*Proc. Natl. Acad. Sci. USA*
Vol. 84, pp. 113–117, January 1987
Cell Biology

# Insulin rapidly stimulates phosphorylation of a 46-kDa membrane protein on tyrosine residues as well as phosphorylation of several soluble proteins in intact fat cells

H. U. HÄRING*†, M. F. WHITE‡, F. MACHICAO*, B. ERMEL*, E. SCHLEICHER*, AND B. OBERMAIER*

*Institut für Diabetesforschung, Kölner Platz 1, 8000 Munich 40, Federal Republic of Germany; and ‡Joslin Diabetes Center, Harvard Medical School, Boston, MA 02215

*Communicated by Edmond H. Fischer, August 22, 1986*

**ABSTRACT** **It is speculated that the transmission of an insulin signal across the plasma membrane of cells occurs through activation of the tyrosine-specific receptor kinase, autophosphorylation of the receptor, and subsequent phosphorylation of unidentified substrates in the cell. In an attempt to identify possible substrates, we labeled intact rat fat cells with [$^{32}$P]orthophosphate and used an antiphosphotyrosine antibody to identify proteins that become phosphorylated on tyrosine residues in an insulin-stimulated way. In the membrane fraction of the fat cells, we found, in addition to the 95-kDa β-subunit of the receptor, a 46-kDa phosphoprotein that is phosphorylated exclusively on tyrosine residues. This protein is not immunoprecipitated by antibodies against different regions of the insulin receptor and its HPLC tryptic peptide map is different from the tryptic peptide map of the insulin receptor, suggesting that it is not derived from the receptor β-subunit. Insulin stimulates the tyrosine phosphorylation of the 46-kDa protein within 150 sec in the intact cell 3- to 4-fold in a dose-dependent way at insulin concentrations between 0.5 nM and 100 nM. The insulin effect starts after 30 sec, is maximal at 150 sec, and declines to almost basal values by 5 min. Furthermore, the antiphosphotyrosine antibody precipitated at least five proteins in the soluble fraction of the fat cell. Insulin (0.5 nM, 100 nM) stimulated within 2 min the $^{32}$P incorporation into a 116-kDa band, a 62-kDa band, and three bands between 45 kDa and 50 kDa 2- to 10-fold. We suggest that the 46-kDa membrane protein and possibly also the soluble proteins are endogenous substrates of the receptor tyrosine kinase in fat cells and that their phosphorylation is an early step in insulin signal transmission.**

Understanding of the mechanism of cellular insulin action was substantially improved by the observation of Kasuga *et al.* (1) in 1982 that insulin stimulates the phosphorylation of the 95-kDa β-subunit of the insulin receptor in intact cells. Subsequently, it was shown that the tyrosine kinase phosphorylating the insulin receptor is an intrinsic part of the β-subunit of the receptor itself (2–5) and it was proposed that the transmission of an insulin signal across the plasma membrane occurs through activation of this kinase (1–12). Furthermore, it was shown *in vitro* that the isolated receptor kinase undergoes insulin-stimulated autophosphorylation and that this activates the ability of the kinase to phosphorylate other protein substrates (10, 13). These observations are the basis of the hypothesis that insulin signal transmission is a sequence of steps, including insulin binding to the α-subunit of the receptor, activation of the tyrosine kinase in the β-subunit, autophosphorylation of the β-subunit, and finally phosphorylation of a cellular protein substrate. Even though the phosphorylation of the β-subunit could be shown in many intact cells (1, 7–10, 14) and even though phosphorylation of many protein substrates by the solubilized and purified receptor kinase was demonstrated *in vitro* (13, 15–20), a substrate phosphorylation in the intact cell at tyrosine residues could not yet be demonstrated. Recently, White *et al.* (21) succeeded in identifying a 185-kDa protein in the well-differentiated FAO hepatoma cell line that appears to be a substrate of the insulin receptor kinase in this cell type.

In this study an antiphosphotyrosine antibody prepared as described by Pang *et al.* (22) was used to immunoprecipitate specifically tyrosine phosphorylated proteins in the hepatoma cell. We used this antiphosphotyrosine antibody to search for a substrate of the insulin receptor kinase in the rat adipocyte and found that several proteins are precipitated that become phosphorylated in an insulin-dependent way.

## MATERIALS AND METHODS

**Materials.** Porcine insulin was purchased from Novo Industrie (Bagsvaerd, Denmark), [$^{32}$P]orthophosphate was from New England Nuclear, aprotinin, phenylmethylsulfonyl fluoride, leupeptin, and pepstatin were from Sigma, and Triton X-100 and all reagents for $NaDodSO_4$/polyacrylamide gel electrophoresis ($NaDodSO_4$/PAGE) were from Bio-Rad; all other reagents were of the best grade commercially available.

