysis. Although IRS-1 was not normally detected in Grb-2 precipitates from mice treated with vehicle or estradiol (Fig. 4B), in some instances extended exposure (~5 min) of immunoblots to film revealed a weak IRS-1 band in Grb-2 precipitates from estradiol-treated mice (data not shown). In uterine extracts collected 6 h after treatment with estradiol or vehicle, no major tyrosine-phosphorylated bands were detected in Grb-2 precipitates (Fig. 4B). Likewise, Tyr(P) immunoprecipitates from extracts of control or estradiol-treated mice showed no detectable Grb-2 immunoreactivity (data not shown). In accord with our above findings, there was an increase in immunoreactive IRS-1 and p85 in Tyr(P) precipitates following estrogen exposure (data not shown). Syp immunoprecipitates from control or estradiol-treated mice revealed negligible amounts of IGF-1R (β-subunit) and IRS-1 immunoreactivity (Fig. 4C). An increased amount of a tyrosine-phosphorylated 120-kDa band was associated with Syp immunoprecipitates following treatment with estradiol (Fig. 4C); the identity of this band has not been determined.

The Tyr(P) content of the uterine 52- and 66-kDa Shc proteins was not changed by 12 h after treatment of ovariectomized mice with estradiol (data not shown). Additionally, immunoreactive Grb-2 was not observed in Shc precipitates prepared at various times up to 12 h after treatment with estradiol (data not shown). At each time period examined, Shc protein levels were not significantly altered by treatment with estradiol (data not shown). Given that the IGF-1R and IRS-1 bound p85, but not Shc, Grb-2, and/or Grb-10, following estrogen exposure, we also utilized IGF-1m/m mice to determine whether the weak activation of uterine IGF-1R observed after treatment of these mice with estradiol results in a correspondingly low level of p85 associated with the IGF-1R or IRS-1. Immunoblots of IGF-1R immunoprecipitates from mutant mice obtained 6 h after treatment (data not shown). Additionally, immunoreactive Grb-2 was not observed in Shc precipitates prepared at various times up to 12 h after treatment with estradiol (data not shown). At each time period examined, Shc protein levels were not significantly altered by treatment with estradiol (data not shown). Given that the IGF-1R and IRS-1 bound p85, but not Shc, Grb-2, and/or Grb-10, following estrogen exposure, we also utilized IGF-1m/m mice to determine whether the weak activation of uterine IGF-1R observed after treatment of these mice with estradiol results in a correspondingly low level of p85 associated with the IGF-1R or IRS-1. Immunoblots of IGF-1R immunoprecipitates from mutant mice obtained 6 h after treatment with estradiol revealed only a minor increase in p85 immunoreactivity when compared with that of vehicle-treated IGF-1m/m mice; an estrogen-dependent increase in the IGF-1R-associated p85 was readily detected in wild-type mice (Fig. 5A). The binding of p85 to IRS-1 was negligible in IGF-1m/m mice after treatment with either estradiol or vehicle; by contrast, a marked increase in the interaction of p85 with IRS-1 was observed in wild-type mice following estradiol exposure (Fig. 5B).

