A Synthetic Peptide Derived from a COOH-terminal Domain of the Insulin Receptor Specifically Enhances Insulin Receptor Signaling*

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The role of the insulin receptor COOH-terminal domain in the regulation of insulin signal transduction was explored with a variety of synthetic peptides. One of the peptides, termed peptide HC, whose structure corresponds to residues 1293–1307 of the insulin proreceptor sequence, enhanced insulin-stimulated autophosphorylation of the insulin receptor in cell-free systems and in semipermeabilized Chinese hamster ovary (CHO) cells that had been transfected with an expression plasmid encoding the human insulin receptor (CHO/HIIRc) at concentrations where there was no detectable effect on basal autophosphorylation levels or on receptor dephosphorylation. A lipophilic analogue of peptide HC, stearyl peptide HC, added to intact CHO/HIIRc cells enhanced significantly insulin-stimulated insulin receptor autophosphorylation while having no effect on ligand-stimulated receptor phosphorylation in CHO cells overexpressing either the IGF-1 receptor or epidermal growth factor receptor. Addition of stearyl peptide HC to CHO/HIIRc cells resulted in a 2.4 ± 0.3-fold increase in the amount of insulin-stimulated phosphatidylinositol 3-kinase detected in anti-IRS-1 immunoprecipitates and a 2.1 ± 0.6-fold increase in the levels of tyrosine phosphorylation of mitogen-activated protein kinase in response to insulin. Finally, a derivative of peptide HC coupled to a biotin moiety was prepared and showed to bind with the β-subunit of the wild-type insulin receptor and a truncated receptor that lacks 43 amino acids from its carboxyl terminus. However, there was little binding, if any, of the peptide with the IGF-1 receptors or the epidermal growth factor receptors. Taken together, our data demonstrate that a pentadecapeptide related to the carboxyl terminus of the insulin receptor binds to the insulin receptor β-subunit and that this interaction may contribute to the increased receptor’s intrinsic activity and signal transduction.

The insulin receptor is structurally related to the insulin-like growth factor-1 (IGF-1)1 receptor (1–3), both having tyrosine kinase function with very similar properties. Highly overlapping yet different responses are elicited upon activation of these receptors with their specific ligand (4). Both insulin and IGF-1 can bind to the receptor for the other with a much lower affinity. Although controversial (5), it is thought that the differences in metabolic activities and mitogenic effects of insulin and of IGF-1 are correlated with differences in the carboxyl-terminal domains of insulin and IGF-1 receptor β-subunits, whereby their association with distinct signaling proteins may contribute to specific cellular activities. Indeed, in cells expressing a chimeric IGF-1 receptor in which its carboxyl-terminal domain is replaced by that of the insulin receptor, the stimulation of glycerol synthesis and mitogen-activated protein (MAP) kinase pathway are correlated with the activities of the insulin receptor (6). Likewise, substitution of the insulin receptor carboxyl terminus with that of the IGF-1 receptor severely affects insulin-stimulated mitogenic responses (7, 8).

Thus, it appears that kinase regulation by sequences within the COOH terminus may, in part, be involved in defining the specificity of downstream signaling events. Of interest is the fact that antipeptide antibodies directed against epitopes in the region 1294–13172 of the insulin receptor inhibit in vitro insulin receptor kinase activity toward an exogenous substrate (9). This region in particular is poorly conserved between the two receptors, and the insulin receptor sequence contains a serine residue at position 1315; phosphorylation of that residue after exposure to phosphor-ester (10, 11) provides the potential for modulation of insulin receptor signaling through its carboxyl-terminal tail. These studies thus suggest that the differences in this region of the carboxyl-terminal domains of the insulin receptor and IGF-1 receptor may be functionally significant.

As an initial step in delineating those features of the insulin receptor that are responsible for the specific nature of insulin signaling, we have synthesized a series of peptides whose sequences correspond to COOH-terminal regions that are found in human and rodent insulin receptors but not in the IGF-1 receptors (12, 13) and showed that peptide HC (amino acid residues 1293–1307) was able to bind to the insulin receptor, thereby potentiating the ability of insulin to activate specifically receptor tyrosine kinase and diverse signaling pathways.

MATERIALS AND METHODS

Transfection and Cell Culture—CHO cell lines used in this study have been previously described (14–17). These include the CHO cell lines stably co-transfected with a plasmid containing a neomycin resistance gene driven by a SV40 promoter and a plasmid encoding the normal human insulin receptor without exon 11 (CHO/HIIRc), the truncated; Fmoc, N-(9-fluorenyl)methoxycarbonyl; PVDF, polyvinylidene difluoride.

