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PHOSPHORYLATION OF PHOSPHOPROTEIN PHOSPHATASE INHIBITOR-2 (I-2) IN RAT FAT CELLS

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SUMMARY: Fat cells were incubated with $^{32}P_i$ for 2 h before the [^{32}P]I-2 was immunoprecipitated, subjected to SDS/PAGE, and detected by autoradiography. [^{32}P]I-2 (Mr = 32,000) was not recovered when excess purified I-2 was added with the antiserum or when nonimmune serum was used. Immunoprecipitated I-2 was heat-stable, inhibited phosphatase activity, and could be synergistically phosphorylated by casein kinase II and F_A/GSK-3. Several times more [^{32}P]phosphoserine than [^{32}P]phosphothreonine was found in I-2 from ^{32}P-labeled cells. Insulin increased the ^{32}P-content of I-2 by as much as 40%, suggesting that phosphorylation of I-2 might be involved in the effect of insulin on stimulating protein dephosphorylation.

Type I phosphatases are sensitive to inhibition by 2 heat stable proteins, inhibitor 1 (I-1) and I-2 (1,2). I-1 inhibits only after it has been phosphorylated by cAMP-dependent protein kinase (cAdPK); whereas, I-2 does not need to be phosphorylated to inhibit (3). In fat cells, isoproterenol and insulin have been reported to increase and decrease, respectively, the phosphorylation of I-1 (4). Effects of hormones on I-2 phosphorylation have not been reported.

I-2 and the catalytic subunit of Type I phosphatase can exist in a 1 : 1 complex, refered to as Fc'M or the Mg:ATP-dependent phosphatase (5). This complex may be the native form of the phosphatase. Phosphatase activity is

ABBREVIATIONS USED: BSA, bovine serum albumin; cAdPK, cAMP-dependent protein kinase; EGTA, [ethylenebisoxyethylenenitrilo]tetraacetic acid; I-1 and I-2, phosphoprotein phosphatase inhibitors 1 and 2, respectively; PAGE, polyacrylamide gel electrophoresis; PS, phosphoserine; PT, phosphothreonine; and SDS, sodium dodecyl sulfate.

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increased when Fc'M is incubated with Mg:ATP and a protein factor, F_A (1,2,5). F_A appears to be identical to GSK-3, a protein kinase that phosphorylates and inactivates glycogen synthase (6). Phosphorylation of a threonine residue in I-2 by this kinase, denoted F_A/GSK-3 in the present report, activates the phosphatase (1,2). I-2 may also be phosphorylated by casein kinase II and cAdPK (7,8). In these cases phosphorylation occurs on serines and does not directly affect phosphatase activity. However, phosphorylation by casein kinase II enhances activation of the phosphatase by F_A/GSK-3 (7).

In fat cells, insulin stimulates the phosphorylation of some proteins and promotes the dephosphorylation of others (9-11). One hypothesis is that insulin-stimulated phosphorylation of key regulatory proteins in the cell triggers dephosphorylation. I-2 is a candidate for such a regulatory protein, and in the present experiments we have investigated the effects of insulin on the phosphorylation of I-2.

MATERIALS AND METHODS

Incubation of Cells and Preparation of Samples for Immunoprecipitations. Adipocytes were prepared by collagenase digestion of rat epididymal adipose tissue and suspended in medium composed of 3% BSA in Krebs-Ringer buffer containing 0.2 mM phosphate (11). In most experiments, 5 ml of cells (0.5 g) were incubated with $^{32}P_i$ (1-2 mCi/tube) for 2 h. Insulin was added at the appropriate time before the incubations were terminated by homogenizing the cells in 0.8 ml buffer (0°) containing 100 mM KF, 10 mM EDTA, 2 mM EGTA, 2 mM potassium phosphate, 50 mM Tris/HCl (pH 7.8 at 21°) and the following protease inhibitors: 10 mM benzamidine, 0.2 mM phenylmethylsulfonylflouride, 0.1 mM leupeptin, and 0.05 mM Nα-p-tosyl-L-lysinechloromethylketone. Homogenates were centrifuged at 10,000 x g for 30 min. Because it provided a much cleaner immunoprecipitation, extracts were routinely subjected to heat-treatment before incubation with antibodies. Triton X-100 was added to 0.1% before the extracts (0.5 ml) were added to thick-walled glass tubes (Corex, No. 8441) that had been preheated in a boiling water bath. In this way the extracts quickly reached sufficient temperature to denature most proteins. The samples were incubated at 100° for 5 min, then cooled and vigorously mixed before centrifugion at 10,000 x g for 10 min to remove precipitated proteins. Essentially the same amount of the ^{32}P-labeled I-2 was immunoprecipitated from heat-treated extracts as from nonheated extracts (see later, Fig. 1).

Immunoprecipitation of I-2. Antiserum was prepared by immunizing guinea pigs with skeletal muscle I-2 (7), and antibodies were affinity-purified by using an agarose-I-2 column as described previously (12). I-2 was immunoprecipitated following essentially the same procedures previously described (11), except that different antibodies were used.

Electrophoretic Analyses. SDS/PAGE was performed using a modification of the method of Laemmli (13). Samples were applied to slab gels formed with a linear gradient (from 5% to 20%) of acrylamide. After the gels were dried,

radioactive bands were located by autoradiography with Kodak XAR-5 film. ^{32}P Autoradiograms were scanned for optical density and relative amounts of ^{32}P were estimated from peak areas.

Phosphoamino acid analysis of the immunoprecipitated [^{32}P]I-2 after SDS/PAGE was performed by the method of Cooper et al (14).

