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# Insulin-stimulated protein phosphorylation in 3T3-L1 preadipocytes

(ribosomal proteins/epidermal growth factor/insulin receptor/phosphoproteins)

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**ABSTRACT** **A protein of molecular weight 31,000 became labeled with $^{32}P$ within 5 min after addition of insulin to differentiated 3T3-L1 preadipocytes previously incubated for 55 min with $^{32}P_i$. The effect was mimicked by antisera directed against the insulin receptor and was eliminated by anti-insulin antiserum. Incorporation of $^{32}P$ into this protein was more than 20-fold greater in insulin-treated cells than in cells not exposed to the hormone. At concentrations greater than required with insulin, epidermal growth factor and serum (1–5%) also stimulated phosphorylation whereas *l*-isoproterenol, a β-adrenergic agonist that increases intracellular accumulation of cyclic AMP, was without effect. The 31,000-dalton protein has been tentatively identified as ribosomal protein S6 by two-dimensional polyacrylamide gel electrophoresis. Incorporation of $^{32}P$ into S6 could be detected within the same time period (5 min) and at the same insulin concentrations (0.1–1.0 nM) as are required to stimulate hexose uptake in both 3T3-L1 cells and mature mammalian adipocytes. The mechanism by which this phosphorylation either mediates or reflects the intracellular actions of insulin remains to be elucidated.**

During differentiation, the murine preadipocyte cell line 3T3-L1 acquires many of the morphological and biochemical properties of mature mammalian adipocytes (1–6) including a high concentration of cell surface insulin receptors (7, 8) and an insulin-sensitive hexose transport system (9, 10). Because the acute effects of insulin on carbohydrate and lipid metabolism in liver, muscle, and adipose tissue impinge, directly or indirectly, on enzyme systems known to be regulated by protein phosphorylation (11, 12), the cultured 3T3-L1 cells provide a unique system for (*i*) investigating insulin-mediated protein phosphorylation in intact cells and (*ii*) correlating covalent modification of proteins with the physiological effects of insulin.

## MATERIALS AND METHODS

Porcine insulin was a gift from M. Root (Eli Lilly). Carrier-free $^{32}P_i$ ($H_3{}^{32}PO_4$) (285 Ci/mg; 1 Ci = $3.7 \times 10^{10}$ becquerels) was obtained from New England Nuclear, RNase and papain were from Worthington, bovine serum albumin was from Armour Pharmaceutical (Kankakee, IL), cytochalasin B and 1-methyl-3-isobutylxanthine were from Aldrich, acrylamide and bisacrylamide were from Eastman, and ultrapure urea and sucrose were from Schwarz/Mann. Epidermal growth factor was purchased from Collaborative Research (Waltham, MA); anti-porcine insulin serum was a gift from Peter Wright (University of Indiana School of Medicine); rabbit anti-insulin receptor serum was a gift from S. Jacobs and P. Cuatrecasas (Burroughs Wellcome, Research Triangle Park, NC). X-Omat film was purchased from General Electric Co. All other chemicals were from Sigma.

**Cell Culture.** The 3T3-L1 cells, originally obtained from Howard Green, were grown and maintained in Dulbecco's modified Eagle's medium (GIBCO) containing 10% fetal calf serum (GIBCO) and 2 mM glutamine. Differentiation was enhanced by the addition of 0.5 mM 1-methyl-3-isobutylxanthine and 0.25 μM dexamethasone to confluent monolayers for 48 hr (7). Cells were routinely used 3–5 days after withdrawal of the drugs.