**Methods.** *Isolation of fat cells and labeling with [$^{32}$P]orthophosphate.* Rat adipocytes were freshly isolated as described (23). After isolation by collagenase, the cells were washed four times in phosphate-free Krebs/Ringer/Hepes buffer. Aliquots of 15 ml of adipocytes (4.5–5.5 × $10^6$ per ml) were subsequently labeled for 90 min in phosphate-free Krebs/Ringer/Hepes buffer containing carrier-free [$^{32}$P]orthophosphate (0.3–0.8 mCi/ml; 1 Ci = 37 GBq) at 37°C. Insulin (0.5–100 nM) was omitted or added and the incubation was continued after addition of insulin for 30–300 sec. The experiments were stopped quickly by addition of a cold stopping solution followed by immediate freezing in liquid nitrogen. The stopping solution contained phosphatase inhibitors (vanadate, 2 mM; sodium fluoride, 100 mM; sodium pyrophosphate, 10 mM) and protease inhibitors (aprotinin, 1000 trypsin-inhibiting units/liter; phenylmethylsulfonyl fluoride, 5 mM; pepstatin, 2 μM; leupeptin, 2 μM; leucin, 10 mM). The cells were subsequently solubilized by repeated freezing and thawing. The homogenate was then centrifuged at 200,000 × *g* for 50 min. The fat layer was discarded. The liquid supernatant and the pellet were further processed as described below.

*Immunoprecipitation of phosphoproteins in the particulate fraction of fat cells.* The pellet of the 200,000 × *g* centrifugation was solubilized in buffer containing 1% Triton X-100

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

†To whom reprint requests should be addressed.

(by volume) as well as all of the above listed protease and phosphatase inhibitors in the same concentrations as above. The samples were centrifuged again at 200,000 × *g* for 40 min to remove insoluble material. The supernatant was subsequently immunoprecipitated either with antiphosphotyrosine antibodies prepared according to the method of Pang *et al.* (22) or with antiinsulin receptor antibodies obtained from patients, respectively prepared by immunization of rabbits with synthetic peptides with the amino acid sequences surrounding Tyr-1122, Tyr-1150, and Tyr-1151 as well as Tyr-960 of the receptor β-subunit. After incubation with the extract at 4°C for 2 hr, the antibodies were immobilized on Pansorbin (Calbiochem) and the precipitates were washed three times with a solution containing 50 mM Hepes, 1% Triton X-100, and 0.1% $NaDodSO_4$. The proteins were eluted by addition of *p*-nitrophenol phosphate or eluted directly by addition of Laemmli buffer (24). The eluted proteins were reduced with 100 mM dithiothreitol, unless otherwise stated, and the phosphoproteins were separated by $NaDodSO_4$/PAGE on gradient gels. The phosphoproteins were identified by autoradiography of the stained and dried gels using Kodak X-Omat film and an intensifying screen. The bands identified by the autoradiography were cut from the gel and radioactivity was counted in a scintillation counter.

*Immunoprecipitation of phosphoproteins from the soluble fraction of fat cells.* The proteins in the supernatant of the first 200,000 × *g* centrifugation were precipitated with trichloroacetic acid 2% (by volume), final concentration. The sediment was washed four times with trichloroacetic acid 2% (by volume) and then redissolved by careful neutralization with NaOH. The redissolved proteins were then immunoprecipitated and further processed as described above for the solubilized membrane proteins.

*Identification of phosphoamino acids.* The phosphorylated bands in the dried gel containing ≈2500 cpm were excised and washed with dioxane, followed by washing with methanol and 10% methanol, respectively. The gel fragments were placed into 500 μl of 6 M HCl and hydrolyzed for 2 hr at 110°C. The samples were then diluted with 2 ml of water, lyophilized, and redissolved in 30–50 μl of water. Electrophoresis was performed on Whatman 3 MM paper at pH 3.5 with pyridine/acetic acid/water, 5:50:945 (vol/vol) for 90 min at 1 kV. Phosphoamino acid standards were localized with ninhydrin; $^{32}P$-labeled amino acids were localized by autoradiography.

