Phosphorylation of Tyrosyl Residues 350/354 of the β-Adrenergic Receptor Is Obligatory for Counterregulatory Effects of Insulin*

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Insulin stimulates a loss of function and increased phosphorytyrosine content of the β2-adrenergic receptor in intact cells, raising the possibility that the β2-receptor itself is a substrate for the insulin receptor tyrosine kinase. Phosphorylation of synthetic peptides corresponding to cytoplasmic domains of the β2-adrenergic receptor by the insulin receptor in vitro and peptide mapping of the β2-adrenergic receptor phosphorylated in vivo in cells stimulated by insulin reveal tyrosyl residues 350/354 and 364 in the cytoplasmic, C-terminal region of the β2-adrenergic receptor as primary targets. Mutation of tyrosyl residues 350, 354 (double mutation) to phenylalanine abolishes the ability of insulin to counterregulate β-agonist stimulation of cyclic AMP accumulation. Phenylalanine substitution of tyrosyl reside 364, in contrast, abolishes β-adrenergic stimulation itself.

The counterregulatory effects of insulin and catecholamines on carbohydrate and lipid metabolism are well known, whereas the molecular details of insulin regulation of G-protein-linked pathways remain unknown. Upon ligand binding, the insulin receptor displays tyrosine kinase activity which is critical to signal propagation (1). G-protein-linked receptors (like the β2-adrenergic receptor, β2AR), in contrast, activate adenyl cyclase via Gs and are phosphorylated during agonist-induced desensitization (2, 3). We demonstrated recently that the well known counterregulatory actions of insulin included loss of function and increased phosphorylation of the β2-adrenergic receptor (4). In the current study the structural basis for these counterregulatory effects of insulin exerted on the β2-adrenergic receptor is explored.

MATERIALS AND METHODS

Preparation of Recombinant β2AR and Insulin Receptor—Recombinant hamster β2-adrenergic receptor was expressed using the baculovirus-Sf9 insect cell expression system (5) and purified by afinity, HPLC, and lectin chromatography (6). Recombinant human insulin receptor (rIR) was purified by lectin chromatography (7) from Chinese hamster ovary (CHO) T cells, which stably expresses the human insulin receptor (8), or from COS-1 cells, which were transiently transfected with the human insulin receptor CDNA (9).

Phosphorylation of β2AR in Vivo—In vivo, DDT_MF-2 hamster vas deferens smooth muscle cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), DMEM containing 0.5% fetal bovine serum and [32P]orthophosphate (1 mCi/ml) for 4 h at 37 °C. At the end of the 4-h incubation, insulin or vehicle was added as indicated in the figure legends. To terminate phosphorylation, cells were washed and then lysed. The lysis buffer was composed of Triton X-100 (1%), sodium dodecyl sulfate (0.1%), dithiothreitol (6.0 mM), aprotinin (5 µg/ml), leupeptin (5 µg/ml), bacitracin (100 µg/ml), benzamidine (100 µg/ml), sodium orthovanadate (2 mM), NaCl (150 mM), EDTA (5 mM), NaF (50 mM), sodium pyrophosphate (40 mM), K2HPO4 (50 mM), sodium molybdate (10 mM), and Tris-HCl (20 mM, pH 7.4). Immunoprecipitation was performed in the lysis buffer (10). Each sample was preceeded with nonimmune serum-protein A-g agarose complex for 2 h prior to immunoprecipitation with anti-receptor antibody CM-4 (4, 10). The immunoprecipitated proteins were denatured for 5 min at 95 °C and then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% (w/v) acrylamide gels (4). Phosphorylated proteins were made visible by exposing the dried gel to X-Omat AR film (Kodak).

Stoichiometry of Phosphorylation—In vivo, duplicate cultures of DDT_MF-2 smooth muscle cells were labeled metabolically with [32P]orthophosphate as described above. After 4 h of labeling, the medium was aspirated. One culture was washed, lysed, and used as the source for determination of receptor number by ICYP binding and isolation of the labeled β2AR by immunoprecipitation. The replicate culture was washed and total protein precipitated with 0.5 M perchloric acid. The precipitate was collected by centrifugation and the supernatant neutralized with KOH. The specific activity of the [32P]ATP in the supernatant was determined as described by England and Walsh (11). With the determination of the specific activity of the cellular [32P]ATP pool, the derivative amount of labeled phosphate incorporated into receptor protein, and the amount of receptor, the stoichiometry of the phosphorylation was calculated as moles of phosphate/μg of β2AR.