The numbering system used is the minus exon 11 variant of the insulin receptor according to Ullrich et al. (1).
cated insulin receptor lacking 43 amino acids at the carboxyl terminus (CHO/HIRc, EGF receptor, CHO/IGF-1 receptor (CHO/IGF-1R), CHO/HIRc and CHO/443 cells were a gift from Dr. Morris F. White (Joslin Diabetes Center, Boston, MA), CHO/EGFR cells from Dr. Roger J. Davis (University of Massachusetts, Worcester, MA), and CHO/IGF-1R cells from Dr. Derek LeRoith (National Institutes of Health, Bethesda, MD). CHO cells overexpressing both the insulin receptors and the EGF receptors (CHO/EGF) were generated as follows. The expression plasmid containing the cDNA for the human insulin receptor, pcSVSHIRc, was kindly provided by Dr. Peter A. Wilden (University of Missouri, Columbia, MO). The plasmid containing hygromycin resistant gene pSVHPP (ATCC, Bethesda, MD) was linearized with BamHI. Five µg of pcSVSHIRc (Life Technologies Inc.) and 0.5 µg of linearized pSVHPP were co-transfected into 2.75 × 10^6 CHO/HIRc cells (2.75 × 10^6 cells/dish) by using a Gene Pulser (Bio-Rad) at 300 V and 960 microfarads. Aliquots of cells were then diluted and plated in multiwell culture dishes. After 24 h, cells were exposed to 300 µg/ml hygromycin, which has been tested to be 100% lethal after 5 days in nontransfected controls. Colonies resistant to hygromycin were identified after 5 days and allowed to grow for another 10 days. Cells were then passaged and tested for the expression of insulin receptor. Four independent CHO/EGF clones were obtained that expressed large numbers of both the EGF and insulin receptors. All cell lines were grown on tissue culture plates in F-12 medium containing 10% fetal bovine serum and maintained in a humidified incubator with 5% CO2 at 37°C. Cell culture reagents were purchased from Life Technologies, Inc. The approximate number of receptors on each cell line was determined by cell surface labeling by Scatchard analyses of competitive binding studies with labeled ligand and the homologous unlabelled peptide.

Peptide Synthesis—All peptides used in this study (see Fig. 1A for amino acid sequences) were prepared by solid phase synthesis employing Fmoc chemistry on an Applied Biosystems 430A automatic peptide synthesizer as described (18). Peptide HC was modified to increase its lipophilicity by incorporation of the C-18 aliphatic stearic acid (Sigma) at the amino terminus using the following conditions for automation on the peptide synthesizer: a portion of the protected peptide resin was treated with 20% piperidine to remove the NH2-terminal Fmoc protection group, washed extensively with N-methyl-pyrrolidone, and dried. Stearic acid, which had been converted into the symmetrical anhydride, reacted with the peptide-bound resin, as described (18). The release from the solid phase support matrix and deprotection of amino acid side chains were accomplished by treating the fatty acylated peptide on the resin with 83.5% trifluoroacetic acid containing 4.5% phenol, 4% thioanisole, and 2% ethanethiol. Diethyl ether was used at 0°C to remove the unreacted reagents. Stearyl peptide HC was loaded onto a C18 column (4 × 250 mm; Vydac, Hesperia, CA). After liquid chromatography and purified as follows. Solvent A contained 10 mM trifluoroacetic acid, and solvent B contained 10 mM trifluoroacetic acid, 80% acetonitrile. A linear gradient was run over 60 min of 20–80% solvent B. The flow rate was 1 ml/min. The eluant was monitored at 210 nm. The composition of the stearyl peptide HC was confirmed by mass spectral analysis. Biotinylated HC peptide (SSHCQREEAGGRDGGDGGGGGQSR; Ref. 21) or stearyl peptide HC (SSHCQREEAGGRDGGDGGGGGQSR; Ref. 21) was added to cell cultures prior to the beginning of the experiment. Whole cell extracts were prepared and clarified; cell lysates were immunoprecipitated with a monoclonal anti-insulin receptor antibody (αIR; clone 29B4, Oncogene Science, Uniondale, NY) adsorbed on protein A/protein G-agarose (Oncogene Science). Proteins were analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions followed by autoradiography.