**Labeling with $^{32}P_i$.** Conditions were based upon the methods developed for rat epididymal adipocytes (13, 14). Monolayers of cells were washed three times with 10 ml of warm (37°C) Krebs–Ringer bicarbonate-Hepes buffer (KRB-Hepes) (120 mM NaCl/4.75 mM KCl/1.2 mM $MgSO_4$/1.2 mM $CaCl_2$/24 mM $NaHCO_3$/10 mM Hepes, pH 7.5) containing 2.5 mM glucose and 10 units of penicillin (GIBCO) and 10 μg of streptomycin per ml. Following this, 5 ml of the KRB-Hepes buffer containing 2% bovine serum albumin (dialyzed against 0.15 M NaCl) and $^{32}P_i$ (200 μCi/ml) were added to each plate and the cells were returned to the incubator for 50 min. For most experiments, cells were then treated with insulin (0.07–7.3 nM) for 10 min. Where indicated, bacitracin (1.5 mM) was added 20 min prior to the addition of insulin to inhibit proteolysis of the hormone (7). A separate monolayer (100 mm) containing approximately 3–4 × $10^6$ cells was used for each experimental variable presented. For experiments using single-dimension analysis, 5–10% of the processed sample (approximately 50 μg of protein) was applied to the gel. The total extract derived from one monolayer was used in the two-dimensional analyses.

**Sample Preparation.** At the conclusion of the 60-min exposure to $^{32}P_i$, monolayers were washed three times with 10 ml of cold (4°C) KRB-Hepes buffer and homogenates were prepared in the following ways:

*Homogenization in $Mg^{2+}$-free buffer.* Washed cells were scraped into 1.0–2.0 ml of cold 50 mM Tris·HCl, pH 7.0, 50 mM benzamidine·HCl/10 mM EDTA/100 mM NaF/5 mM dithiothreitol/0.25 M sucrose.¶ The cell suspension was then pipetted into a polypropylene test tube, placed on ice, and homogenized with a Brinkman Polytron (75 V/10 sec). Under these conditions, >90% of the cells were broken and the nuclei remained intact. The homogenate was centrifuged at 1000 × $g_{max}$ for 15 min to remove nuclei and unbroken cells. The supernatant fluid was adjusted to 2% sodium dodecyl sulfate ($NaDodSO_4$), 10 vol of acetone (at −20°C) was added, and the preparation was allowed to stand for 16 hr at −20°C to ensure complete precipitation of acetone-insoluble protein. The precipitate was collected by centrifugation at 5000 × $g_{max}$ and washed successively with 10-ml portions of acetone (4°C), 95% ethyl alcohol, chloroform/methanol (1:1, vol/vol), 95% ethyl alcohol, anhydrous diethyl ether/methanol (1:1, vol/vol), and anhydrous diethyl ether. The pellet was then dried and dissolved in the $NaDodSO_4$ sample buffer described by Laemmli

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Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

¶ All buffers were adjusted to their final pH at 23°C.

