Insulin Action Inhibits Insulin-like Growth Factor-II (IGF-II) Receptor Phosphorylation in H-35 Hepatoma Cells

IGF-II RECEPTORS ISOLATED FROM INSULIN-TREATED CELLS EXHIBIT ENHANCED IN VITRO PHOSPHORYLATION BY CASEIN KINASE II

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Insulin caused a rapid, dose-dependent increase in the binding of 125I-insulin-like growth factor-II (IGF-II) to the surface of cultured H-35 hepatoma cells. The [32P]phosphate content of the IGF-II receptors, immunoprecipitated from extracts of H-35 cell monolayers previously incubated with [32P]phosphate for 24 h, was decreased after brief exposure of the cells to insulin. Analysis of tryptic digests of labeled IGF-II receptors by bidimensional peptide mapping revealed that the decrease in the content of [32P]phosphate occurred to varying degrees on three tryptic phosphopeptides. Thin layer electrophoresis of an acid hydrolysate of isolated IGF-II receptors revealed the presence of [32P] phosphoserine and [32P]phosphothreonine. Insulin treatment of cells caused a decrease in the labeled phosphoserine and phosphothreonine content of IGF-II receptors.

The ability of a number of highly purified protein kinases (cAMP-dependent protein kinase, protein kinase C, phosphorylase kinase, and casein kinase II) to catalyze the phosphorylation of purified IGF-II receptors was examined. Casein kinase II was the only kinase capable of catalyzing the phosphorylation of the IGF-II receptor on serine and threonine residues under the conditions of our assay. Bidimensional peptide mapping revealed that the kinase catalyzed phosphorylation of the IGF-II receptor on a tryptic phosphopeptide which comigrated with the main tryptic phosphopeptide found in receptors obtained from cells labeled in vivo with [32P]phosphate. IGF-II receptors isolated by immunoadsorption from insulin-treated H-35 cells were phosphorylated in vitro by casein kinase II to a greater extent than the receptors isolated from control cells. Similarly, IGF-II receptors from plasma membranes obtained from insulin-treated adipocytes were phosphorylated by casein kinase II to a greater extent than the receptors from control adipocyte plasma membranes. Thus, the insulin-regulated phosphorylation sites on the IGF-II receptor appear to serve as substrates in vivo for casein kinase II or an enzyme with similar substrate specificity.

Previous studies have shown that the IGF-II receptor is among a number of membrane proteins that are rapidly exposed on the cell surface membrane of target cells in response to insulin. This increase in the cell surface concentration of IGF-II receptors is caused by their redistribution from an intracellular membrane pool into the plasma membrane (1–3). The molecular mechanism which mediates this redistribution is unknown. However, it has been shown that IGF-II receptors continually recycle in the absence of ligand (4). Thus, an increase in the exocytosis of the receptor, a decrease in its rate of internalization, or a combination of both, could cause an increase in the steady-state cell surface concentration of this molecule.

In an effort to gain insight into the mechanisms which maintain the basal steady-state distribution of IGF-II receptors, we have studied the phosphorylation state of these receptors in plasma membranes and low density microsomes from rat adipocytes treated with or without insulin. We reported that IGF-II receptors derived from the adipocyte plasma membrane were phosphorylated to a higher stoichiometry than the receptors derived from the low density microsomes (5). Furthermore, insulin added to intact cells caused a marked decrease in the overall phosphorylation level of receptors in the plasma membrane with a time course that closely paralleled the increase in the cell surface receptor concentration. We have proposed that this insulin-mediated dephosphorylation of the IGF-II receptor may decrease its rate of internalization and thus leads to the increase in the steady-state cell surface number of receptors caused by this hormone (5). To test this hypothesis more detailed information on the characteristics of IGF-II receptor phosphorylation is required. In this paper we present data which indicate that in another cell type, the H-35 hepatoma cell, the effect of insulin to increase the number of IGF-II receptors on the cell surface was also accompanied by a decrease in the phosphorylation state of the receptor. Isotopic labeling experiments with [33P]phosphate revealed that both in freshly isolated adipocytes and in H-35 hepatoma cells, the IGF-II receptor was phosphorylated on serine and threonine residues. Furthermore, we report that purified casein kinase II catalyzed the phosphorylation of isolated IGF-II receptors in vitro. The phosphorylation of IGF-II receptors derived from plasma membranes obtained from insulin-treated adipocytes or from insulin-treated H-35 cells was increased compared to the phosphorylation of receptors obtained from control cells.