In Vitro Phosphorylation of the Insulin Receptor—Preparation of plasma membranes from CHO/HIRc cells and the partial purification of the insulin receptors by chromatography on WGA-bound agarose were essentially as described (17). WGA-purified insulin receptors were precipitated with 100 µl protein aprotinin, 2 mM phenylmethylsulfonylfluoride, 1 mM benzamidine, 8 µg/ml aprotenin, 2 µg/ml leupeptin), and the cell lysates were incubated for 1 h on ice. After centrifugation (17,000 g, 20 min) to remove cell debris, the clarified supernatant from CHO/HIRc cell lysates was incubated with αIR antibody, while monoclonal anti-phosphotyrosine (αpY; clone 4G10, Upstate Biotechnology Inc., Lake Placid, NY) antibody was used with clarified CHO/EGFR, CHO/EGFR, and CHO/443 cell lysates. After an overnight incubation at 4°C, the immune complexes were precipitated with G-agarose beads. The phosphate-labeled proteins were washed, and the antibody receptor complexes were eluted with Laemmli sample buffer containing 5% 2-mercaptoethanol. After SDS-polyacrylamide gel electrophoresis, proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes (Novex) and probed with polyclonal αpY antibody (UBI). The immunoprecipitates from CHO/443 and CHO/EGFR cells were immunoblotted with a polyclonal antibody raised against a peptide corresponding to residues 1293–1306 of the insulin receptor (αCT-IR; Ref. 21) or αEGFR antibody (PC19-2; Oncogene Science). The immunoblots were visualized by using anti-rabbit immunoglobulin G coupled to horseradish peroxidase and the enhanced chemiluminescence (Amer sham) detection kit. Quantification was performed using ImageQuant™ software (version 3.3) on a Molecular Dynamics Densitometer (Sunnyvale, CA).

Phosphatidylinositol 3'-Kinase Assay—In vitro phosphorylation of phosphatidylinositol was carried out in immune complexes as described previously (22). Confluent cultures of CHO/HIRc cells were serum-starved for 16 h, incubated for 1 h with 50 µM stearyl peptide HC, and then treated without or with 3 mM insulin for 1 min. The cells were lysed in 20 mM Tris-Cl, pH 8.0, containing 137 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 10% glycerol, 1% Nonidet P-40, 150 µM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 2 mM dithiothreitol. The cell lysates were clarified by centrifugation and incubated with polyclonal IRS-1 antibody (UBI) overnight at 4°C. Following precipitation and washes, the immune complexes were incubated with 20 µg of phosphatidylinositol 3'-kinase (10 µl 400 µg/ml in a buffer containing 20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.4 mM EGTA, and 10 mM MgCl2) for 20 min at room temperature. Reactions were stopped with 20 µl of 6 M HCl and 160 µl of chloroform/methanol (1:1) and centrifuged. Aliquots of the lower organic phase were applied to a silica gel TLC plate (Merck) that had been treated with 1% potassium oxalate in 40% methanol. TLC plates were developed in chloroform/methanol/water/ammonia (60:47:36:12, v/v/v/v).
purified insulin receptors were preincubated with insulin (100 nM) and 50 µM of the indicated peptides for 10 min at room temperature. The phosphorylation reaction was then initiated by adding [γ-32P]ATP for 5 min as described under “Materials and Methods.” Proteins were analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions, and the levels of 32P associated with the receptor β-subunit were determined by Betagener counting of the dried gels. Each value is expressed relative to that determined in the absence of peptide. Numbers in parentheses refer to the number of independent observations. *, p < 0.01 versus no peptide. B, a dose-response curve for the stimulation of insulin receptor autophosphorylation by peptide HC was generated by the experimental protocol described above. Bars represent the mean ± range of a representative experiment performed in duplicate. The autoradiogram of the 32P-labeled receptor β-subunit is shown in the inset. Comparable results were obtained in a separate experiment.

FIG. 1. Effect of synthetic peptides on insulin-stimulated autophosphorylation of partially purified insulin receptors. A, WGA-purified insulin receptors were preincubated with insulin (100 nM) and 50 µM of the indicated peptides for 10 min at room temperature. The phosphorylation reaction was then initiated by adding [γ-32P]ATP for 5 min as described under “Materials and Methods.” Proteins were analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions, and the levels of 32P associated with the receptor β-subunit were determined by Betagener counting of the dried gels. Each value is expressed relative to that determined in the absence of peptide. Numbers in parentheses refer to the number of independent observations. *, p < 0.01 versus no peptide. B, a dose-response curve for the stimulation of insulin receptor autophosphorylation by peptide HC was generated by the experimental protocol described above. Bars represent the mean ± range of a representative experiment performed in duplicate. The autoradiogram of the 32P-labeled receptor β-subunit is shown in the inset. Comparable results were obtained in a separate experiment.