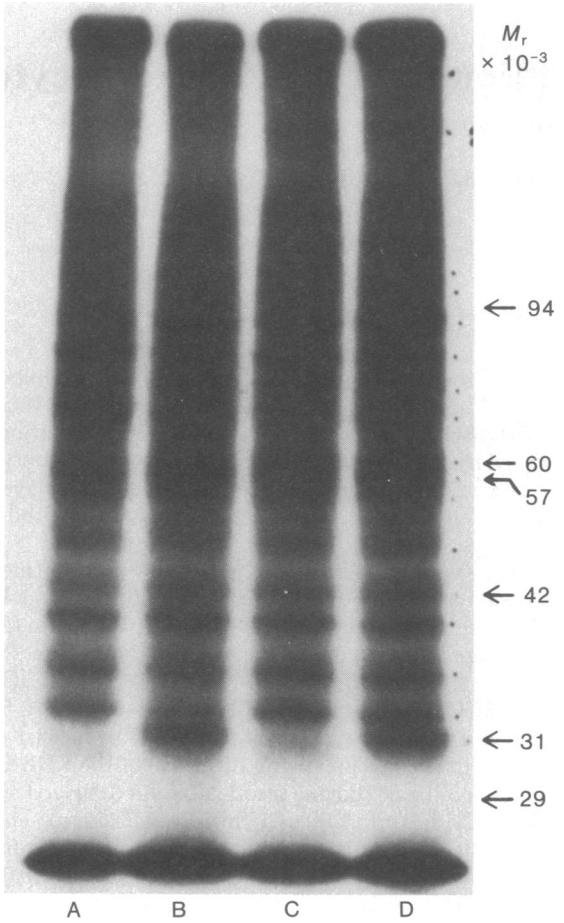


FIG. 1. NaDodSO$_4$/polyacrylamide gel electrophoresis of [$^{32}$P]phosphoproteins after treatment with insulin and isoproterenol. Monolayers of cells were washed and incubated for a total of 60 min with $^{32}$Pi (200 $\mu$Ci/ml). After 50 min, insulin (7.3 nM) was added and the cells were returned to the incubator for 10 min. Isoproterenol (1.0 $\mu$M) was added for the last 5 min of the incubation with $^{32}$Pi. After incubation, cells were washed and homogenized in the absence of added $Mg^{2+}$. The 15% trichloroacetic acid-precipitable radioactivity applied to each lane was $3.0 \times 10^4$ cpm. Lanes: A, no additions; B, insulin (1 milliunit/ml; 7.3 nM; 40 ng/ml); C, isoproterenol (1 $\mu$M); D, insulin (7.3 nM) followed 5 min later by isoproterenol (1.0 $\mu$M) for 5 min. Electrophoresis was performed in a resolving gel composed of 8.3% acrylamide and 0.23% bisacrylamide; the stacking gel was 2.5% acrylamide/0.52% bisacrylamide. After electrophoresis, the gels were stained, dried, and exposed to Kodak X-Omat R film for 15 hr with an intensifier screen. The molecular weight markers used were carbonic anhydrase (29,000), ovalbumin (42,000), pyruvate kinase (57,000), and phosphorylase b (94,000).

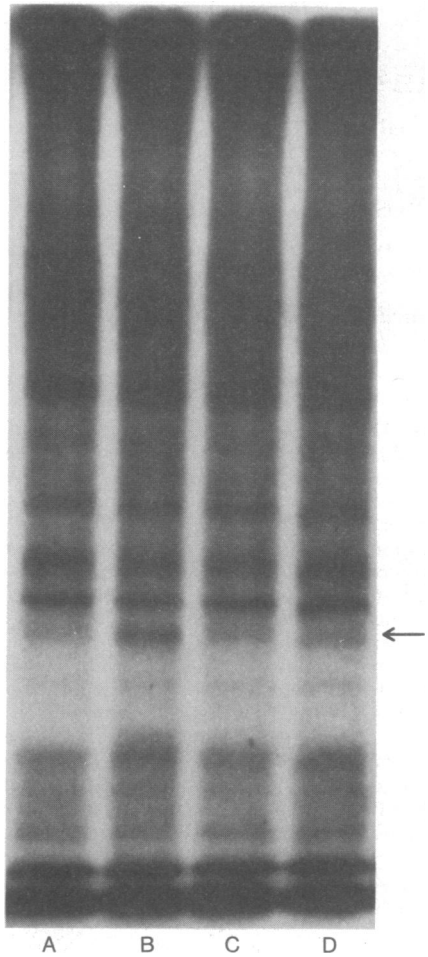


FIG. 2. Effect of anti-insulin antiserum on $^{32}$P incorporation into proteins of insulin-treated cells. Samples were prepared as described in the legend to Fig. 1. Lanes: A, no additions; B, insulin (7.3 nM); C, anti-insulin antiserum (the final dilution, 1:2500, is an amount sufficient to block the effect of 0.37 $\mu$M insulin on hexose uptake); D, insulin and antiserum were combined (4°C/10 min) and then added to the cells. Insulin, antiserum, or both were incubated with the cells for 10 min. Equal amounts of 15% trichloroacetic acid-precipitable radioactivity ($2.0 \times 10^4$ cpm) were added to each lane. The resolving gel of the NaDodSO$_4$/polyacrylamide system was composed of 12% acrylamide and 0.108% bisacrylamide. The stacking gel was as described in the legend to Fig. 1. After electrophoresis gels were stained, dried, and exposed for 20 hr to X-Omat R film with an intensifier screen. The arrow points to the 31,000-dalton protein.