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1 The abbreviations used are: IGF-II, insulin-like growth factor-II; NaDodSO4, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, (ethylenebis(oxyethylene-nitri1o)ltetraacetic acid.

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These results suggest that the insulin-sensitive phosphorylated sites on the receptor may be phosphorylated in vivo by casin kinase II or an enzyme with similar substrate specificity.

MATERIALS AND METHODS

Cell Cultures—Rat H-35 hepatoma cells (gift from Dr. Gerald Litwack, Temple University) were grown as monolayers in Dulbecco’s modified Eagle’s medium containing 5% calf serum, 1000 units/ml of penicillin, and 100 μg/ml of streptomycin. Upon confluence, the cells were subcultured and seeded in 22.6-mm wells for binding studies or in 100-mm dishes for isotopic labeling with 32P-6-phosphate. Dulbecco’s modified Eagle’s medium, supplemented with 25 mM Hepes, pH 7.4.

Measurement of 32P-IGF-I and Anti-IGF-II Receptor IgG Binding—After 24 h in phosphate-free, serum-free medium, cells were stimulated by adding insulin (porcine, Lilly) at the concentrations indicated in the figure. After 10 min at 37°C, the medium was then replaced with 2 ml of Dulbecco’s phosphate-free, serum-free Dulbecco’s modified Eagle’s medium, supplemented with 25 mM Hepes, pH 7.4.

Phosphopeptide Mapping and Phosphoamino Acid Analysis—Gel slices corresponding to the IGF-I receptor band were excised and ethylmorpholine-acetate for another 2 h at 37°C. The combined eluates contained more than 90% of the radioactivity. After drying, the phosphopeptides were resuspended in 7 μl of ice-cold perfominc acid, in order to convert cysteine residues into their stable cysteic acid derivatives (8). After 150 min at 0°C, the acid was diluted with 250 μl of ice-cold water, frozen, and lyophilized to dryness. The phosphopeptides were then resuspended in 30% formic acid and spotted on a cellulose-coated thin layer plate (Machery-Nagel). The tryptic phosphopeptides were separated by two-dimensional peptide mapping. The number of spots resolved by two-dimensional peptide mapping.

For phosphoamino acid analysis, the tryptic peptides eluted from the polyacrylamide gel pieces were lyophilized to dryness and resuspended in 500 μl of 6 N HCl. Hydrolysis was performed for 3 h at 110°C, after which the hydrolysates were dried and resuspended in 1 mg/ml of phosphoserine, phosphothreonine, and phosphotyrosine (Sigma). Electrophoresis was then performed on cellulose-coated thin layer plates using acetic acid/pyridine/water (10:1:189 by volume). After drying, the phosphoamino acids were visualized by ninhydrin staining and autoradiography.

RESULTS

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Phosphorylation of IGF-II Receptor with Purified Protein Kinases—The IGF-II receptors from approximately 1 X 10^6 H-35 cells or from rat adipocyte plasma membranes (see below) were solubilized in 1 ml of lysis buffer and immunosorbed onto 5 μl of packed Affi-Gel 10 resin. The resin was washed three times with 1 ml of lysis buffer, three times with 1 ml of 25 mM Hepes, pH 7.4, 1.5% Triton X-100, 1% bovine serum albumin, 50 mM NaCl, 5 mM EDTA and then three times with 1 ml of 20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA. The resin was then suspended in 20 μl of a phosphorylation buffer containing 50 mM Tris, pH 7.5, 1 mM diithiothreitol (0.1% mercaptoethanol for casin kinase II), 1 mM EDTA, 1 mM EGTA, 6 mM MgCl2 (or 3 mM CaCl2 + 25 μM phosphatidylserine for protein kinase C), and 0.1 mM NaCl (for casin kinase II). The enzymes were then added to the resin, and phosphorylation reactions were immediately started with the addition of 2 μl of γ-[32P]ATP stock (200 μCi, 10 μCi/ml, Amersham Corp.). After 60 or 90 min, the reactions were stopped by boiling the samples for 5 min. After boiling the samples for 1 min, the IGF-II receptor was separated on NaDodSO4, 6% polyacrylamide slab gels. Electrophoresis was monitored using prestained high molecular weight markers (Bethesda Research Laboratories) and allowed to continue until the 42-kDa marker (ovalbumin) reached the bottom of the gel. This allowed the electroelution of the unreacted γ-[32P]ATP and a better separation of the IGF-II receptor from the top of the separating gel.