Tyrosine Phosphorylation of MAP Kinases—Cells were serum-starved for 4 h and subsequently incubated in the absence or presence of 50 µM stearyl peptide HC for 1 h prior to the addition of 100 nM insulin for 1 min. After lysis of the cells in radioimmune precipitation buffer, clarified cell extracts were subjected to immunoprecipitation with monoclonal αPY antibody. Immunoprecipitates were then electrophoresed on SDS-polyacrylamide gels under reducing conditions, and Western immunoblotting was performed on a PVDF membrane using a monoclonal MAP kinase antibody that reacts with the 44-kDa Erk-1 and, to a lesser extent, the 42-kDa Erk-2 protein (sc-94, Santa Cruz Biotechnology, Santa Cruz, CA) followed by detection with the ECL chemiluminescence detection system.

Chemical Cross-linking Protocol—Semipermeabilized cell monolayers were incubated with insulin (100 nM), IGF-1 (100 nM) or both insulin (100 nM) and EGF (5 nM), as indicated, in the presence of 50 µM biotinylated peptide HC with or without a 10-fold excess of unmodified peptide HC for 15 min at 6 °C. The phosphorylation reaction was initiated by the addition of 100 µM ATP and 4 mM MnCl2. Fifteen min later, 0.2 mM of the homobifunctional cross-linking reagent bis(2-(suc-cinimidooxy)carboxyl/oxo)ethyl)sulfone (BSO-COES; Pierce) was added for 15 min. Cells extract were prepared, and the clarified cell lysates were immobiloprecipitated with αRI, monoclonal anti-IGF-1 receptor (αIGF-1R, clone 3B7, Santa Cruz), or polyclonal anti-EGF receptor (αEGFR, UBI) antibodies adsorbed on protein A/protein G-agarose. The immunoprecipitated proteins were electrophoresed on SDS-polyacrylamide gels under reducing conditions; the proteins were electrotransferred to PVDF membrane and probed with enzyme-linked streptavidin (Vector Laboratories Inc., Burlingame, CA, or subjected to immunoblotting with polyclonal αPY, α-CR-IR, αEGFR, or a polyclonal antibody raised against a peptide corresponding to the major autophosphorylation domain of both the insulin receptor and IGF-1 receptor (α-G86.2, kindly provided by C. Ramachandran, Merck Frost Canada). The blots were visualized using the ECL chemiluminescence detection system.

Statistical Analysis—Significant differences were determined by an analysis of variance coupled to Fisher’s PLSD test for multiple mean comparison using StatView 4.01 (Abascan Concepts, Inc.).

RESULTS

Peptide HC Enhances Insulin-stimulated Autophosphorylation of the Insulin Receptor—In order to examine the ability of the COOH-terminal domain of the insulin receptor to modulate insulin receptor autophosphorylation in vitro, the peptides HC, HC scramble, HC-N5, and CT-24 were prepared, based on the corresponding sequences that are present in the insulin receptor but not in the receptor for IGF-1 (Fig. 1A). A 98-amino acid COOH-terminal fragment of the insulin receptor (residues 1245–1343) has previously been shown to stimulate the autophosphorylation activity of the insulin receptor nearly 3-fold (23). Using partially purified insulin receptors from CHO/HIRc cells, peptide HC (based on the corresponding amino acid residues 1293–1307) increased the insulin-stimulated receptor autophosphorylation by nearly 2-fold (Fig. 1B), while having no effect on basal receptor autophosphorylation activity (data not shown). As shown in Fig. 1B, preincubation with peptide HC increased insulin-stimulated receptor autophosphorylation in a concentration-dependent manner, with a maximum ranging between 50 and 100 µM. Two structural variants of peptide HC (HC scramble and HC-N5) were used and have been found to be inactive. In contrast to the enhancing effect of peptide HC on insulin receptor autophosphorylation, CT-24 peptide exerted an inhibitory activity.