*Tryptic peptide mapping.* Tryptic peptide mapping by HPLC was performed as described (25). Fixed, stained, destained, and dried polyacrylamide gel fragments containing the phosphoprotein bands located by autoradiography were washed for 12 hr at 37°C with 20 ml of 10% methanol. The adsorbent paper was removed from the gel fragment. The gel was dried at 70°C for 60 min and rehydrated in 2 ml of 50 mM $NH_4HCO_3$ containing 100 μg of L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin. The mixture was incubated for 12 hr at 37°C, the gel fragment was removed, and the supernatant was clarified by centrifugation. The supernatant was lyophilized and the residue was dissolved in 25–50 μl of 0.1% trifluoroacetic acid and again clarified by centrifugation and filtration. The phosphopeptides were separated by using an LKB HPLC system equipped with an Ultropac column TSK ODS-120 T, 5-μm column. Phosphopeptides applied to the column were eluted at a flow rate of 0.5 ml/min with water containing 0.05% trifluoroacetic acid and modified with acetonitrile, also containing 0.05% trifluoroacetic acid. Radioactivity in 0.1-ml to 0.11-ml fractions eluted from the reverse-phase column was measured in 3 ml of scintillation mixture.

## RESULTS

**Immunoprecipitation of Tyrosine Phosphate-Containing Proteins from the Particulate Fraction of Fat Cells.** Rat adipocytes labeled with [$^{32}P$]orthophosphate were stimulated with insulin (100 nM) for 150 sec. Lanes A and B of Fig. 1 show an autoradiogram of phosphoproteins isolated from these fat cells with the antiphosphotyrosine antibody and analyzed under reducing conditions (100 mM dithiothreitol) by PAGE. After 12 hr of exposure of the polyacrylamide gel to the film, one strongly phosphorylated band corresponding to a molecular mass of 46 kDa is detected. In lane A no insulin was added, whereas in lane B the intact cell was stimulated for 150 sec with insulin (100 nM). Here also appears a faint 95-kDa band corresponding to the known size of the β-subunit of the insulin receptor (6, 8). Insulin increased the $^{32}P$ incorporation into the 46-kDa band in six separate experiments 3- to 6-fold within 150 sec. If an aliquot of the phosphoproteins was analyzed under nonreducing conditions (lanes C and D, Fig. 1), the phosphorylated band appears in the same position, suggesting that the protein is not derived from a disulfide-linked polymeric complex as it is the case for the insulin receptor (1, 6).

To analyze the phosphoamino acid composition of the 46-kDa protein, the insulin stimulated band was excised from the gel, the protein was hydrolyzed, and the phosphoamino acids were separated by paper electrophoresis. Fig. 1, lane E, shows an autoradiogram of the one-dimensional separation of the phosphoamino acids. $^{32}P$ incorporation occurs exclusively in tyrosine residues. Thus, this finding demonstrates an insulin-stimulated tyrosine phosphorylation of a membrane protein in the intact adipocyte aside from the insulin receptor itself.

To test the possibility that the 46-kDa protein is derived from the insulin receptor, we precipitated the phosphorylated membrane proteins of the fat cell also by a polyclonal antiinsulin receptor serum. Fig. 2 shows an autoradiogram of an experiment in which the phosphoproteins were precip-

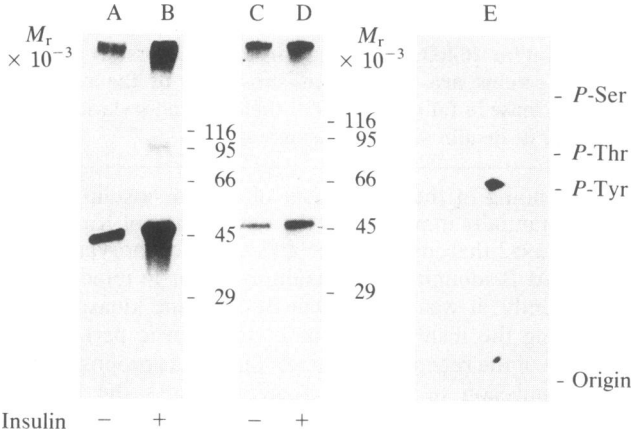


FIG. 1. Fat cells (2 × 15 ml, 4.5–5.5 × $10^6$ per ml) were labeled with [$^{32}P$]orthophosphate (0.6 mCi/ml), incubated without or with insulin (100 nM), and subsequently processed. Phosphoproteins from the particulate fraction solubilized by Triton X-100 were then immunoprecipitated with antiphosphotyrosine antibody. Lane A, phosphorylation in the absence of insulin; lane B, after addition of 100 nM insulin for 150 sec; lanes C and D, aliquots of the samples of lanes A and B, respectively, that were analyzed under nonreducing conditions; lane E, autoradiogram of phosphoamino acids from the 46-kDa band. The 46-kDa protein band in the dried gel containing ≈2500 cpm was excised and further processed. Phosphoamino acid standards were localized with ninhydrin; $^{32}P$-labeled amino acids were localized by autoradiography. The experiments shown in lanes A and B were reproduced five times; those in lanes C–E were reproduced two times.