The kinases tested were: cAMP-dependent protein kinase catalytic subunit (Sigma), phosphoflavine kinase (Sigma), protein kinase C from bovine retina (9), and caskin kinase II from rabbit skeletal muscle (Kim, unpublished). Except when otherwise noted, the kinase reactions were incubated for 60 min.

In some experiments, IGF-II receptors from adipocyte plasma membranes were used as a substrate for casin kinase II. For these studies, cells were isolated by collagenase digestion from rat epididymal fat pads as described by Rodbell (11). Cells were then treated with 10 μM insulin for 10 min at 37°C, after which the plasma membranes were separated by differential centrifugation as previously described (12), but in a buffer containing phosphatase inhibitors (5). The membranes (500 μg) were solubilized and the IGF-II receptor was immunosorbed and used as a substrate for casin kinase II as described above. To quantitate the relative amounts of receptor present in the membrane or insulin-treated cell membranes, IGF-II receptor was transferred onto nitrocellulose paper (0.45 μM, Schleicher & Schuell) immediately after electrophoresis. The immunoblot membranes were then treated sequentially with anti-IGF-II receptor IgG and 125I-protein A (Du Pont-New England Nuclear) as previously described (5).

Reagents—Rat IGF-II was purified from the conditioned medium of Buffalo rat liver (BRL-3A) cells as described (19). Purified IGF-II was labeled with 125I (Amersham Corp.) using Enzymobeads (Bio-Rad) to a specific activity of 80–80 Ci/g. Insulin (porcine) was a gift from Dr. Ronald Chance (Lilly).

RESULTS

It was important to assess whether or not the effect of insulin on IGF-II receptor phosphorylation occurred in cell types other than the rat adipocyte. The H-35 hepatoma cell
has been shown to respond to insulin with increases in the uptake of IGF-II and incorporation of [³H]thymidine into DNA (14). Fig. 1 shows an experiment in which H-35 cells were treated with different concentrations of insulin for 10 min at 37 °C. Following this incubation the cells were washed with ice-cold buffer and assayed for radioactivity on ice, and the specific binding of ¹²⁵I-IGF-II was measured. Because at this low temperature endocytosis and exocytosis of receptors is blocked, the results reflect the number of IGF-II receptors present on the cell surface membrane. Insulin caused an approximately 2-fold increase in the binding of IGF-II to the surface of these cells (Fig. 1A). The insulin-induced increase in the binding of IGF-II could be due to an increase in the number of receptors on the cell surface or to an increase in the affinity of the receptor for IGF-II. To distinguish between these two possibilities, the effect of insulin on the binding of an anti-IGF-II receptor polyclonal antibody to the surface of the cells was investigated. Fig. 1B shows that the binding of the antibody to insulin-treated cells was increased approximately 2-fold compared to controls. These results indicate that the increased binding of ¹²⁵I-IGF-II is due to an increase in the number of IGF-II receptors on the cell surface in response to insulin.

In order to investigate if this effect of insulin was accompanied by a change in the phosphorylation state of the IGF-II receptor, H-35 cells were labeled to constant specific activity with [³²P]phosphate. Cells were then stimulated with insulin for 10 min and the IGF-II receptor from cell extracts was immunoprecipitated and separated on a 6% polyacrylamide gel. Fig. 2 shows an autoradiograph of such an experiment. A 250-kDa phosphopeptide, corresponding to the molecular weight of the IGF-II receptor, was the major band observed in five successive experiments. The lower molecular weight bands that were detected in addition to the 250-kDa IGF-II receptor band showed variation from one experiment to another and were very prominent in one experiment in which phenylmethylsulfonyl fluoride was omitted. Therefore, they most probably represent proteolytic fragments of the receptor, which contain the phosphorylated sites within the molecule. The amount of ³²P associated with the IGF-II receptor was quantitated by Cerenkov counting. In this specific experiment, 2003 and 1758 cpn were obtained in the receptors from control or insulin-treated cells, respectively. The bands were digested with trypsin and 20% of the sample (400 and 350 cpn for control or insulin, respectively) was applied to the thin layer plates. The phosphopeptides were resolved by electrophoresis and chromatography as described under "Materials and Methods." The origin is indicated (O).
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much lower stoichiometry (data not shown).