Next, peptide HC was tested for its ability to enhance insulin-stimulated insulin receptor autophosphorylation in semipermeabilized cells. This treatment gives small molecules free access to the intracellular milieu and yet retains much of the membrane architecture of the whole cells (17). In control cells, insulin (100 nM) alone caused a 2.5 ± 0.2-fold increase in receptor phosphorylation (Fig. 2). Similar to the effect in cell-
free systems, 50 μM peptide HC doubled the insulin-stimulated insulin receptor autophosphorylation when compared with semipermeabilized cells incubated with insulin alone. This potentiating effect was maintained after incorporation of a stearyl moiety at the NH₂ terminus of peptide HC. Neither peptide had any effect on the insulin receptor autophosphorylation in unstimulated cells (data not shown).

To explore the possibility that peptide HC enhances the insulin receptor autophosphorylation by causing a decrease in the dephosphorylation of the receptor β-subunit, we have employed a pulse-chase technique using semipermeabilized CHO/HIRc cells. In this cell model, it has been previously demonstrated that the active, phosphotyrosine-containing insulin receptor is dephosphorylated rapidly by protein-tyrosine phosphatases (17). As shown in Fig. 3, less than 45% of the total phosphotyrosyl insulin receptors present remained after a 3-min dephosphorylation reaction. Using 25 μM peptide HC, it was found that peptide HC failed to prevent the loss of 32P from the phosphorylated receptors (Fig. 3). This lack of effect was observed in the presence of peptide HC up to 100 μM (data not shown). These studies were extended with the use of 3S-peptide I, a tris-sulfotyrosyl peptide, known to inhibit protein-tyrosine phosphatases (18). In contrast to our findings with peptide HC, 3S-peptide I caused a 85% decrease in the dephosphorylation of the insulin receptor (Fig. 3). This suggests that the increase in insulin receptor autophosphorylation by peptide HC cannot be accounted for by an altered receptor dephosphorylation.

Effect of Stearyl Peptide HC on Receptor Tyrosine Phosphorylation in Intact Cells—The incorporation of a fatty acid moiety at the NH₂ terminus of peptide HC dramatically increased its lipophilicity (data not shown). Indeed, this approach has been successfully used in our laboratory, where we showed that stearyl 3S-peptide I was a potent inhibitor of insulin receptor dephosphorylation in intact cells (18, 24).

To investigate the effect of peptide HC in intact cells, stearyl peptide HC was prepared and added to CHO/HIRc cells. The extent of ligand-stimulated tyrosine phosphorylation of the insulin receptors was then compared with that of vanadate, a known inhibitor of protein-tyrosine phosphatases. CHO/HIRc cells were exposed to a range of concentrations of stearyl peptide HC or 1 mM vanadate for 1 h, and stimulated with 100 nM insulin for 1 min. The phosphorylation reaction was terminated with liquid nitrogen. The cells were lysed, and the insulin receptors were immunoprecipitated with a βIR and protein A/protein G-agarose. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis, visualized, and quantitated with a Betagen counter. The data are presented as Betagen counts and represent the average ± S.E. from two to three separate experiments where each point was determined using two dishes.

![Fig. 2. Tyrosine phosphorylation of insulin receptors in semipermeabilized cells treated with peptide HC.](image)

![Fig. 3. Lack of inhibition of insulin receptor dephosphorylation by peptide HC.](image)

![Fig. 4. Insulin-stimulated autophosphorylation of insulin receptor in cells treated with stearyl peptide HC.](image)
the insulin receptor by insulin. Neither the basal nor insulin-stimulated receptor autophosphorylation was affected by stearic acid (data not shown).

To determine the specificity of stearyl peptide HC, CHO cells expressing large numbers of receptors for IGF-1 (CHO/IGF-1R cells) or EGF (CHO/EGFR cells) were incubated with 50 μM stearyl peptide HC for 1 h, followed by the addition of their cognate ligand. In contrast to the insulin receptor, there was no enhancement in the ligand-stimulated autophosphorylation of the IGF-1 receptor (Fig. 5A) or the EGF receptor (Fig. 5B). Inhibition of cellular protein-tyrosine phosphatases by vanadate led to an increase in ligand-stimulated receptor autophosphorylation in both cell lines.

To gain further insight into the selective activation of insulin receptor functions by peptide HC, CHO cells expressing large numbers of both insulin receptors and EGF receptors were generated (CHO/EI cells) and treated with 50 μM stearyl peptide HC; then receptor phosphorylation was assessed following the addition of insulin and EGF. The results shown in Fig. 6 indicate that stearyl peptide HC caused a 1.92 ± 0.07-fold increase in insulin receptor autophosphorylation in insulin-stimulated CHO/EI cells but was without effect on ligand-stimulated EGF receptor phosphorylation.