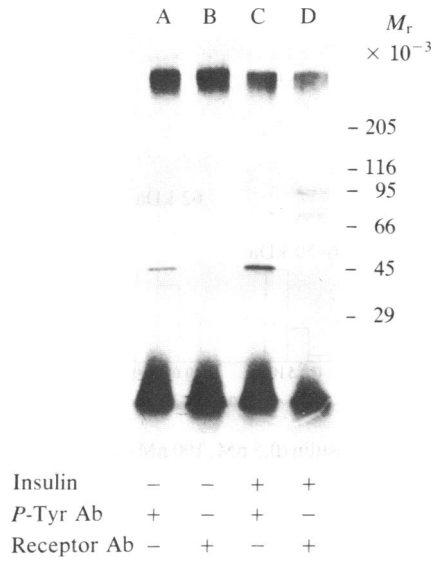


FIG. 2. Fat cells (2 × 15 ml, 4.5–5.5 × $10^6$ per ml) were labeled with [$^{32}$P]orthophosphate (0.3 mCi/ml), incubated without or with insulin (100 nM), and subsequently processed. Phosphoproteins solubilized by Triton X-100 were then immunoprecipitated with antiphosphotyrosine antibody (Ab) or antiinsulin receptor antibody. Lanes A and B, phosphoproteins from cells that were not stimulated; lanes C and D, phosphoproteins from cells that were stimulated with insulin (100 nM) for 150 sec. Lanes A and C show immunoprecipitates with the antiphosphotyrosine antibody; lanes B and D show immunoprecipitates with receptor antibody. The experiments shown were reproduced two times.

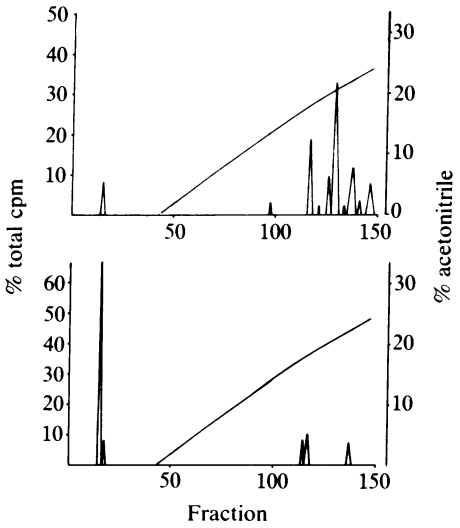


FIG. 3. Tryptic peptide mapping of the 46-kDa phosphoprotein and the 95-kDa receptor subunit by HPLC was performed as described (25). Insulin receptor from adipocytes was phosphorylated *in vitro* as described (8) and identified on the gel by autoradiography. Gel fragments containing the 46-kDa phosphoproteins shown in Fig. 1 and gel fragments containing the β-subunit of insulin receptor were incubated with trypsin for 12 hr; the supernatant was lyophilized and dissolved in 50 μl of 0.1% trifluoroacetic acid. The phosphopeptides were separated by using an LKB HPLC system. (*Lower*) Elution profile of the phosphopeptides of the 46-kDa protein. (*Upper*) Elution profile of the phosphopeptides of the 95-kDa protein. Data from one experiment are shown.

itated in parallel either by the antiphosphotyrosine antibody (lanes A and C) or by the antireceptor antibody (lanes B and D). The gel was exposed for 12 hr to the film. The 46-kDa band is not precipitated by the antireceptor antibody, which precipitated only a faint 95-kDa receptor band (lane D), suggesting that the 46-kDa protein is immunologically different from the insulin receptor. The 95-kDa band that is not precipitated by control serum (data not shown) was identified earlier as the β-subunit of the insulin receptor in adipocytes (8). In agreement with earlier results (8), its $^{32}$P labeling after insulin stimulation of the intact fat cell for only 150 sec is barely detectable after 12 hr of exposure to the film. In contrast, the 46-kDa protein becomes strongly labeled in the same time. As the possibility exists that the 46-kDa phosphoprotein is derived from a part of the receptor β-subunit that is not recognized by the polyclonal receptor antibody, we used also three peptide antibodies prepared against the amino acid sequences surrounding Tyr-960, Tyr-1122, and Tyr-1150–1151 in the β-subunit of the insulin receptor. No significant precipitation of the 46-kDa band was found with these antibodies (data not shown). To gain further information about a possible relation of the 46-kDa band to the receptor β-subunit, we excised the 46-kDa band, prepared a tryptic digest and analyzed the phosphopeptides by HPLC. Fig. 3 shows that the elution profile is different from the profile of a tryptic digest of a 95-kDa subunit of the insulin receptor from adipocytes phosphorylated *in vitro* that was used as a control. Therefore, it appears that the 46-kDa protein is not derived from the β-subunit of the insulin receptor of the adipocytes but is a distinct cellular protein.