(15). An alternative procedure, direct lysis of the cells in the NaDodSO$_4$ buffer described by Benjamin and Singer (13), gave similar results.

*Homogenization in $Mg^{2+}$-containing buffer.* Cells from washed plates were scraped into 1.0–2.0 ml of 50 mM Tris·HCl, pH 7.0, 10 mM benzamidine·HCl/1 mM ethylene glycol bis($\beta$-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid/1.5 mM MgCl/100 mM NaF/5 mM dithiothreitol/0.25 M sucrose and homogenized in ice by using a Brinkmann Polytron (75 V/10 sec). Unbroken cells and nuclei were removed by centrifugation at $1000 \times g_{max}$ for 15 min. Deoxycholate (final concentration, 0.5%) was added to the supernatant fluid, and the slightly clarified supernatant was recentrifuged at $15,000 \times g_{max}$ for 15 min. The $15,000 \times g_{max}$ supernatant fluid was centrifuged at $65,000 \times g_{max}$ for 60 min and the pellet was either solubilized directly in NaDodSO$_4$ sample buffer or extracted with glacial acetic acid as described below.

*Acetic acid extraction.* Washed cells were scraped into the $Mg^{2+}$-free buffer (see above), homogenized, and centrifuged at $1000 \times g_{max}$ for 15 min. The supernatant fluid was adjusted to a final concentration of 100 mM $MgCl_2$, and 2 vol of glacial acetic acid was slowly added with stirring on ice. Stirring was continued for 60 min and the extract was centrifuged for 40 min at $15,000 \times g_{max}$. The resultant supernatant fluid was dialyzed overnight against 1000 vol of 1% acetic acid, lyophilized, and dissolved in urea sample buffer (8 M urea/10% 2-mercaptoethanol/10% glycerol/1% acetic acid). Samples prepared by this method were used for two-dimensional NaDodSO$_4$/polyacrylamide gel electrophoresis at pH 5.0.

**Polyacrylamide Gel Electrophoresis and Autoradiography.** One-dimensional electrophoresis was performed according to Laemmli (15), with the addition of 1 mM 3-mercaptopropionic acid to the upper electrode buffer (16). The two-dimensional NaDodSO$_4$ electrophoresis at pH 5.0 was performed according to Gorenstein and Warner (17). Acrylamide and bisacrylamide concentrations are given in the figure legends. Gels were stained