Activation of Phosphatidylinositol 3-Kinase Activity—An immediate effect of insulin receptor activation is the phosphorylation of IRS-1, one of the major substrates of insulin receptor kinase. Tyrosine-phosphorylated IRS-1 can associate with and stimulate PI 3-kinase activity (25, 26). An analysis of PI 3-kinase activity in anti-IRS-1 immunoprecipitates revealed that insulin increased by 5.7 ± 0.4 times the incorporation of 32P into phosphatidylinositol when compared with unstimulated CHO/HIRc cells (Fig. 7). In agreement with our findings with the insulin receptor autophosphorylation, stearyl peptide HC caused a further 2.4 ± 0.3-fold increase in PI 3-kinase activity in insulin-stimulated cells (Fig. 7) but was without effect on basal PI 3-kinase activity (data not shown). Neither the basal nor insulin-stimulated PI 3-kinase activity was affected by 50 μM stearic acid (data not shown).

Insulin-stimulated Tyrosine Phosphorylation of MAP Kinase—MAP kinase activity is rapidly stimulated in response to insulin and other growth factors via a mechanism that involves both tyrosine and serine/threonine phosphorylation of the enzyme itself (27, 28). In order to determine whether stearyl peptide HC affects tyrosine phosphorylation of MAP kinase, αPY immunoprecipitates from CHO/HIRc cells incubated under various conditions were analyzed by Western blot with an anti-MAP kinase antibody. As shown in Fig. 8, insulin alone led to a 2.3 ± 0.3-fold increase in phosphorylation of MAP kinase (and its activity thereof), when compared with control unstimulated cells, while the addition of 50 μM stearyl peptide HC caused a further 2.1 ± 0.6-fold increase in MAP kinase phosphorylation in cells stimulated with insulin.

Binding of Biotinylated Peptide HC to the Insulin Receptors—To determine whether the effect of peptide HC on insulin stimulation of receptor autophosphorylation and signaling were due to a direct association between peptide HC and a domain of the receptor, semipermeabilized CHO/EI cells were incubated in the presence of 50 μM biotinylated peptide HC and then exposed to 0.2 mM BSO-COES, a bifunctional cross-linking reagent. Extracts were prepared from insulin- and EGF-stimulated CHO/EI cells and subjected to immunoprecipitation with either αIR or αEGFR antibodies. Immunoprecipitates were analyzed for biotinylation by blotting with enzyme-conjugated streptavidin. A clear signal could be detected in αIR immunoprecipitates (Fig. 9A). We observed a ~95-kDa biotinylated protein, suggesting binding of biotinylated peptide HC to the β-subunit of the insulin receptor with no modification of the receptor α-subunit (Fig. 9A, lane 1). Cross-linking of the biotinylated streptavidin reagent with the IRS-1 subunit of the insulin receptor resulted in a ~300-kDa complex (Fig. 9A, lane 2).
Peptide Activation of Insulin Receptor Functions

Fig. 7. Effect of stearyl peptide HC on activation of PI 3-kinase by insulin. Serum-starved monolayers of CHO/HIRc cells, pretreated with (+) or without (−) 50 μM stearyl peptide HC for 1 h, were stimulated or not stimulated with 3 nM insulin for 1 min. Cell lysates were immunoprecipitated with an anti-IRS-1 antibody, the immune pellets were washed, and the associated PI 3-kinase activity was assayed in vitro by [32P]incorporation into phosphatidylinositol. The resulting phosphatidylinositol 3-phosphate was resolved by thin layer chromatography (inset). Data from the inset are presented as -fold stimulation above control (treated with vehicle alone) and represent the average ± range from two independent experiments.

Fig. 8. Effect of stearyl peptide HC on insulin-stimulated tyrosine phosphorylation of MAP kinase. Serum-starved monolayers of CHO/HIRc cells, pretreated with (+) or without (−) 50 μM stearyl peptide HC for 1 h, were stimulated or not stimulated with 100 nM insulin for 1 min and then lysed as described under “Materials and Methods.” The cell lysates were immunoprecipitated with anti-PY antibodies or with receptor-specific antibodies. The immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis and probed with horseradish peroxidase-conjugated streptavidin (panel A) or subjected to immunoblotting with αPY, αCT-IR, or αEGFR antibodies (panels B, C, and D, respectively). The specific complex is indicated by the asterisk. The positions of the molecular weight markers are shown (K).