The dose dependency of the insulin effect on the phosphorylation of the 46-kDa protein was studied in the experiment shown in Fig. 4. Fat cells were stimulated for 150 sec with increasing concentrations of insulin. The autoradiogram shows that the insulin effect is dose dependent at physiological concentrations and follows the known concentration dependency of insulin binding to the receptor. Fig. 5 shows the time course of the insulin effect on the phosphorylation of the 46-kDa protein. The effect starts at 30 sec after addition of insulin, reaches a maximum around 150 sec, and declines by 5 min to almost basal values.

**Immunoprecipitation of Tyrosine Phosphate-Containing Proteins in the Soluble Fraction of Fat Cells.** $^{32}$P-labeled fat cells were stimulated with insulin (0.5 nM, 100 nM) for 2 min and further processed as described in *Methods*. Fig. 6 shows an autoradiogram of an immunoprecipitate of phosphopro-

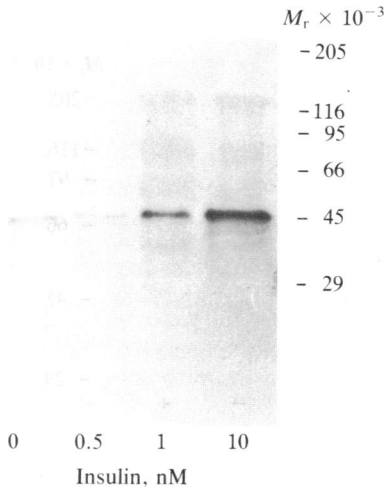


FIG. 4. Autoradiogram showing a dose–response curve of the insulin effect on phosphorylation of the 46-kDa protein. Insulin in the concentrations indicated was added to the cells for 150 sec after prelabeling with [$^{32}$P]orthophosphate (0.3 mCi/ml). The cells were then processed further. As a modification to the experiments shown in Figs. 1 and 2, the phosphoproteins were eluted from the immunoprecipitate with *p*-nitrophenol phosphate before addition to Laemmli buffer. The experiment was reproduced two times.

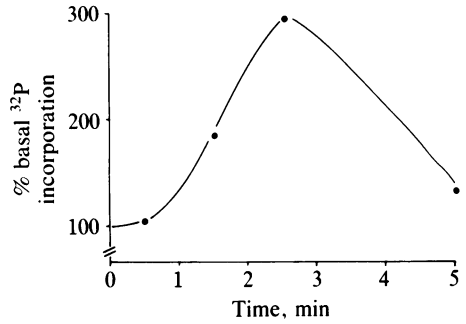


FIG. 5. Time course of the insulin effect on the phosphorylation of the 46-kDa protein. The prelabeled fat cells (0.3 mCi/ml; 5 × 15 ml, 4.5–5.5 × $10^6$ per ml) were stimulated with insulin (100 nM) for 30–300 sec as indicated on the *x* axis. The cells were then processed further. The 46-kDa bands identified by autoradiography were cut from the gel and radioactivity was counted. Mean values of two experiments are shown.

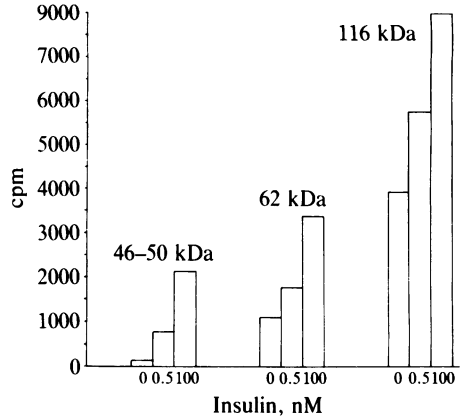


FIG. 7. Effect of insulin (0.5 nM, 100 nM) on the $^{32}$P incorporation into the 46- to 50-kDa bands, the 62-kDa band, and the 116-kDa band. The columns show mean values of three separate experiments.