Phosphorylation of Synthetic Peptide Substrates—Peptides corresponding to each cytoplasmic domain of the β2AR harboring a tyrosyl residue were synthesized, purified by HPLC, and subjected to phosphorylation by rIR. The replicate cultures were incubated with [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 concentrated Laemmli sample buffer. Phosphopeptides were separated by Tricine gel electrophoresis (12, 13). After fixing for 30 min, the gel was subjected to autoradiography for 30 min. For quantitation, the radioactive bands were identified in the gel and then excised. Phosphopeptide incorporation was estimated from quantitation of Cerenkov radiation ([32P] window) in the gel piece. The data shown are from a single experiment, replicated

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¶ The abbreviations used are β2AR, β2-adrenergic receptor; HPLC, high performance liquid chromatography; rIR, recombinant human insulin receptor; CHO, Chinese hamster ovary; DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; SH2, Src homology 2...
Fig. 1. Topological model of β2-adrenergic receptor, highlighting candidate tyrosyl residues for insulin-stimulated phosphorylation. A, model of β2AR organization and location of synthetic sequences used as substrates for insulin-stimulated rIR-catalyzed phosphorylation of the β2AR and as precursors for tryptic fragments used as markers for peptide mapping of the labeled tyrosyl residues. B, synthetic peptides used to map cytoplasmic tyrosyl residues for phosphorylation by activated tyrosine kinase growth factor receptors. Six sequences were selected to be used as substrates for insulin-stimulated rIR-phosphorylation. The peptides were designed as substrates for phosphorylation as well as a source of markers for reverse-phase HPLC and two-dimensional mapping of tryptic fragments. The derivative peptide fragments are displayed.

Once with similar results.

Reverse phase HPLC of Tryptic Phosphopeptides—32P-Labeled β2AR immunoprecipitated from metabolically labeled DDT, MF-2 cells were separated on SDS-PAGE as described above. Synthetic peptides containing tyrosine residues 350, 354, and 364 were phosphorylated in vitro with [γ-32P]ATP and rIR, and then separated on Tricine gels. The bands corresponding to β2AR 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 (7). 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) at a flow rate of 200 μl/min. Fractions were collected at 1-min intervals and Cerenkov radiation (14P window) measured for each fraction.

Two-dimensional Peptide Mapping—Tryptic digestion of β2-adrenergic receptor phosphorylated in vivo as well as of synthetic peptides phosphorylated in vitro was performed as described above. The tryptic eluates from the HPLC peaks were separated in two dimensions on cellulose, thin-layer plates. Aliquots (10 μl) of tryptic eluates were spotted onto the TLC plates and electrophoresed at 1000 V for 60 min in pH 3.9 buffer (formic acid/glacial acetic acid/water; ratio 50:156:1794). Following electrophoresis, the plate was air-dried overnight and subjected to chromatography at a right angle to the direction of electrophoresis in a phosphochromatography buffer (1-butanol/pyridine/acetic acid/water; ratio 15:10:3:12). The plates were dried and the peptides identified by autoradiography.

Site-directed Mutagenesis—Single mutation of tyrosyl residue 364 and the double mutation of tyrosyl residues 350 and 354 to phenylalanine in the hamster β2AR CDNA was performed using the Transformer Mutagenesis® kit (Clontech), according to the manufacturer's suggested protocols. The sequences of the mutagenic primers were as follows: Y350F/Y354F, AGAACGCCTGCTTGCAAGCTTCTACCA; Y354F, CAAACAGACCTTGTGGAAGGAGGC. Suspected mutations were confirmed by direct DNA sequencing of the mutant plasmid DNA. The mutant and wild-type cDNAs were then subcloned into the expression vector pcMV5 for subsequent transfection of CHO cells.