FIG. 4. Grb-2 and Syp do not associate with the uterine IGF-1R and IRS-1 following estradiol exposure. Tissue extracts were prepared from CD-1 castrates 6 h after treatment with 1 μg of estradiol (E) or vehicle (C). Panel A, immunoprecipitates (IP) of the IGF-1R and IRS-1 as well as supernatants of uterine extracts (Ext) were separated by SDS-15% PAGE and immunoblotted with antibody to Grb-2 or Syp. Grb-2 (panel B) or Syp (panel C) immunoprecipitates were separated by SDS-7.5% PAGE or SDS-15% PAGE and immunoblotted with antibody to phosphotyrosine (P-Tyr), IRS-1, IGF-1R, Grb-2, or Syp. In panel A, the weak doublet observed in IGF-1R and IRS-1 precipitates was also detected in nonspecific IgG control precipitates, and the smaller protein (~25 kDa) in this doublet migrated slightly faster than the immunoreactive Grb-2 (~27 kDa) observed in uterine extracts (lane 7). In addition, Western blots of the nonspecific IgG, IGF-1R, and IRS-1 precipitates incubated with only the secondary antibody revealed a doublet with the same migration pattern (data not shown). These data suggest that the bands comprising this doublet are not Grb-2-specific and are likely contributed by the small chain component of the IgG molecule. In Fig. 4C, the identity of the Syp-associated, 120-kDa Tyr(P) band has not been determined. Molecular mass markers are expressed in kDa. Results presented are representative of three separate experiments.
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To determine whether in vivo activation of the uterine IGF-1R by a cognate ligand elicits similar intracellular signaling events as those observed following treatment with estradiol, mice were treated with long R3 IGF-1, a congener of human IGF-1 that has relatively low affinity to IGF-1-binding proteins (42). As expected, the Tyr(P) content of the IGF-1R as well as that of IRS-1 increased rapidly after administration of long R3 IGF-1 to mice (Fig. 6). This polypeptide also increased the amount of p85 in precipitates of the receptor or IRS-1 (Fig. 6). Long R3 IGF-1 did not induce the decrease in IRS-1 mobility that was observed following treatment with estradiol (Fig. 6). These data confirm that ligand-induced activation of the uterine IGF-1R kinase results in tyrosine phosphorylation of IRS-1 and formation of an IRS-1-p85 complex.

The Increase in PI 3-Kinase Association with the Uterine IGF-1R after Estrogen Exposure Is Dependent on IRS-1—To determine whether the estradiol-induced interaction of p85 with the IGF-1R and IRS-1 coincides with the association of receptor and IRS-1 with phospholipid kinase activity, IGF-1R and IRS-1 precipitates from control and estradiol-treated mice were subjected to an in vitro PI 3-kinase assay. In accord with a hormone-dependent increase in binding of p85 with the IGF-1R and IRS-1 (Fig. 3, A and B), the association of PI 3-kinase activity with the receptor and docking protein increased approximately 4- and 6-fold, respectively, after treatment with estradiol (Fig. 7A). Since estradiol apparently stimulates the formation of a complex in the uterus containing the IGF-1R, IRS-1, and PI 3-kinase, we examined whether p85 and IRS-1 each bind to independent populations of the IGF-1R or whether a IGF-1R, IRS-1, and p85 complex predominates after estradiol treatment. To resolve this, IGF-1R precipitates were obtained from uterine extracts that were depleted of most of the IRS-1 by two successive precipitations with anti-IRS-1 antibody or rabbit IgG. Supernatants immunodepleted (ID) of IRS-1 or control (CN) supernatants were precipitated with anti-IGF-1R antibody. Panel B, immunoprecipitates were separated by SDS-7.5% PAGE and immunoblotted with antibody to phosphotyrosine (P-Tyr), p85, IRS-1, or p85. Molecular masses are expressed in kDa. Results are representative of four separate experiments.

The estradiol-stimulated interaction of PI 3-kinase (p85) with the IGF-1R occurs primarily in a complex that also contains IRS-1. Uterine extracts were obtained from ovariectomized CD-1 mice 6 h after treatment with 1 μg of estradiol (E) or vehicle (C). Panel A, IGF-1R and IRS-1 immunoprecipitates (IP) were analyzed for PI 3-kinase activity. In addition, aliquots of tissue extracts were subjected to two rounds of precipitation with anti-IRS-1 antibody or rabbit IgG. Supernatants immunodepleted (ID) of IRS-1 or control (CN) supernatants were precipitated with anti-IGF-1R antibody. Panel B, immunoprecipitates were separated by SDS-7.5% PAGE and immunoblotted with antibody to phosphotyrosine (P-Tyr), IRS-1, IGF-1R, or p85. Panel C, additional IP were assayed for PI 3-kinase activity. PI-3P, phosphatidylinositol 3-monophosphate. Molecular mass markers are expressed in kDa. Data are representative of four separate experiments.