teins from the soluble fraction of the fat cells. Several labeled bands are detectable. Addition of insulin (0.5 nM, 100 nM) for 2 min increases the $^{32}$P incorporation into a band of 116 kDa, a band of 62 kDa, and three bands between 46 and 50 kDa. Fig. 7 shows the increase of $^{32}$P incorporation after insulin stimulation (0.5 nM, 100 nM) in three separate experiments. The insulin-induced increase in all bands is 3- to 10-fold within 2 min. In one experiment, insulin was added for 2 and for 5 min. It was found that the $^{32}$P incorporation in all bands decreased at the later time point.

## DISCUSSION

The antiphosphotyrosine antibody precipitates several phosphoproteins in the particular fraction and the soluble fraction of rat fat cells. In the particulate fraction, insulin stimulates $^{32}$P incorporation into a 46-kDa protein as well as into a 95-kDa protein, which is the β-subunit of the insulin receptor of the fat cell (8). In the soluble fraction of the fat cells, insulin increases the $^{32}$P incorporation in phosphoprotein bands of 116 kDa and 62 kDa as well as three protein

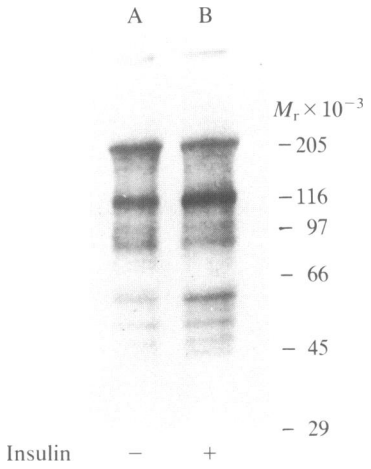


FIG. 6. Immunoprecipitation of phosphoproteins from the soluble fraction of $^{32}$P-labeled fat cells. Fat cells were labeled with [$^{32}$P]orthophosphate (0.3 mCi/ml; 2 × 15 ml, 4.5–5.5 × $10^6$ per ml), incubated without or with insulin (100 nM), and subsequently processed. The supernatant of a 200,000 × *g* centrifugation was then precipitated with 2% trichloroacetic acid (by volume) and redissolved by titration with NaOH. The proteins were then immunoprecipitated. Lane A, phosphorylation in the absence of insulin; lane B, phosphorylation in the presence of 100 nM insulin. The experiment was reproduced two times and mean values of these experiments are shown in Fig. 7.

bands in the molecular mass region between 45 and 50 kDa. To evaluate a possible significance of these effects as signal-transmitting reactions, the time course and concentration dependency of the insulin effect on the phosphorylation of these proteins in intact cells are important. The data show that these effects are rapid—a 3- to 10-fold stimulation occurs within 2 min showing that the insulin effect on phosphorylation of these proteins precedes or parallels the fastest known insulin effects on fat cell metabolism. For comparison, stimulation of the glucose transport system starts in the intact fat cell after an initial lag time of almost 1 min and reaches its maximum no earlier than 4 min after addition of insulin to the cell (23, 26). Furthermore, the effects can be demonstrated at physiological insulin concentrations, showing that the two basic criteria of a putative signal-transmitting reaction are fulfilled.

Information about the identity of these proteins is at present insufficient and allows only speculations. The 46-kDa protein appears to be a membrane protein that becomes phosphorylated exclusively on tyrosine residues. Even though the possibility that the 46-kDa protein is derived from the β-subunit of the insulin receptor cannot be completely ruled out, our results are not in favor of this origin of the protein. Antibodies against different domains of the insulin receptor do not precipitate the 46-kDa protein. The HPLC of the tryptic digest of the 46-kDa protein appears to be different from the HPLC of a tryptic digest of the insulin receptor that was phosphorylated *in vitro*. Another observation argues also against the possibility that the 46-kDa protein is derived from the insulin receptor. The phosphoamino acid composition of the β-subunit of the insulin receptor in intact fat cells is not known. The low levels of $^{32}$P incorporation into the receptor β-subunit that are found if intact fat cells are labeled did not allow analysis of the phosphoamino acids. However, an analogous situation, as in intact FAO hepatoma cells, may be expected whereby the insulin receptor in the intact cell is not only phosphorylated on tyrosine residues but also incorporates $^{32}$P predominantly in serine and, to some extent, also in threonine residues (7, 27, 28); in contrast, the 46-kDa protein is exclusively phosphorylated on tyrosine residues and a basal tyrosine phosphorylation is already found before stimulation of the cell with insulin. If the 46-kDa protein is not derived from the insulin receptor, several other proteins in this molecular mass range exist that would be possible candidates. These proteins include the glucose transport protein (29) as well as actin, which is a substrate of the isolated insulin receptor kinase *in vitro* (17). However, thus far we are unable to precipitate the 46-kDa protein with antibodies against these two proteins. Hence, further studies are needed to elucidate the nature of the 46-kDa protein.

