G-protein-linked receptors and intrinsic tyrosine-kinase growth receptors represent two prominent modalities in cell signaling. Cross-regulation among members of both receptor superfamilies has been reported, including the counter-regulatory effects of insulin on β-adrenergic catecholamine action. Cells stimulated by insulin show loss of function and increased phosphotyrosine content of β2-adrenergic receptors. Phosphorylation of tyrosyl residues 350/354 of β2-adrenergic receptors is obligatory for counter-regulation by insulin (Karoor, V., Baltensperger, K., Paul, H., Czech, M., and Malbon, C. C. (1995) J. Biol. Chem. 270, 25305-25308), suggesting the hypothesis that G-protein-linked receptors themselves may act as substrates for the insulin receptor and other growth factor receptors. This hypothesis was evaluated directly using recombinant human insulin receptor, hamster β2-adrenergic receptor, and an in vitro reconstitution and phosphorylation assay. Insulin is shown to stimulate insulin receptor-catalyzed phosphorylation of the β2-adrenergic receptor. Phosphoamino acid analysis establishes that insulin receptor-catalyzed phosphorylation of the β2-adrenergic receptor in vitro is confined to phosphotyrosine. High pressure liquid chromatography and two-dimensional mapping reveal insulin receptor-catalyzed phosphorylation of the β2-adrenergic receptor at residues Tyr141, Tyr202, Tyr205, Tyr350, Tyr354, and Tyr364, known sites of phosphorylation in response to insulin in vivo. Insulin-like growth factor-I receptor as well as the insulin receptor displays the capacity to phosphorylate the β2-adrenergic receptor in vitro, establishing a new paradigm, i.e. G-protein-linked receptors acting as substrates for intrinsic tyrosine kinase growth factor receptors.
porated into the rIR β-subunit itself.

Phosphoamino Acid Analysis of βAR—rIR (5 μl, 100–200 fmol) from CHO-T cells or from COS-1 cells was reconstituted with rIR (20 μl, 10–20 pmol) in the absence or the presence of insulin (100 nm) and incubated for 30 min at 22°C with [γ-32P]ATP (5 μM) as described above. Phosphorylated proteins were then separated by SDS-PAGE and visualized by autoradiography of the dried gel. The regions of interest were excised from the gel, rehydrated, and subjected to acid hydrolysis in 6 M HCl (7, 12). Phosphoamino acids were separated by thin-layer electrophoresis at pH 3.5 and visualized by autoradiography as described (4, 9). Proportional amounts of radioactivity as detected in the rehydrated gel pieces were resolved and detected.

Phosphorylation of Synthetic Peptide Substrates—Peptides corresponding to the cytoplasmic domains of the βAR displaying phosphorylation of tyrosyl residues in vivo (6) were synthesized, purified by HPLC, and subjected to in vitro phosphorylation by rIR in the absence or the presence of insulin (100 nm). The peptide sequences employed as internal standards for the HPLC and high voltage electrophoresis after tryptic digestion were as follows: L339, LCLRRSSKKAYGNQYS-NSNGKTD; T362, TDYMGAEASGCQGOE; R62, RLOTVTVNYFITS-LACAD; Y132, YIATSPKYSOLLTKNKAR; and I135, ITSPKYSOLLTKALKAR. Partially purified rIR (wheat germ agglutinin extracts from CHO-T cells) was incubated in the absence or the presence of insulin (100 nm), 10 μM [γ-32P]ATP, and the synthetic peptides at the concentrations indicated for 30 min at 22°C. The reaction was stopped by adding an equal volume of 2× concentrated Laemmli sample buffer. Phosphopeptides were separated by Tricine gel electrophoresis (13).