Hormonal Activation of Uterine IGF-1R Also Stimulates Tyrosine Phosphorylation of IRS-2—Since IRS-2 is a potential substrate of the IGF-1R tyrosine kinase (43), we examined this protein for changes in Tyr(P) content at various times after treatment with estradiol. Tyrosine-phosphorylated IRS-2 was

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**Fig. 6.** Long R3 IGF-1 stimulates tyrosine phosphorylation of the uterine IGF-1R and IRS-1 and their interaction with p85. Ovariectomized CD-1 mice were treated with three successive injections, 5 min apart, of 200 μg of long R3 IGF-1 (D) or vehicle (C), and uteri were obtained 5 min after the last treatment. IGF-1R and IRS-1 immunoprecipitates (IP) from tissue extracts were separated by SDS-7.5% PAGE and immunoblotted with antibody to phosphotyrosine (P-Tyr), p85, IGF-1R, or IRS-1. Molecular masses are expressed in kDa. Results are representative of three separate experiments.

**Fig. 7.** The estradiol-stimulated interaction of PI 3-kinase (p85) with the IGF-1R occurs primarily in a complex that also contains IRS-1. Uterine extracts were obtained from ovariectomized CD-1 mice 6 h after treatment with 1 μg of estradiol (E) or vehicle (C). Panel A, IGF-1R and IRS-1 immunoprecipitates (IP) were analyzed for PI 3-kinase activity. In addition, aliquots of tissue extracts were subjected to two rounds of precipitation with anti-IRS-1 antibody or rabbit IgG. Supernatants immunodepleted (ID) of IRS-1 or control (CN) supernatants were precipitated with anti-IGF-1R antibody. Panel B, immunoprecipitates were separated by SDS-7.5% PAGE and immunoblotted with antibody to phosphotyrosine (P-Tyr), IRS-1, IGF-1R, or p85. Panel C, additional IP were assayed for PI 3-kinase activity. PI-3P, phosphatidylinositol 3-monophosphate. Molecular mass markers are expressed in kDa. Data are representative of four separate experiments.
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FIG. 8. Estradiol increases tyrosine phosphorylation of uterine IRS-2 and its association with the IGF-1R and p85. At various times after injection of 1 μg of estradiol (E) or vehicle (C), tissue extracts were collected from CD-1 castrates. IRS-2 (panel A), IGF-1R (panel B), and p85 (panel C) immunoprecipitates (IP) were separated by SDS-7.5% PAGE and immunoblotted with antibody to phosphotyrosine (P-Tyr), IRS-2, or p85. In panel C, nonspecific IgG served as a control. Molecular mass markers are expressed in kDa. Results are representative of four (A and B) or two (C) separate experiments.

The present data support and extend these earlier findings by showing that estradiol stimulates the formation of an IGF-1R-IRS-1 p85 3-kinase signaling complex in the mouse uterus, which is analogous to the insulin-induced formation of an insulin receptor-IRS-1 p85 3-kinase ternary complex in Chinese hamster ovary cells (45) and rat HTC hepatoma cells (46). We also demonstrate that the estradiol-enhanced tyrosine phosphorylation of IGF-1R and IRS-1, as well as formation of a p85-IRS-1 complex, in the uterum of IGF-1m/m mice was very weak. This suggests that the estradiol-stimulated uterine synthesis of IGF-1 observed in rodents (5, 6) is impaired in the IGF-1m/m mouse and underscores the importance of IGF-1 in mediating this estradiol-induced signaling pathway. Other receptor tyrosine kinases and cytokine receptors, along with their receptor-associated kinases, are present in the uterus and may be activated by estradiol (2, 3, 47); however, our cumulative findings indicate that it is the uterum IGF-1R tyrosine kinase that mediates the estrogen-stimulated tyrosine phosphorylation of IRS-1 and the subsequent binding of IRS-1 to PI 3-kinase.