Insulin treatment caused a significant decrease in the phosphorylation of tryptic phosphopeptides 1 and 3, identified in control cells, as well as the virtual disappearance of phosphopeptide 2. These experiments indicate that in these cells the effect of insulin to increase the cell surface number of IGF-II receptors is accompanied by a decrease in the phosphorylation state of the receptor. This decrease is not observed exclusively on one tryptic peptide, indicating that insulin can decrease the 32P content of the IGF-II receptor on several of its phosphorylation sites.

The identity of the phosphorylated amino acids in 32P-labeled IGF-II receptors was studied by thin layer electrophoresis of an acid hydrolysate of the receptor. Both [32P]phosphoserine and [32P]phosphothreonine could be detected in receptors derived from control cells (Fig. 3). In contrast, the analysis of the labeled receptors from insulin-treated cells revealed only traces of phosphothreonine. It appears from these experiments that the insulin-mediated decrease in IGF-II receptor phosphorylation occurs on both serine and threonine residues.

As an approach toward identifying the enzymes that are involved in the phosphorylation and dephosphorylation of the IGF-II receptor, the ability of a number of highly purified protein kinases to catalyze phosphorylation of the purified receptor was tested. Among several enzymes employed (cAMP-dependent protein kinase, protein kinase C, phosphorylase kinase, and casein kinase II), only casein kinase II was able to catalyze the phosphorylation of IGF-II receptors. Fig. 4 shows the result of an experiment in which casein kinase II was incubated for 30 min with an IGF-II receptor immunoprecipitate and [γ-32P]ATP. The proteins were then separated on a 6% polyacrylamide gel. Autoradiography of such a gel revealed one phosphorylated band of 250 kDa, which corresponds to the molecular weight of the IGF-II receptor (A). As described under "Materials and Methods," electrophoresis of these samples was allowed to continue until the 43-kDa molecular weight marker reached the bottom of the gel. For this reason, the autophosphorylated β subunit of the casein kinase II, which has a molecular weight of 25 kDa, cannot be observed in these autoradiograms. Under these conditions, the phosphorylation stoichiometry of the receptor by the purified casein kinase II was approximately 20%. These data were derived from the intensity of the silver-stained receptor immunoprecipitated from unlabeled cells under similar experimental conditions and the specific activity of the [γ-32P]ATP employed in the phosphorylation reaction (10 μCi/mmole). Phosphoamino acid analysis of the IGF-II receptor band revealed the presence of [32P]phosphoserine but predominantly [32P]phosphothreonine (B).

It has been previously shown that the ability of the casein kinases to phosphorylate exogenous substrates is markedly dependent on the previous phosphorylation state of the substrate employed (15-17). Thus, we investigated whether the changes in the phosphorylation state of the IGF-II receptor produced by insulin could alter its ability to serve as a substrate for casein kinase II. An experiment was performed in which the IGF-II receptor was immunoprecipitated from either control or insulin-treated H-35 cells and then incubated with casein kinase II and [γ-32P]ATP for different times. The results of such an experiment are shown in Fig. 5. It can be seen that the rate of 32P incorporation into IGF-II receptors was significantly increased when the receptors were obtained from insulin-treated cells compared to control cells.

It was previously reported that insulin markedly decreased the phosphorylation of the IGF-II receptor derived from isolated adipocyte plasma membranes (5). The 32P content of the IGF-II receptors derived from insulin-treated adipocyte

FIG. 3. Phosphoamino acid analysis of the IGF-II receptor isolated from H-35 cells. Cell monolayers were incubated in the presence of [32P]phosphate and treated with 10⁻⁷ M insulin as described in the legend to Fig. 2. The IGF-II receptor was immunoprecipitated, separated by polyacrylamide gel electrophoresis, and digested with trypsin. The eluted phosphopeptides were hydrolyzed for 3 h at 110 °C and electrophoresed at pH 3.5 on cellulose-coated thin layer plates. The positions of the phosphoamino acids, identified by ninhydrin staining, are indicated. SER(P), phosphoserine; THR(P), phosphothreonine; TYR(P), phosphotyrosine.