FIG. 9. Cross-linking of biotinylated peptide HC to semipermeabilized CHO/EI cells using BSO-COES. Digitonin-permeabilized monolayers of CHO/EI cells were incubated with 100 nM insulin and 5 nM EGF in the presence of 50 μM biotinylated peptide HC with (+) or without (−) 500 μM unmodified peptide HC for 15 min at 6 °C. The cells were then phosphorylated with ATP/Mn2+ and treated with 0.2 mM BSO-COES. Cell extracts were prepared and subjected to immunoprecipitation (Ip) with αIR or αEGFR antibodies. The immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis and probed with horseradish peroxidase-conjugated streptavidin (panel A) or subjected to immunoblotting with αPY, αCT-IR, or αEGFR antibodies (panels B, C, and D, respectively). The specific complex is indicated by the asterisk. The positions of the molecular weight markers are shown (K).

Earlier studies point to the fact that the specificity of insulin and IGF-1 signaling is generated at the receptor level, where defined receptor subdomains interact with specific sets of regulatory molecules, some of which are common to both receptors (29–36) whereas others are possibly unique. There are regions of the β-subunit carboxyl-terminal domain of both the insulin and IGF-1 receptors, showed that the three cell lines expressed similar numbers of receptors (data not shown). Taken together, these data demonstrate that peptide HC binds specifically to the insulin receptor β-subunit in a region other than the carboxyl-terminal 43 amino acids.

DISCUSSION

The results presented here further support the concept that insulin signal transduction is mediated via receptor subdomain-specific interactions. The specific subdomain of the insulin receptor that mediates its signaltransduction appears to be the carboxyl-terminal 43 amino acids, which is consistent with the findings of previous studies. The data also suggest that insulin signal transduction is mediated via receptor subdomain-specific interactions, which are distinct from those involved in the receptor kinase domain.

In conclusion, the data presented here provide further support for the concept that insulin signal transduction is mediated via receptor subdomain-specific interactions. The specific subdomain of the insulin receptor that mediates its signaltransduction appears to be the carboxyl-terminal 43 amino acids, which is consistent with the findings of previous studies. The data also suggest that insulin signal transduction is mediated via receptor subdomain-specific interactions, which are distinct from those involved in the receptor kinase domain.
Peptide Activation of Insulin Receptor Functions

FIG. 10. Specific binding of biotinylated peptide HC to the insulin receptors. Digitonin-permeabilized CHO/D43 cells, CHO/EI cells, and CHO/IGF-1R cells were incubated with either 100 nM insulin (lanes 1 and 2) or 100 nM IGF-1 (lane 3) in the presence of 50 μM biotinylated peptide HC for 15 min at 6 °C. The cells were then phosphorylated with ATP/Mn2+ and treated with 0.5 μM BSO-COES. Cell extracts were prepared and subjected to immunoprecipitation (Ip) with αIR or αIGF-1R antibodies. The immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis and probed with horseradish peroxidase-conjugated streptavidin. The asterisk denotes peptide binding to receptor β-subunits. Lane 1, CHO/D43 cells; lane 2, CHO/EI cells; lane 3, CHO/IGF-1R cells.

activity of purified insulin receptors but has no effect on the IGF-1 receptor activity (23). The role of the carboxyl-terminal domain of the insulin receptor in transducing insulin signals has been restricted, in most part, to the use of deletion mutant receptors. Several studies have reported that a region within the carboxyl terminus downstream of residue 1274 of the insulin pror receptor sequence has defined effects on kinase function by promoting both catalytic efficiency and stability of the receptor tyrosine kinase activity (37–43).

In the present study, we have observed that a specific pentadecapeptide whose amino acid sequence is derived from the carboxyl-terminal region of the insulin receptor (residues 1293–1307) enhances insulin-stimulated receptor autophosphorylation under a number of experimental conditions. This peptide, termed peptide HC, has been used along with other peptides related to the COOH terminus of the receptor to characterize its “specific” effect on kinase function. A truncated peptide (HC-N5) and an analog of peptide HC with a scrambled sequence lack the ability to enhance autophosphorylation, and on this basis we conclude that primary sequence requirements do participate in peptide HC response. Consistent with our results, Kaliman et al. (9) have recently shown that binding of antipeptide antibodies to the COOH-terminal domain of the insulin receptor modulated receptor-mediated substrate phosphorylation. They reported that antipeptide antibodies against the sequence 1294–1317 (encompassing peptide HC sequence) inhibit receptor kinase activity, possibly because binding of antipeptide antibodies causes perturbation of the COOH-terminal domain and affects exogenous substrate phosphorylation. Thus, this region of the receptor appears to be involved in regulation of insulin receptor kinase activity.