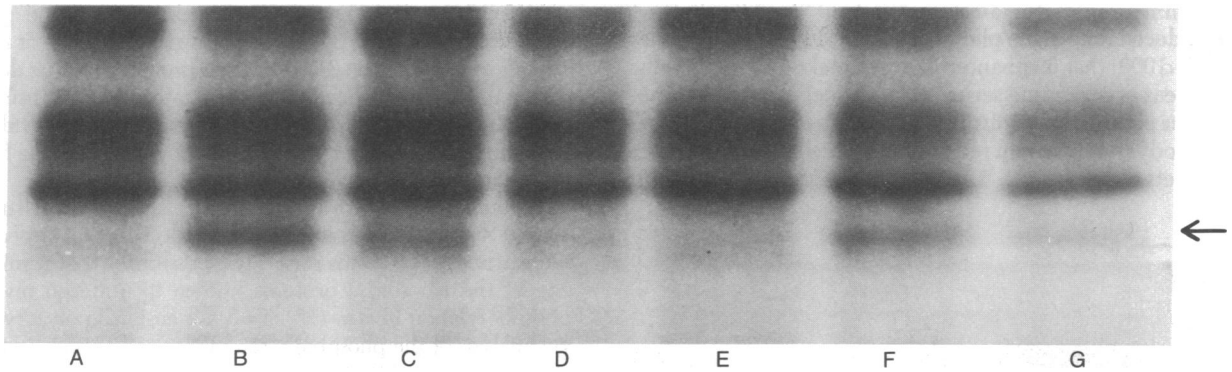


FIG. 3. Effects of epidermal growth factor, cytochalasin B, and anti-insulin-receptor antibody on [$^{32}$P]phosphoprotein formation. Samples were prepared as described in the legend to Fig. 1. Lanes: A, no additions; B, insulin (40 ng/ml); C, epidermal growth factor (1 $\mu$g/ml); D, epidermal growth factor (1 ng/ml); E, cytochalasin B (4 $\mu$M); F, cytochalasin B (4 $\mu$M) and insulin (7.3 nM; 40 ng/ml); G, anti-insulin-receptor antibody (final dilution, 1:1000). Incubations with cytochalasin B were for 15 min; all other incubations were for 10 min. The trichloroacetic acid-precipitable radioactivity applied to each lane was $5 \times 10^4$ cpm. Electrophoresis was performed in a resolving gel composed of 8.5% acrylamide and 0.153% bisacrylamide; the stacking gel was as in Fig. 1. After electrophoresis, gels were stained, dried, and exposed to X-Omat R film for 20 hr with an intensifier screen. The arrow points to the 31,000-dalton protein.

in 40% methanol/10% acetic acid containing 0.04% Coomassie brilliant blue and destained in 20% methanol/10% acetic acid. One-dimensional $NaDodSO_4$ gels were bathed in 20% methanol/10% acetic acid/10% glycerol for 1 hr prior to drying. SaranWrap was placed over dried gels to prevent the x-ray film emulsion from coming into contact with the glycerol. Autoradiography was performed with Kodak X-Omat R film with a Quanta II intensifier screen (Du Pont).

## RESULTS

When differentiated 3T3-L1 cells were incubated for 60 min with $^{32}P_i$, the addition of insulin for the final 10 min resulted in the appearance of a $^{32}$P-labeled protein of molecular weight 31,000 (Fig. 1). The overall incorporation of $^{32}P_i$ into cellular protein was not altered by this brief exposure to insulin. The radioactive band resolved by 8.5% $NaDodSO_4$/polyacrylamide gel electrophoresis was eliminated by prior treatment of the cell extract with papain or by incubation of the fixed gel with 0.1 M NaOH for 1 hr at 90°C (during which the stained protein pattern remained intact). The ability of insulin to promote phosphorylation was eliminated by the addition of anti-insulin antiserum (Fig. 2) and was unaffected by either sufficient cytochalasin B (4 $\mu$M) to reduce glucose transport to less than 5% of control values (Fig. 3) (10) or by cycloheximide at concentrations (5 $\mu$g/ml) that inhibit protein synthesis more than 90% (10). When added without insulin, none of these agents affected protein phosphorylation. At high concentrations (1.0 $\mu$g/ml), epidermal growth factor, previously shown to interact with specific cell membrane receptors on 3T3-L1 cells (8), elicited some simulation of $^{32}$P incorporation into the 31,000-dalton protein (Fig. 3) as did 1.0–5.0% dialyzed fetal calf serum (data not shown). The addition of 1 $\mu$M *l*-isoproterenol (Fig. 1), alone or in combination with insulin, had no detectable effect on this phosphorylation. [Isoproterenol (1–2 $\mu$M) stimulates the adenylate cyclase of differentiated 3T3-L1 cells approximately 15-fold and leads to a 25-fold increase in intracellular cyclic AMP within 5 min of addition (9).] It should be noted that, although isoproterenol had no effect on the phosphorylation of the 31,000-dalton protein, it did enhance the radioactivity associated with another protein of molecular weight 60,000 (Fig. 1). This effect of isoproterenol may be similar to that previously reported to occur with a protein of molecular weight 65,000–70,000 in rat epididymal fat cells (14, 18, 19).