FIG. 4. Phosphorylation of the IGF-II receptor by purified casein kinase II. The IGF-II receptors from approximately 10⁷ cells were immunoadsorbed on anti-IGF-II receptor IgG covalently attached to Affi-Gel 10. After extensive washing, the resin (5 μl) was incubated with approximately 3 ng of purified casein kinase II and [γ-32P]ATP, as described under "Materials and Methods." The reaction was ended by the addition of Laemmli sample buffer, and the IGF-II receptor was separated on a 6% polyacrylamide slab gel. Electrophoresis was monitored using prestained molecular weight standards and was allowed to continue until the 42-kDa marker (ovalbumin) reached the bottom of the gel. A is an autoradiograph of the dried gel obtained from this experiment. No phosphorylated bands were detected in experiments in which the casein kinase II was the only component omitted from the reaction (data not shown). The amino acid residues phosphorylated by casein kinase II were identified by thin layer electrophoresis of an acid hydrolysate of the receptor as described under "Materials and Methods." B shows an autoradiograph of the dried thin layer plate. The positions of the phosphoamino acids are indicated. SER(P), phosphoserine; THR(P), phosphothreonine; TYR(P), phosphotyrosine. Similar results were obtained in six successive experiments.
membranes was only 30–50% of that observed in receptors derived from control cells. This effect was larger than the one observed when studying the total cellular receptor pool in H-35 cells. For this reason, the ability of casein kinase II to catalyze phosphorylation of IGF-II receptors immunoprecipitated from control or insulin-treated adipocyte plasma membranes was investigated.

The amount of receptors present in the immunoprecipitate was quantitated by immunoblotting because plasma membranes from insulin-treated cells contain 2–3-fold more receptors than membranes from control cells. Fig. 6 shows the autoradiograph of an experiment in which immunoprecipitates from control or insulin-treated plasma membranes were phosphorylated in the presence of casein kinase II. The immunoprecipitated IGF-II receptors were separated on a 6% polyacrylamide slab gel and then transferred onto nitrocellulose. Following autoradiography, the nitrocellulose was incubated with anti-IGF-II receptor IgG and 125I-protein A. A 3-fold increase in the [32P]phosphate content of IGF-II receptors isolated from insulin-treated cells and incubated with [γ-32P]ATP and casein kinase II was observed. The number of receptors estimated by the amount of 125I associated with the receptor band was only 2-fold higher. These results indicate that insulin treatment of intact fat cells results in a 50% increase in the amount of [32P]phosphate that can be incorporated into plasma membrane IGF-II receptors in vitro by purified casein kinase II.

Figs. 5 and 6 show that IGF-II receptors from insulin-treated cells were phosphorylated in vitro by casein kinase II to a higher stoichiometry than those receptors obtained from control cells. To further understand the nature of this effect, the tryptic phosphopeptide maps of IGF-II receptors obtained from control or insulin-treated cells, and subsequently phosphorylated in vitro by casein kinase II, were compared. In Fig. 7A and B, the positions of the main tryptic phosphopeptides detected in receptors obtained from in vivo labeled cells (Fig. 2) are indicated. It can be observed that a significant proportion of the radioactivity incorporated into IGF-II receptors obtained from control cells by purified casein kinase II (Fig. 7A) was found in a tryptic phosphopeptide which had identical electrophoretic and chromatographic mobility as phosphopeptide 1 detected in the IGF-II receptor obtained from 32P-labeled cells (Fig. 2). Phosphopeptides 2 and 3 were not detectably phosphorylated by casein kinase II in receptors from control cells. In addition, casein kinase II phosphorylated a number of sites which were not detected in vivo. The phosphopeptide map of the receptor obtained from insulin-treated cells (Fig. 7B) was markedly different. In this case, two of the phosphopeptides (1 and 2) had identical mobilities to those found in IGF-II receptors from in vivo labeled control cells (Fig. 2). In addition, several phosphopeptides, which were not detected in control cell-derived, casein kinase II-phosphorylated IGF-II receptors nor in receptors obtained from cells labeled in vivo with [32P]phosphate, could be observed.