The antiphosphotyrosine antibody precipitates in the soluble fraction of fat cells at least five insulin-stimulated phosphoprotein bands. Again, there is at present no conclusive information about the nature of these phosphoproteins. However, the 116-kDa protein could be identical with a phosphoprotein band of 120 kDa, which was observed in the FAO hepatoma cell (21). Phosphorylation of a protein of this molecular mass range was also observed in a cell-free system using a Triton X-100 extract of rat hepatocyte plasma membranes purified by wheat germ agglutinin chromatography (19, 20) as well as in a wheat germ agglutinin extract of human placenta membranes (unpublished data). The nature of this phosphoprotein in all these systems is unknown. We have preliminary data showing that the 116-kDa protein as well as the other soluble proteins contain phosphorylated serine residues as well, suggesting that these proteins are either serine-specific protein kinases that undergo an insulin-stimulated autophosphorylation on serine residues or that these proteins are substrates for serine-specific kinases. This parallels the situation found for the β-subunit of the insulin receptor, which is phosphorylated on serine and threonine residues as well (7, 27, 28), even though the purified insulin receptor kinase phosphorylates exclusively on tyrosine residues (2). This led to the speculation that the insulin signal transmission involves on the postreceptor level also the activation of a serine kinase that is able to phosphorylate the β-subunit of the insulin receptor (6). There is now some evidence that one serine kinase that can phosphorylate the insulin receptor β-subunit is the protein kinase C (30, 31), which appears to inhibit the receptor tyrosine kinase, possibly by modulation of the affinity of the ATP-binding site of the enzyme (32). In an attempt to find a protein that might link the receptor tyrosine kinase to these serine/threonine phosphorylation reactions, we have studied also the interaction of a partially purified preparation of insulin receptor from FAO hepatoma cells with purified preparations of serine/threonine kinases known to phosphorylate glycogen synthase (18). We found that the isolated insulin receptor tyrosine kinase was able to phosphorylate the calmodulin-dependent protein kinase in this *in vitro* system at tyrosine residues (18). Therefore, it will be interesting to determine if one of the phosphoprotein bands in the molecular mass range around 50 kDa is identical with a subunit (33) of the calmodulin-dependent protein kinase. Similarly, a relationship of the phosphoproteins found here to insulin-stimulated phosphoproteins found earlier (34–36) as well as to the recent finding of Lawrence *et al.* (37) is possible and must be evaluated.

In summary, the results show that other proteins aside from the β-subunit of the insulin receptor become phosphorylated in the intact cell on tyrosine residues and that insulin in physiological concentrations rapidly increases the $^{32}P$ incorporation in these proteins. The data suggest that some of these proteins might be direct substrates of the insulin receptor kinase and that their phosphorylation is involved in insulin action.

We thank Dr. C. R. Kahn for the gift of receptor antibodies and Dr. O. Rosen for the gift of an antibody against the glucose transport protein. Furthermore, we thank J. Mushack, E. Seffer, and R. Mayer for expert technical assistance and Prof. O. Wieland for critical reading of the manuscript.