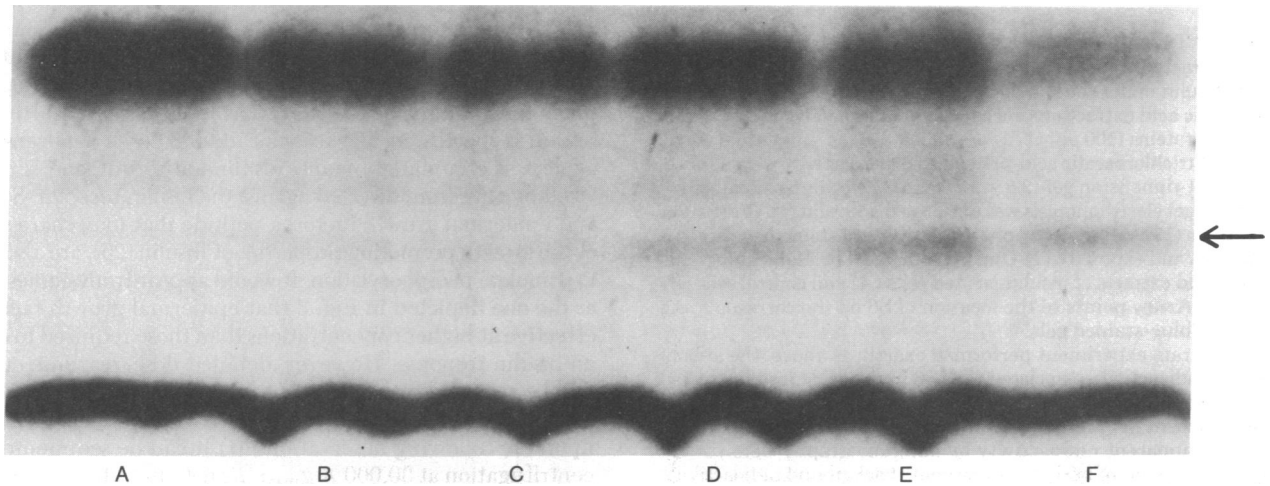


FIG. 4. Effect of insulin concentration on [$^{32}$P]phosphoprotein formation. Cells were incubated with $^{32}P_i$ at 200 $\mu$Ci/ml for a total of 60 min. After 30 min of incubation, bacitracin (1.5 mM) was added and 20 min thereafter other additions were made. Lanes: A, none; B, 0.037 nM insulin (5 microunits/ml); C, 0.073 nM insulin; D, 0.29 nM insulin; E, 0.73 nM insulin; F, 7.3 nM insulin (1 milliunit/ml). Incubations were terminated after 10 min and the cells were homogenized in buffer containing $Mg^{2+}$. Equal amounts of 15% trichloroacetic acid-precipitable material were applied to each lane ($5.0 \times 10^4$ cpm). Electrophoresis was performed in a resolving gel composed of 8.5% acrylamide and 0.153% bisacrylamide; the stacking gel composition is given in Fig. 1. The dried gel was exposed, for 12 hr, to X-Omat R film and an intensifier screen. The arrow points to the 31,000-dalton protein.

Antiserum prepared against purified rat liver insulin receptors induced detectable phosphorylation (Fig. 3) at the same dilution (1:1000) that it enhanced hexose uptake in 3T3-L1 cells (20) whereas nonimmune serum at this concentration had no effect. This observation confirms and extends existing evidence that antibody to the insulin receptor can simulate many of the acute effects of insulin (21, 22). It is not yet known, however, whether the correlation of S6 phosphorylation and glucose uptake is the same for the anti-insulin-receptor and for insulin. Phosphorylation of the 31,000-dalton protein could be detected within 5–10 min of the addition of 5 microunits of insulin per ml (0.037 nM) (Fig. 4), a concentration at the lower end of the physiological range for mature adipocytes as well as for differentiated 3T3-L1 cells.