DISCUSSION
The data presented in this paper indicate that the effect of insulin to cause an increase in the cell surface number of IGF-II receptors in H-35 hepatoma cells was accompanied by a decrease in the phosphorylation state of this receptor. This decrease had been previously observed to occur in isolated rat adipocytes, where IGF-II receptors continually recycle be-
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FIG. 7. Phosphopeptide mapping of the IGF-II receptor phosphorylated in vitro with casein kinase II. IGF-II receptors were immunoprecipitated from control (A) or insulin-treated (B) H-35 cells and subsequently phosphorylated with casein kinase II as described in the legend to Fig. 6 and under “Materials and Methods.” Tryptic digestion and separation of the phosphopeptides was done as described under “Materials and Methods.” The numbers indicate the positions of the tryptic phosphopeptides identified in Fig. 2.

Both in H-35 cells (Fig. 3) and in freshly isolated adipocytes labeled with 32P (data not illustrated) the amino acid residues on the IGF-II receptor that were found to be phosphorylated were phosphoserine and phosphothreonine. Upon exposure of cells to insulin, a significant decrease in the 32P-phosphate content of the IGF-II receptor was observed, to be interpreted that the purified kinase cannot phosphorylate those sites that are already phosphorylated on the receptor (5). The insulin-mediated decrease in the 32P-phosphate content of the IGF-II receptor from H-35 cells was consistent but small. This small decrease may be due to the fact that the total IGF-II receptor pool from H-35 hepatoma cells was immunoprecipitated in these experiments. Thus, analogous to observations in adipocytes insulin may cause a selective decrease in the phosphorylation of receptors present exclusively in the H-35 plasma membrane, which may comprise a small proportion of the total receptor population. Methods to purify plasma membranes and intracellular membranes from H-35 cells are not yet available.

Both in H-35 cells (Fig. 3) and in freshly isolated adipocytes labeled with 32P (data not illustrated) the amino acid residues on the IGF-II receptor that were found to be phosphorylated were phosphoserine and phosphothreonine. Upon exposure of cells to insulin, a significant decrease in the 32P-phosphate content of the IGF-II receptor was observed, together with a smaller decrease in 32P-phosphoserine. Previously it has been shown that, upon incubation of isolated adipocyte plasma membranes with [y-32P]ATP, the activity of a kinase which catalyzed the phosphorylation of IGF-II receptors on tyrosine residues (18) could be detected. Furthermore, this tyrosine phosphorylation reaction was decreased in membranes isolated from insulin-treated cells. For this reason, it was interesting to investigate if the tyrosine phosphorylation of the IGF-II receptor, which occurs in isolated membranes incubated with [y-32P]ATP, could also be detected in receptors obtained from intact cells labeled in vivo with [32P]phosphate. Under the experimental conditions employed, a significant amount of phosphotyrosine on the 32P-labeled receptor could not be detected (Fig. 3). This negative result could be due to several technical reasons, such as the loss of receptor tyrosine phosphate during cell disruption or to the low recovery of tyrosine phosphate during acid hydrolysis. Alternatively, the tyrosine kinase activity of the adipocyte membrane may be under tight regulatory control in intact cells by a mechanism that is lost upon disruption of the cells. These possibilities are currently being explored in an effort to gain insight into the physiological significance of IGF-II receptor tyrosine phosphorylation.