Reverse-phase HPLC of Tryptic Phosphopeptides—rIR radiolabeled in vitro by rIR with [γ-32P]ATP were separated on SDS-PAGE (6). Synthetic peptides containing tyrosine residues 141, 350, 354, and 364 were labeled in vitro with [γ-32P]ATP and separated on Tricine gels. The bands corresponding to rIR or the synthetic peptides were excised from the gels and treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (40 μg/ml) for 18 h at 37°C (9). The trypsin eluate was then separated on a microbore HPLC (Applied Biosystems) using a 220-mm Aquapore OD-300 column and a gradient of acetonitrile (0–50% in 45 min) in 0.1% trifluoroacetic acid at a flow rate of 200 μl/min. Fractions were collected at 1-min intervals, and Cerenkov radiation ([32P] window) was measured for each fraction. The standards for tryptic digests of L339 and T362 reported earlier (6) are included for reference.

Two-dimensional Peptide Mapping—Tryptic digestion of rIR (64). The phosphorylated rIR present in the IR preparations was observed occasionally as shown here. Insulin-stimulated phosphorylation of rIR was observed in reconstitutions with lectin-purified 1R from either CHO-T cells or a second source, SV40-transformed African green monkey kidney COS-1 cells, which were transiently transfected with the human insulin receptor cDNA (not shown).

Phosphoamino acid analysis of rIR and rIR products from an in vitro phosphorylation reaction revealed phosphorylation labeling of the rIR β-subunit, whether performed with rIR expressed in CHO-T (Fig. 1, lanes 1 and 2) or COS-1 (lanes 3 and 4) cells. The rIR β-subunit was predominantly phosphorylated on tyrosine residues (Fig. 2, lanes 5 and 6), providing an internal control. Some phosphoseryl in the rIR β-subunit was detected, which is due to low level serine kinase activity of the IR tyrosine kinase (9). Similarly, upon overexposure of the autoradiography, some phosphoserine in the rIR β-subunit was detected, which is consistent with the hypothesis that the insulin receptor directly interacts with and phosphorylates the rIR β-subunit.

We explored whether the β2-adrenergic receptor was a substrate for a second growth factor–activated tyrosine kinase receptor, the IGF-I receptor, which is structurally closely related to the IR (14). We assayed the ability of insulin to stimulate phosphorylation of rIR in vitro in reconstitution studies with 1R purified from human osteogenic sarcoma cell extracts (Fig. 3, lanes 2–5) and compared it with the phosphorylation of rIR by rIR (Fig. 3, lane 1, and Fig. 1). The 1R–R phosphorylated rIR in response to stimulation by high concentrations (1 μM) of insulin (lane 3). 1R–R-dependent phosphorylation of the 45,000 M, protein was absent in the reconstitutions devoid of rIR (lanes 4 and 5). A small amount of phosphorylation was observed in the lectin-purified extract rich in 1R in the absence of hormonal stimulation (lane 3), which may reflect phosphorylation catalyzed by platelet-derived growth factor receptor, which was activated (autophosphorylated) in this fraction (see *, Fig. 3, lanes 2–5). These observations demonstrate that tyrosine kinase growth factor receptors, in addition to the IR, can catalyze specific phosphorylation of rIR in response to insulin.

In an effort to explore the site(s) for insulin-stimulated, rIR–rIR catalyzed phosphorylation, recently we prepared synthetic peptides corresponding to cytoplasmic regions of the β2AR that
The synthetic peptides were designed not only to probe all cytosolic tyrosyl residues available for phosphorylation by IR but also to provide a source of tryptic fragments in which the candidate sites for tyrosine kinase phosphorylation were imbedded (6). Maps of tryptic digestions might permit analysis of the sites phosphorylated on the β2AR in response to insulin in vitro (Fig. 4). Tryptic digests of peptides phosphorylated in vitro by rIR in response to insulin provided markers for HPLC analysis (Fig. 4, A–C). The retention times for the tryptic fragments subjected to HPLC separation agreed well with the retention times calculated from the sequence information (not shown). For tryptic digests of phosphorylated β135 peptide, uncleaved peptide was detected routinely (*, Fig. 4A). Tryptic digests of phosphopeptide Y132 display the same mobility as the fragments of I135 (not shown), i.e., fraction 4 (Fig. 4A). The Y132 peptide was shown previously to be the preferred substrate for insulin-stimulated, rIR-catalyzed phosphorylation (6). Using these labeled standards, we established that β2AR reconstr-
tated in vitro with rIR in the presence of insulin was phosphorylated predominantly on peptides harboring residues Tyr131/Tyr141, Tyr350/Tyr354, and Tyr364 (Fig. 4D). Phosphorylation of the β2AR by rIR in vitro was not detected in the absence of insulin.