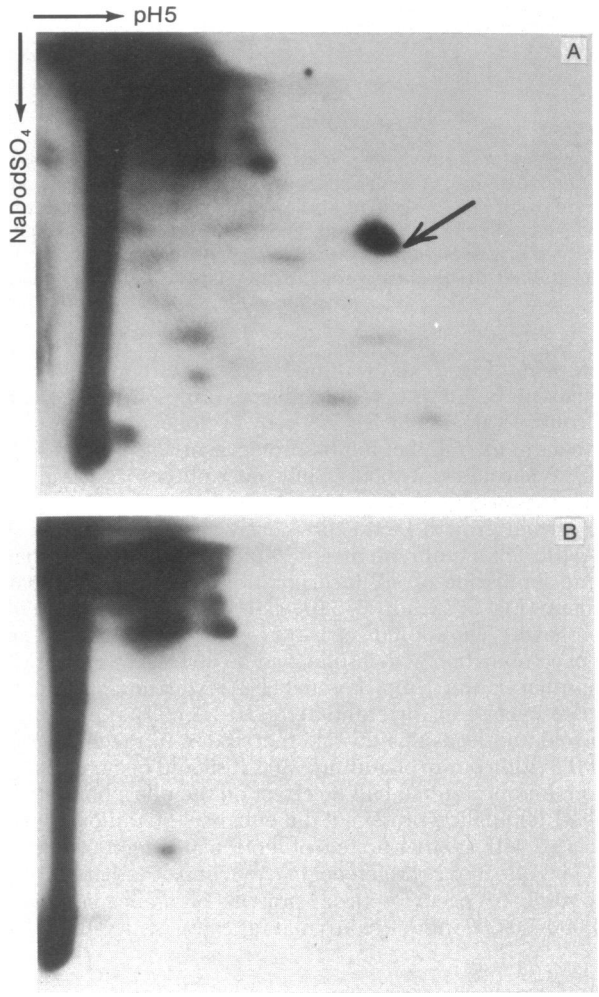


FIG. 5. Two-dimensional polyacrylamide gel electrophoresis of acid-extracted cell homogenates. Samples of cells treated with or without insulin under the standard labeling conditions were prepared by the acetic acid extraction procedure. Nonradioactive mouse L cell ribosomal proteins (100 $\mu$g) (17) were added to each sample and equal amounts of trichloroacetic acid-precipitable radioactivity were applied to each first-dimension gel ($2.5 \times 10^5$ cpm). Two-dimensional polyacrylamide gel electrophoresis was performed according to Gorenstein and Warner (17). After electrophoresis, gels were stained, encased in plastic bags, and exposed to X-Omat R film for 24 hr. Radioautograms of gels of acid extracts of insulin-treated cells (*A*) and control cells (*B*) are shown. Arrow points to the location of S6 on the corresponding Coomassie blue-stained gels.

In a separate experiment performed exactly as above, the area of increased radioactivity associated with S6 in the gel of insulin-treated cells and an equivalent area from the control gel were cut out. In addition, adjacent gel pieces equal in size to the former two but containing no apparent radioactivity by autoradiography were cut out of equivalent areas of both gels to estimate background radioactivity. Gel pieces were digested in 1.0 ml of 30% $H_2O_2$ (50°C, 5 hr) and then assayed directly for radioactivity in 10 ml of scintillation fluid (Triton/toluene, 3:7, vol/vol) containing 4 g of Omnifluor per liter. The radioactivity in the S6 region of the insulin-treated cells was 1374 cpm, compared to 89 cpm in the gel of untreated cells. Background radioactivity was 23 and 30 cpm in the insulin-treated and control runs, respectively.

When extracts of insulin-treated cells were centrifuged at $30,000 \times g_{max}$ for 60 min, the $^{32}P$-labeled protein appeared in the washed pellet. This raised the possibility that it might be one of the ribosomal proteins known to undergo reversible phosphorylation *in vivo* (23). Two-dimensional polyacrylamide gel analysis of the phosphoproteins extracted in 66% acetic acid (Fig. 5) indicated that the principal insulin-induced phosphoprotein moved to the location of phosphorylated S6 which is slightly to the left and a little higher than the stained, nonphosphorylated protein (24). When this radioactive spot was cut out of the second-dimension gel and subject to electrophoresis in an 8.5% $NaDodSO_4$ gel (see Fig. 1), it comigrated with the radioactive band initially detected by this technique. A direct assessment of the radioactivity associated with S6 revealed that it was 20-fold greater in insulin-treated cells than in control cells (see legend, Fig. 5). However, the stoichiometry of phosphate incorporation and the extent of net phosphorylation have not yet been determined. Careful examination of the radioautograms in Fig. 5 also reveals some other differences between insulin and control cells although none compares to the contrast apparent in the region of S6. Additional evidence to support the identification of the insulin-directed phosphoprotein as S6 was obtained after sucrose density gradient analysis (17) of polyribosomes prepared from insulin-treated cells. One-dimensional $NaDodSO_4$/polyacrylamide gel analysis of the isolated 40S and 60S peaks confirmed the presence of the 31,000-dalton $^{32}P$-labeled protein in the 40S particle.