Exposure of 32P-labeled cells to insulin caused a marked decrease in the phosphothreonine and phosphoserine content of the IGF-II receptor. Thus, insulin may cause the inhibition of a serine/threonine kinase or the activation of a serine/threonine phosphatase, or both. To gain insight into the enzymes that may catalyze phosphorylation of IGF-II receptors in vivo, the ability of a number of purified serine/threonine kinases to phosphorylate the immunoprecipitated purified IGF-II receptor was tested. Among several enzymes tested, only casein kinase II was able to phosphorylate the immunoprecipitated receptor in vitro. Analysis of a tryptic digest of the receptor phosphorylated by casein kinase II revealed that this enzyme phosphorylated the receptor on several sites. One of the peptides observed comigrated on electrophoresis and chromatography with a tryptic peptide derived from the receptor obtained from cells labeled in vitro with [32P]phosphate, suggesting that the receptor may be phosphorylated in vivo by casein kinase II or a similar enzyme. Phosphoamino acid analysis of the receptors obtained from intact cells revealed a predominance of phosphoserine over phosphothreonine, whereas the receptor labeled in vitro with casein kinase II contained predominantly phosphothreonine over phosphoserine. These results are consistent with the interpretation that the purified kinase cannot phosphorylate those sites that are already phosphorylated on the receptor when it is extracted from the cell (phosphoserine residues), but can readily phosphorylate those sites that are unoccupied with unlabeled phosphate (phosphothreonine residues). This interpretation is also consistent with the finding that the receptors obtained from insulin-treated cells, which contain less phosphate than controls, are phosphorylated to a greater extent in vitro by casein kinase II. However, it is also possible that other kinases, in addition to casein kinase II or a casein kinase II-like enzyme, may phosphorylate the receptor on serine residues in intact cells.

Relatively little is known about the physiological role of casein kinase II. This kinase has been identified in numerous mammalian and avian cells, in association with nuclei, membranes, mitochondria, and ribosomes (reviewed in Ref. 19). Casein kinase II has been shown to be involved in the phosphorylation of other insulin-sensitive phosphoproteins such as glycogen synthase (20) and phosphatase inhibitor 2 (21). These possibilities are currently being explored in an effort to gain insight into the physiological significance of IGF-II receptor tyrosine phosphorylation.

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substrates by casein kinase II (21, 24).

More recently casein kinase II has been shown to be associated with brain and liver coated vesicles, where it has been shown to phosphorylate stoichiometrically the β-light chain of clathrin (25). This finding raises the possibility that casein kinase II may play a role in regulating the interactions among coated vesicle proteins or between the proteins which undergo endocytosis through coated pits. The IGF-II receptor may be similar to other membrane receptors (reviewed in Ref. 26) in that its internalization may involve its movement and concentration into coated pits. It is possible that phosphorylation of the receptor by the casein kinase II present in the coated pit might be important in anchoring the receptor to this structure, although no direct supporting data for this postulate is available.

An interesting finding presented in this report is that the amount of casein kinase II-catalyzed phosphorylated incorporated into IGF-II receptors was significantly greater when receptors from insulin-treated cells were used as the substrate. It has been shown that the casein kinases recognize specific sequences in acidic protein substrates which are affected by the state of prior phosphorylation of the molecule. In the case of phosphotyrosine, optimal incorporation of 32P is achieved when the substrate is partially dephosphorylated (27). Studies with variant cases have shown that the ability of casein kinase II to phosphorylate a specific site is greatly affected by the phosphorylation of the sequence surrounding the site (reviewed in Ref. 19). The tryptic phosphopeptide maps of IGF-II receptors obtained from insulin-treated cells and phosphorylated in vitro by casein kinase II revealed a number of phosphopeptides which were not detected in receptors derived from control cells and phosphorylated with this enzyme. Thus, it appears that the insulin-mediated decrease in the phosphorylation of IGF-II receptors on specific sites enhances the ability of casein kinase II to catalyze phosphorylation of neighboring sites within the molecule. In addition, two of the sites which are dephosphorylated upon insulin treatment in vivo (peptides 1 and 2, Fig. 2B) appear to be substrates for casein kinase II. Therefore, the increased phosphorylation of the IGF-II receptor from insulin-treated cells by casein kinase II may also be partially due to the replacement of the phosphopeptide groups on the dephosphorylated amino acids with [32P]phosphate in vitro. Studies to directly determine whether or not casein kinase II or a similar enzyme directly participates in IGF-II receptor phosphorylation and its modulation by insulin in vivo are presently being conducted.

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Note Added in Proof—During the review of this manuscript, two groups found that deduced amino acid sequences of the IGF-II receptor from human (28) and rat (29) cDNA clones exhibit high identity to the bovine cation-independent mannose-6-phosphate (Man-6-P) receptor (30). The two receptors were shown to be the same protein (29). The deduced amino acid sequences of the cytoplasmic domains of the IGF-II/Man-6-P receptors from these species reveal several highly conserved characteristic casein kinase II phosphorylation sites (29).

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