Transfection and Cyclic AMP Determination—CHO wild-type cells were co-transfected with either mutant, wild-type, or empty vector expression vector pCMV5 for subsequent transfection of CHO cells. For assay of beta-adrenergic stimulation of cyclic AMP accumulation, cells were suspended in Krebs-Ringer phosphate buffer and treated with the indicated hormones for 15 min at 37°C. The reaction was terminated by the addition of HCl (0.1 N final). Cyclic AMP accumulation was measured using a competition binding assay (14).

RESULTS AND DISCUSSION

In an effort to explore the site(s) for insulin-stimulated phosphorylation of the β2AR, we prepared synthetic peptides to corresponding to each cytoplasmic regions of the β2AR that harbor candidate tyrosyl residues, i.e. Tyr-70, Tyr-132, Tyr-141, Tyr-350, Tyr-354, and Tyr-364, and analyzed their potential as substrates for rIR (Fig. 1). The β2AR peptides were reconstituted with rIR in the absence (not shown) or presence of insulin (100 nm). For the in vitro assay, no labeling of peptides by rIR was observed in the absence of insulin. Insulin-stimulated phosphorylation of the peptides by the rIR was
analyzed phosphorylation of tyrosyl residues 350, 354, and 364 in vivo. C–F, insulin promotes phosphorylation of the β2-adrenergic receptor in metabolically labeled DDT, MF-2 smooth muscle cells. Tryptic peptides of in vitro labeled L339 (harboring Tyr-350 and Tyr-354) and of T362 (harboring Tyr-364) were employed as standards (panels A and B, respectively). Cells metabolically labeled with [32P]orthophosphate (see "Materials and Methods") were incubated for 20 min without (panels C and D) or with (panels E and F) 100 nM insulin. After lysis of the cells, the β2-AR was immunoprecipitated, and the phosphorylated receptor isolated, and then digested with trypsin. Reverse-phase HPLC analysis of the tryptic fragments was performed as described under "Materials and Methods." Chromatograms from two separate experiments, representative of five independent experiments, are displayed. The label in fraction 30 of panel F was observed on occasion, but contained no phosphotyrosine.

compared, after electrophoretic separation of the labeled products from the rIR (Fig. 2A). Insulin stimulated rIR-catalyzed phosphorylation of peptides L339 (Tyr-350 and Tyr-354), T362 (Tyr-364), and to a lesser extent peptides Y132 (Tyr-132 and Tyr-141) and I135 (Tyr-141).

Since peptide L339 contains both Tyr-350 and Tyr-354, it was important to explore if one site, or the other, or both were phosphorylated in vitro by rIR in the presence of insulin. When L339 peptide analogs that carried phenylalanine substitutions at either Tyr-350 or Tyr-354 were reconstituted with rIR in the in vitro system, both analogs showed equally well as substrates for insulin-stimulated phosphorylation (data not shown). Elimination of the YIA sequence of Y132 to create peptide L135 reduced insulin-stimulated, rIR-catalyzed phosphorylation of the residual peptide, suggesting either that Tyr-132 is the site of phosphorylation or that the YIA sequence is required for recognition/phosphorylation of the Tyr-141. The absence of labeling of the E122 peptide containing Tyr-132 and the detection of some label in tryptic fragments containing Tyr-141 supports the latter interpretation. Phosphopeptides from peptides R62 (Tyr-70 and E121 (Tyr-132) could not be detected, although the presence of the unphosphorylated peptides in the gel could be detected by silver staining. Phosphoamino acid analysis of the labeled peptides was performed, and for all phosphopeptides, labeling was confined to phosphotyrosine (not shown). These data demonstrate that residues Tyr-350, Tyr-354, and Tyr-364 (and to a lesser extent Tyr-141) are phosphorylated by the rIR tyrosine kinase in vitro.

The efficacy of peptide phosphorylation was assessed by comparing the amount of phosphate incorporated into each peptide by insulin-stimulated rIR at various concentrations of peptides (Fig. 2B). Peptide L339 was clearly the best substrate for the rIR. The ED50 for insulin-stimulated phosphorylation of L339 peptide was ~100 μM. At 3 mM concentrations of peptide, saturation of rIR-catalyzed phosphorylation for the other peptides was not achieved, precluding the calculation of ED50 values for the other peptides. The rank order of insulin-stimulated phosphorylation for the synthetic peptides employed at 1 mM, from best to worst substrate, was L339 (Tyr-350 and Tyr-354) > T362 (Tyr-364) > Y132 (Tyr-132 and Tyr-141). Phosphorylation of peptides R62 and E122, containing Tyr-70 and Tyr-132, respectively, was not detected.