Although the tyrosine-phosphorylated IGF-1R and IRS-1 have been shown to associate, either directly or indirectly, with Shc, Grb-2, Grb-10, and Syp (14–17, 32), our present data show that IGF-1R/IRS-1 interaction with these SH2 domain-containing proteins was negligible in response to estradiol. These findings suggest that the estrogen-stimulated uterine IGF-1R-IRS-1 3-kinase signal transduction pathway does not proceed through Shc, Grb-2, Grb-10, or Syp. Since the present experimental model examines only estradiol-dependent signaling, it is possible that one or more of these SH2-containing proteins function dependently or independently of IGF-1R and IRS-1 in uterine signaling pathways activated by other hormones.

The present findings show that the estradiol-induced increase in PI 3-kinase associated with the IGF-1R represents PI 3-kinase bound primarily to receptor-IRS-1 complexes. We also have data from IGF-1-deficient mice (48, 49) that reveal a marked reduction in the IGF-1R-associated p85 obtained from uterine extracts of either estradiol- or vehicle-treated animals when compared with that of wild-type mice; this suggests that the association of PI 3-kinase with uterine IGF-1R occurs indirectly by binding to IRS-1. These data are consistent with in vitro studies that examined the role of IRS-1 in mediating the interaction of PI 3-kinase with the IGF-1 and insulin receptors following ligand binding (19, 50). The IGF-1R immunoprecipitates obtained from uterine extracts of control mice or from extracts of estrogen-treated mice initially depleted of IRS-1

2 G. Richards and R. DiAugustine, unpublished observation.
The availability of more IGF-1m/m mice should help determine whether IGF-1R and p85, demonstrating the estrogen-dependent formation of an IGF-1R-IRS-2:p85 complex in the mouse uterus. The rapid stimulation of the Tyr(P) content of IRS-2 and its association with p85 in the uterus following pharmacological administration of long R3 IGF-1 also indicates that this docking protein has the capacity to serve as a substrate for the uterine IGF-1R kinase. These findings suggest that both IRS-1 and IRS-2 are downstream components of the estradiol-induced IGF-1R pathway in the uterus. Although IRS-2 appears to serve as a substrate for the uterine IGF-1R tyrosine kinase, cytokines, such as IL-3, IL-4, and granulocyte-macrophage colony-stimulating factor, stimulate tyrosine phosphorylation of IRS-2 in various lymphohemopoietic cells (52), and estradiol can increase the synthesis of various cytokines, including granulocyte-macrophage colony-stimulating factor (53), in the mouse uterus (47). Thus, a receptor(s), in addition to the IGF-1R, may be mediating the estradiol-induced phosphorylation of IRS-2. The availability of more IGF-1m/m mice should help determine the extent to which IGF-1R contributes to tyrosine phosphorylation of uterine IRS-2 in response to estradiol.

The loss of IRS-2, but not IRS-1, in response to estradiol suggests nonredundant roles for these docking proteins in hormone-stimulated signaling in the uterus. Potential mechanisms for such divergence in regulation of these IRS proteins could include differences in IRS-associated proteins or differences in intracellular or cellular localization within the uterus. As was previously shown for IRS-1 (4), IRS-2 was immunolocalized primarily in the uterine epithelium of estradiol-treated mice, which suggests that estradiol may have differential effects on these proteins located within the same uterine cell type. When 3T3-L1 adipocytes were exposed to insulin for at least 30 min, IRS-1 was down-regulated due to an apparent decrease in the protein half-life (54). In the present study, increased proteolysis of IRS-2 could account for the decrease in levels of this docking protein in response to estradiol.