CT-24 peptide (residues 1300–1323) exerts an inhibitory effect when assayed in vitro. It is interesting to note that the latter peptide contains two tyrosines, corresponding to residues 1316 and 1322 of the second major autophosphorylation domain. It is known that the rate of autophosphorylation with respect to activation of the intrinsic protein kinase can be inhibited by exposure of the insulin receptor to substrate before autophosphorylation is initiated (44, 45). Therefore, one of the contributing factors in the inhibition of insulin receptor autophosphorylation by CT-24 peptide may result from its addition to unphosphorylated receptors and occupation of the substrate binding site. Interestingly, cells expressing a mutated insulin receptor, where tyrosines 1316 and 1322 are replaced by Phe (YPF phosphorylation enhances substrate phosphorylation when compared with wild-type receptor (46, 47). Additional evidence for the modulatory role of these two COOH-terminal tyrosines is provided by Kaliman et al. (9), who show that YPF receptors have a 2-fold higher kinase activity than wild-type receptors and possess an insulin-insensitive conformation that corresponds to an active insulin receptor form. A plausible explanation for these observations is that in the basal state tyrosines 1316 and 1322 help maintain cis-inhibition of the receptor kinase by occupying the substrate binding site. Upon insulin addition, these two tyrosines are disengaged (by conformational change), and trans-autophosphorylation of the autokatalytic domain occurs (48). Hence, insulin-induced conformational change of the COOH terminus has been recently correlated with the ligand’s ability to stimulate receptor autophosphorylation (49).

Here we have introduced stearyl peptide HC in intact CHO/HIR cells and evaluated its influence on insulin receptor functions. Our results show that the addition of stearyl peptide HC in intact cells has an initial effect of enhancing insulin signaling at the level of the insulin receptor itself as well as at several postreceptor targets. This includes the association and activation of PI 3-kinase to phosphorylated IRS-1 and increased levels of tyrosine phosphorylation of MAP kinase in response to insulin. Based on the PI 3-kinase data, one can assume that exposure of cells with stearyl peptide HC results in an increase in the insulin-stimulated tyrosine phosphorylation of IRS-1. Although the effects of peptide HC on kinase function are quantitatively modest (~2-fold), the significance of changes of this magnitude is relevant, since small changes in insulin receptor activity would probably have major consequences on integrated glucose homeostasis and cellular regulation. Because of its lack of effect in the absence of insulin, peptide HC does not appear to mimic the stimulation by insulin but rather affects a sequence of events that is involved in modulation of the in vitro and in vivo insulin receptor activity and functions. It is noteworthy that stearyl peptide HC has no effect on the autophosphorylation of two closely related growth factor receptors. Peptide HC corresponds to a noncatalytic segment of the insulin receptor that contains serine residues 1293 and 1294, thought to be targets for interaction with serine kinase tightly associated to the insulin receptor kinase in an insulin-dependent manner (50) and to be substrates for phosphor ester-stimulated protein kinase C (51). The phosphorylation of this domain is closely linked to inactivation of the insulin receptor kinase (52). These data and ours indicate that this defined region within the carboxyl terminus plays an important role in the molecular regulation of insulin receptor activity and functions.

In this report, we have also shown specific binding of peptide HC with the insulin receptor but not with the IGF-1 or EGF receptors, suggesting that an association between peptide HC and the insulin receptor β-subunit may be required for the increase in receptor kinase activity. One possible consequence of this association is the inhibition of dephosphorylation of the activated insulin receptor, whereby peptide HC occupies a subdomain critical for the interaction of the β-subunit with protein tyrosine phosphatase(s) involved in receptor dephosphorylation. This appears unlikely because of the fact that peptide HC increases insulin-stimulated autophosphorylation of the insulin receptor in cell-free systems and in semipermeabilized cells without an apparently change in receptor dephosphorylation. These results suggest that residues 1293–1307 of the insulin receptor interact with another noncatalytic region of the receptor β-subunit to regulate the phosphotransferase activity. Our findings with the Δ43 mutant receptors support the notion that the important structural determinants that are required for direct binding of peptide HC to the insulin receptor still remain present. Additional studies with a number of cell lines expressing truncated receptors lacking larger deletions will be needed in order to determine where on the receptor...
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peptide HC binds. Finally, experiments are in progress to identify which amino acid residues in the peptide HC are required for the binding to the receptor β-subunit.

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