1. Kasuga, M., Karlsson, F. A. & Kahn, C. R. (1982) *Science* **215,** 185–187.
2. Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D. L. & Kahn, C. R. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 2137–2141.
3. VanObberghen, E., Rossie, B., Kowalski, A., Gazzano, H. & Ponzio, G. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 945–949.
4. Ulrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M. & Ramachandran, J. (1985) *Nature (London)* **313,** 756–761.
5. Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J., Masiarz, F., Kan, Y. W., Goldfine, I. D., Roth, R. A. & Rutter, W. J. (1985) *Cell* **40,** 735–746.
6. Kahn, C. R., White, M. F., Grigorescu, F., Takayama, S., Häring, H. U. & Crettaz, M. (1985) in *Molecular Basis of Insulin Action*, ed. Czech, M. (Plenum, New York), pp. 67–93.
7. Kasuga, M., Zick, Y., Blithe, D. L., Karlsson, F. A., Häring, H. U. & Kahn, C. R. (1982) *J. Biol. Chem.* **257,** 9891–9894.
8. Häring, H. U., Kasuga, M. & Kahn, C. R. (1982) *Biochem. Biophys. Res. Commun.* **108,** 1528–1545.
9. VanObberghen, E. & Kowalski, A. (1982) *FEBS Lett.* **143,** 179–182.
10. Petruzelli, L. M., Ganguly, S., Smith, C. R., Cobb, M. H., Rubin, C. S. & Rosen, O. M. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 6792–6796.
11. Zick, Y., Kasuga, M. & Kahn, C. R. (1983) *J. Biol. Chem.* **258,** 75–80.
12. Avruch, J., Nemenoff, R. A., Blackshear, P. J., Pierce, M. W. & Osothanondh, R. (1982) *J. Biol. Chem.* **257,** 15162–15166.
13. Yu, K. T. & Czech, M. P. (1984) *J. Biol. Chem.* **255,** 5277–5286.
14. Häring, H. U., White, M. F., Kahn, C. R., Kasuga, M., Lauris, V., Fleischmann, R., Murray, M. & Pawelek, J. (1984) *J. Cell Biol.* **99,** 900–908.
15. Grunberger, G., Zick, Y., Roth, J. & Gorden, P. (1983) *Biochem. Biophys. Res. Commun.* **115,** 560–566.
16. Zick, Y., Rees-Jones, R. W., Taylor, S. I., Gorden, P. & Roth, J. (1984) *J. Biol. Chem.* **259,** 4396–4400.
17. Machicao, F., Urumow, T. & Wieland, O. H. (1983) *FEBS Lett.* **163,** 76–80.
18. Häring, H. U., White, M. F., Kahn, C. R., Ahmad, Z., DePaoli-Roach, A. A. & Roach, P. J. (1985) *J. Cell Biochem.* **28,** 171–182.
19. Rees-Jones, R. & Taylor, S. (1985) *J. Biol. Chem.* **260,** 4461–4467.
20. Sadoul, J. C., Pegron, J. F., Ballotti, R., Debant, A., Fehlmann, M. & VanObberghen, E. (1985) *Biochem. J.* **227,** 887–892.
21. White, M. F., Maron, R. & Kahn, C. R. (1985) *Nature (London)* **163,** 76–80.
22. Pang, D. T., Sharma, B. R. & Shafer, J. A. (1985) *Arch. Biochem. Biophys.* **242,** 176–186.
23. Häring, H. U., Biermann, E. & Kemmler, W. (1981) *Am. J. Physiol.* **240,** E556–E565.
24. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
25. White, M. F., Häring, H. U., Kasuga, M. & Kahn, C. R. (1984) *J. Biol. Chem.* **259,** 255–264.
26. Karnieli, E., Zarnowski, M. B., Hissin, P., Simpson, I., Salans, L. B. & Cushman, S. W. (1981) *J. Biol. Chem.* **256,** 4772–4777.
27. Häring, H. U., Kasuga, M., White, M. F., Crettaz, M. & Kahn, C. R. (1984) *Biochemistry* **23,** 3298–3306.
28. White, M. F., Takayama, S. & Kahn, C. R. (1985) *J. Biol. Chem.* **260,** 9470–9478.
29. Lienhard, G. E., Kin, H. K., Ransome, K. J. & Gorga, J. C. (1982) *Biochem. Biophys. Res. Commun.* **105,** 1150–1156.
30. Jacobs, S., Sahoyoun, N. E., Saltiel, A. R. & Cuatrecasas, P. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 6211–6213.
31. Takayama, S., White, M. F., Lauris, V. & Kahn, C. R. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 7797–7801.
32. Häring, H. U., Kirsch, D., Obermaier, B., Ermel, B. & Machicao, F. (1986) *J. Biol. Chem.* **261,** 3865–3875.
33. Ahmad, Z., DePaoli-Roach, A. A. & Roach, P. J. (1982) *J. Biol. Chem.* **257,** 8348–8355.
34. Avruch, J., Leone, G. R. & Martin, D. B. (1976) *J. Biol. Chem.* **251,** 1505–1510.
35. Benjamin, W. B. & Clayton, N. L. (1978) *J. Biol. Chem.* **253,** 1700–1709.
36. Belsham, G. J., Brownsey, R. W. & Denton, R. M. (1982) *Biochem. J.* **204,** 345–352.
37. Lawrence, J. C., Jr., Hiken, J. F., Inkster, M., Scott, C. W. & Mumby, M. C. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 3649–3653.