IRS-2 was dephosphorylated more rapidly than IRS-1 in skeletal muscle of insulin-treated rats and in IGF-1- and insulin-stimulated L6 myoblasts (51). In addition, insulin stimulated the association of PI 3-kinase with both IRS-1 and IRS-2, yet IRS-2 dephosphorylation was regulated, at least in part, by its associated PI 3-kinase activity, whereas IRS-1-associated PI 3-kinase had no apparent effect on IRS-1 Tyr(P) content. In skeletal muscle tissue (51) and uterine tissue (present data), IRS-associated PI 3-kinase may have unique functions depending on the associating docking protein, and this diversity could be due to the existence of multiple isoforms of PI 3-kinase (55). At least five forms of the regulatory subunit for PI 3-kinase have been identified (55–57), and each subtype appears to have different functions (57). In addition, the catalytic subunit of PI 3-kinase possesses both lipid and serine kinase activities (58), and IRS-1 has been shown to serve as a substrate for the serine kinase activity of PI 3-kinase (59). Serine phosphorylation of the IRS proteins represents a potential regulatory mechanism of insulin and IGF-1-stimulated cellular responses, since okadaic acid-, tumor necrosis factor α-, IL-4-, and insulin-enhanced serine phosphorylation of either IRS-1 or IRS-2 attenuated insulin or IGF-1 action in 3T3-L1 adipocytes (60), rat hepatoma Fao cells (61), and bovine fibroblasts (62). The estradiol-induced decrease in IRS-1 and IRS-2 electrophoretic mobility may result from enhanced phosphoserine content of the IRS proteins (50, 60, 61). In the uterus, hormone-dependent serine phosphorylation of the IRS proteins may be important in regulating the signaling capacity of these docking proteins in response to estrogen. The lack of change in IRS-1 and IRS-2 electrophoretic mobility after long R3 IGF-1 treatment, and the estradiol-induced decrease in migration of IRS-1 in IGF-1m/m mice suggest that the hormone-stimulated shift in IRS gel mobility occurs independently of both IGF-1/IGF-1R and tyrosine phosphorylation of IRS-1.

In summary, we show that estradiol stimulates the formation of an IGF-1R-IRS-1-PI3-kinase complex in the mouse uterus. Furthermore, we establish that the estrogen-induced tyrosine phosphorylation of IRS-1 and its association with PI 3-kinase is mediated, for the most part, by the ligand-activated IGF-1R kinase. Tyrosine phosphorylation of IRS-2 and the presence of IRS-2-associated p85 were also stimulated by estradiol, probably as a result of activation of the IGF-1R kinase. The estrogen-stimulated decrease in IRS-2, but not that of IRS-1, suggests that these proteins have divergent roles in hormone-dependent signaling in the uterus. The estradiol-enhanced interaction of p85 (PI 3-kinase) with either IRS-1 or IRS-2 or both of these docking proteins may be pivotal in transducing the hormonal signal for mitogenesis in the uterus. Experiments with mice carrying the IGF-1m/m mutation or null mutations of genes encoding IGF-1 (38–40) or IRS-1 (48, 49) should be informative in determining the relevance of the IGF-1R-IRS-1 PI 3-kinase pathway to estradiol-induced proliferation and examining the role of IGF-1 in stimulating other potential substrates of the IGF-1R kinase, such as Gab-1 (31), IRS-3 (28, 29), and IRS-4 (30), in response to hormone. Additional in vitro experiments evaluating IGF-1-induced proliferation in uterine epithelial cells following systematic inhibition of PI 3-kinase and its major targets, such as AKT (63) and p70s6 kinase (64), should help resolve whether PI 3-kinase and potential downstream intermediates are required for the mitogenic response.

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Insulin-like Growth Factor-1 (IGF-1) Receptor-Insulin Receptor Substrate Complexes in the Uterus: ALTERED SIGNALING RESPONSE TO ESTRADIOL IN THE IGF-1m/m MOUSE

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