The synthetic peptides were designed not only to probe all cytosolic tyrosyl residues available for phosphorylation by rIR, but also to source a number of tryptic fragments in which the candidate sites for tyrosine kinase phosphorylation were imbedded (Fig. 1, A and B). Maps of tryptic digests might permit analysis of the sites phosphorylated on the β2-AR in response to insulin in vivo (Fig. 1). Tryptic digests of peptides phosphorylated in vitro by rIR in response to insulin provided markers for HPLC analysis (Fig. 3, A and B). The retention times for the tryptic fragments subjected to HPLC separation agreed well with the retention times calculated from the sequence information (not shown).

In vivo, metabolic labeling of DDT, MF-2 smooth muscle cells in culture with [32P]orthophosphate revealed the phosphotyrosine content of β2-AR to increase from 0.86 ± 0.10 (basal) to 1.76 ± 0.39 (n = 4) mol/mol receptor in response to insulin (20 min, 100 nM). Some phosphotyrosine was found in tryptic fragments harboring Tyr-350 and Tyr-354 of β2-AR isolated from cells in the absence of stimulation by insulin (Fig. 3, C and D). In the presence of insulin, increased phosphorylation of the β2-AR was observed, confined largely to Tyr-350, Tyr-354, and Tyr-364 (Fig. 3, E and F). Insulin-stimulated phosphorylation displayed two patterns in which labeling occurred either at both Tyr-350/Tyr-354 and Tyr-364 (Fig. 3F) or more prominently at Tyr-350/Tyr-354 with reduced labeling of Tyr-364 (Fig. 3E). Other peaks occasionally observed in the HPLC pro-
files (e.g., fraction 30, Fig. 3D) were subjected to phosphoamino acid analysis and found to contain no phosphorysorine. High voltage electrophoresis followed by thin-layer chromatography of the tryptic fragments confirmed the identity of the HPLC peaks (Fig. 4, A and B) and provided additional markers for analysis of phosphopeptides derived from insulin-stimulated rIR-catalyzed phosphorylation of receptor peptides (Fig. 4, C and D). The two-dimensional analysis confirmed the results of reverse-phase HPLC, establishing that the predominant sites of insulin-stimulated phosphorylation are Tyr-350/Tyr-354, and to a lesser extent Tyr-364.

Site-directed mutagenesis of the tyrosyl residues was performed to test independently the role of Tyr-350/Tyr-354 and Tyr-364 in the regulatory effects of insulin on the β2AR. Tyr-350/Tyr-354 (double substitution) or Tyr-364 were mutated to phenylalanine and the mutant receptor expressed in CHO cells. The β-adrenergic agonist isoproterenol (1 μM) stimulated cyclic AMP accumulation in CHO cells expressing wild-type and Y350F/Y354F mutant receptors, but not the Y364F mutant β2AR (Table I). Tyrosine to phenylalanine substitution of residues 350 and 354 abolishes the ability of insulin to counterregulate β2-agonist-stimulated cyclic AMP accumulation in CHO cells. The Y364F mutation, in contrast, abolishes isoproterenol-stimulated cyclic AMP accumulation itself. These data, gathered from an independent approach, clearly highlight a critical role of Tyr-350/Tyr-354 in expression of the β2AR coupling to Gs (19), much like the Y364F mutations (this study). Phosphorylation of Tyr-350/Tyr-354 by the IR is shown to impair β2AR coupling to Gα (19), much like the Y364F

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**TABLE I**

| Cell line/dose | β2AR mutation | cAMP accumulation pmol/10^6 cells |
|----------------|---------------|----------------------------------|
| CHO pCMV5 Wild-type | 34.2 ± 7.5 | 55.5* | 0.72 | 17.5 |
| CHO pCMV5 Y350F/Y354F | 87.0* ± 7.5 | 82.5* | 64.5* | 25.0 |
| CHO pCMV5 Y364F | 52.5* ± 5.0 | 0.75 | 25.0 |

* Denotes statistical significance from wild-type or from basal cAMP accumulation level (p < 0.05).