High voltage electrophoresis followed by thin-layer chromatography of the tryptic fragments (Fig. 5, A–C) provides additional markers for analysis of phosphopeptides derived from the β2AR phosphorylated by insulin-stimulated rIR using the in vitro reconstitution assay (Fig. 5D). The two-dimensional analysis extends the results of reverse-phase HPLC, establishing that the predominant sites of insulin-stimulated phosphorylation catalyzed by the rIR in vitro are Tyr132/Tyr141, Tyr350/Tyr354, and Tyr364 (Fig. 5D). Peptides harboring Tyr132 and/or Tyr141 are substrates for rIR-catalyzed phosphorylation in response to insulin stimulation, the peptide harboring both Tyr132 and Tyr141 being the preferred substrate (6). The analysis for I135 only is displayed (Fig. 5A), being representative of tryptic fragments from Y132 also, which behave identically with those of I135 in both the HPLC and two-dimensional analyses (not shown). Two-dimensional analysis of tryptic fragments of β2AR phosphorylated by rIR in vitro revealed labeling of Tyr132/Tyr141, as shown here. Thus, in vitro phosphorylation of the β2AR by the IR tyrosine kinase is confined to Tyr132/Tyr141, Tyr350/Tyr354, and Tyr364.

In the current work we exploited the ability to prepare rIR, rbAR, and IGF-IR-enriched fractions from human osteogenic sarcoma cells to directly test the hypothesis that a G-protein-linked receptor can act as a substrate for an intrinsic tyrosine-kinase growth factor receptor. The following observations provide compelling evidence in support of this hypothesis: (i) insulin stimulates rIR-catalyzed phosphorylation of the β2AR in a reconstituted, in vitro assay; (ii) insulin-stimulated phosphorylation of the β2AR in vitro is confined to tyrosyl residues; (iii) the sites phosphorylated in vitro in response to insulin are those phosphorylated in vivo, as determined by structural analysis of β2AR isolated from metabolically labeled cells; (iv) insulin at high concentrations stimulates in vitro phosphorylation of β2AR catalyzed by purified IGF-IR; and (v) mutagenesis of these tyrosyl residues of the β2AR in vivo results in loss of insulin-stimulated counter-regulation of β2AR function (6). In addition, the bradykinin receptor has been shown to be phosphorylated on tyrosyl residues in response to serum (15), and the angiotensin II AT1 receptor has been reported to be a substrate for phosphorylation by the src family of tyrosine kinases (16). Based upon these observations we propose a new paradigm in which two prominent pathways in cell signaling cross-talk to each other at the most proximal point, receptor to receptor.

Interestingly, the sites on the β2AR phosphorylated by the IR and IGF-IR include a well known motif for tyrosine kinase growth factor receptors at Tyr350 (17), a prominent GRB2 site at Tyr350 (6, 18), and a potential SHC binding site at Tyr132 (19). The co-migration of the tryptic fragments harboring Tyr132 and Tyr141 preclude definition of the contribution of each to phosphotyrosine labeling by the IR, either in vitro (present study) or in vivo (6). Insulin-stimulated, IR-catalyzed phosphorylation of peptide Y132, which harbors Tyr132/Tyr141, however, was prominent, whereas that for peptide I135, which lacks Tyr132, was decisively poor (6). Further analysis of this site will be required, because phosphorylation of Tyr132 creates a Src binding site, and this site is conserved among many G-protein-linked receptors, including receptors for neuropeptide Y, tachykinin, A2-adenosine, thyrotropin releasing factor, and serotonin (GeneBank). Although speculation, the full range of adaptor molecules known to play a prominent role in tyrosine kinase receptor signaling may be made available to G-protein-linked receptors, once phosphorylated by a tyrosine kinase activated in response to a growth factor.

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