## DISCUSSION

A protein tentatively identified as ribosomal protein S6 has been shown to incorporate $^{32}P$ after treatment of 3T3-L1 cells with $^{32}P_i$ and physiological concentrations of insulin. The rapidity of this effect and the concentrations of insulin required to induce it are compatible with the conditions needed to demonstrate the insulin receptor-mediated metabolic effects of insulin in adipocytes (11). Both phenomena—$^{32}P$ incorporation into the 31,000-dalton protein and stimulation of hexose transport—occur independently of protein synthesis. The ability of insulin to promote $^{32}P$ incorporation into S6 does not depend upon concomitant hexose transport. The observed effect of insulin is specific in that it is not induced by a $\beta$-adrenergic agonist, is eliminated by anti-insulin antiserum, and is mimicked by antiserum directed against the insulin receptor. Serum and epidermal growth factor, a peptide that may share some of the growth promoting properties of insulin (25), are also able to stimulate phosphorylation. It would appear from studies such as the one depicted in Fig. 3 that epidermal growth factor is effective at higher concentrations than those required to elicit an insulin response. However, detailed dose–response curves will be required to establish the magnitude of this difference in potency. Designation of the protein as ribosomal S6 is based upon the following observations: (*i*) It can be sedimented by centrifugation at $30,000 \times g_{max}$; (*ii*) it comigrates with S6 in a two-dimensional polyacrylamide gel electrophoretic system designed to resolve ribosomal proteins; and (*iii*) it is associated with the 40S ribosomal subunit.

It is not known whether the reactions leading to $^{32}P$ incorporation into S6 are an integral part of the mechanism of insulin action or merely reflect a change in the activity of protein ki-

nases and phosphoprotein phosphatases whose other substrates are not as evident under these experimental conditions. Benjamin and Singer (13, 14) originally reported enhanced phosphorylation of a 130,000-dalton protein in rat epididymal fat cells upon treatment with insulin. Phosphorylation of another protein of molecular weight 62,000 was stimulated by insulin and inhibited by isoproterenol. Avruch *et al.* (19, 26) observed a 30–40% increase in the phosphorylation of a 123,000-dalton protein after exposure of rat epididymal fat cells to insulin. Although these proteins have not been identified, both groups demonstrated cyclic AMP-independent, insulin-directed protein phosphorylation in mammalian fat cells. The dramatic 20-fold alteration reported here was apparently not encountered in mature mammalian adipocytes.

Phosphate incorporation into ribosomal protein S6 is known to be increased by exogenous or endogenous cyclic AMP (23, 27–29, 30) as well as by conditions favoring cell growth (31, 32). The effect of this phosphorylation on ribosomal function is not clear (33). Gressner and Wool (34) reported increased phosphorylation of hepatic ribosomal protein S6 in rats rendered diabetic and partial reversal of this effect by insulin. It is difficult to assess the relationship between those studies with intact animals and the experiments reported here using a different cell type under relatively controlled hormonal conditions *in vitro*. It would seem, however, that S6, like the enzymes of glycogen metabolism (35), is a good candidate for multisite phosphorylation by cyclic AMP-dependent and -independent protein kinases.

Whether phosphate incorporation into the 31,000-dalton protein represents an early effect of insulin or a more distal, indirect one, the magnitude, specificity, and sensitivity of the response to insulin may facilitate elucidation of its mechanism of action.

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