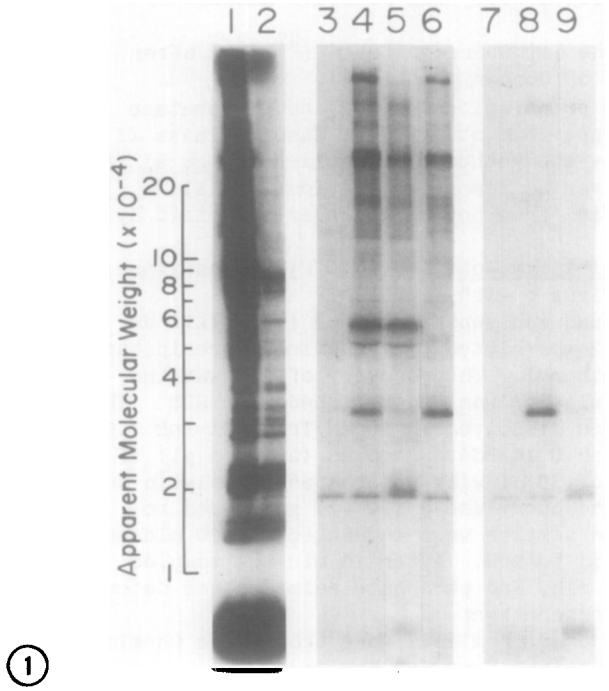
Protein Purification. The same preparations of I-2 and phosphatase catalytic subunit were used as in a previous study (7). Casein kinase II was purified from rabbit reticulocytes by the method of DePaoli-Roach et al (15). F_A/GSK-3 was purified from rabbit liver as described previously (16). Catalytic subunit of cAdPK was purified from bovine heart as described by Peters et al (17).

Measurements of Phosphatase Inhibitory Activity. [^{32}P]Phosphorylase a was prepared by incubating phosphorylase b with [γ-^{32}P]ATP and phosphorylase kinase, then crystallized and separated from [γ-^{32}P]ATP as described by Antonwi et al (18). I-2 was eluted from the immunoprecipitate by incubation at 100° in 1% 2-mercaptoethanol. The recovery of I-2 was the same under these conditions as when elution was accomplished with SDS. The eluted samples were freeze-dried, then dissolved in 50 mM Tris/HCl (pH 7.0), 50 mM 2-mercaptoethanol, 0.1 mM EDTA, and 0.1% BSA. Samples (up to 5 µl) of eluted proteins were incubated in a volume of 30 µl with phosphatase catalytic subunit (10 ng/ml) for 5 min at 23°. [^{32}P]phosphorylase a (10 µl) was added to a final concentration of 2 mg/ml, and samples were incubated for 10 min at 30° before adding trichloroacetic acid to 20%. After 10 min the samples were centrifuged at 13,000 x g for 5 min, and phosphate released was determined by measuring the ^{32}P present in the supernatant.

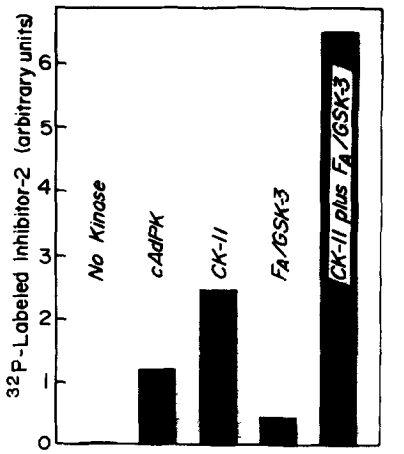
Other Materials. Most commonly used chemicals were from Sigma Chemical Company. $^{32}P_i$ and [γ-^{32}P]ATP were obtained from New England Nuclear.

RESULTS

Extracts were prepared from adipocytes that had been incubated with $^{32}P_i$ for 2 h, a time sufficient to achieve steady state labeling of [γ-^{32}P]ATP and most cellular phosphoproteins (11). Immunoprecipitations were then performed using antiserum against rabbit I-2, and the samples were analyzed by SDS/PAGE followed by autoradiography. As shown in Fig. 1, several ^{32}P-labeled species were detected, including one with the same apparent Mr (32,000) as rabbit muscle I-2 (lane 4). This species was not observed if nonimmune serum was used instead of the antiserum (lane 3), and its immunoprecipitation was blocked by addition of unlabeled rabbit muscle I-2 (lane 5). The protein was also recovered using affinity-purified antibodies (lane 6), and like rabbit muscle I-2, was both heat-stable (lane 8) and synergistically phosphorylated by casein kinase II and F_A/GSK-3 (Fig. 2). In addition, phosphatase inhibitory activity was associated with the immunoprecipitate (Fig. 3). Therefore, it seems clear that the ^{32}P-labeled species of Mr = 32,000 is fat cell I-2.



(1)

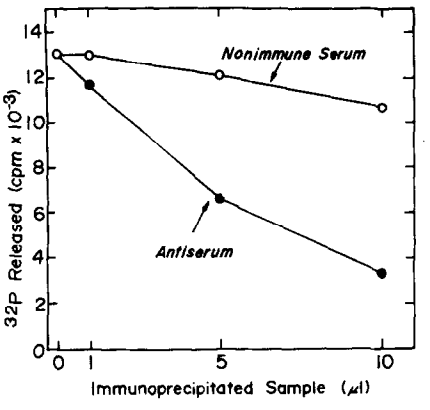


(2)

Figure 1 SDS/PAGE of [^{32}P]I-2 immunoprecipitated from extracts of rat adipocytes. The samples applied to the gel were obtained using 50 µl of boiled (lanes 7-9) and nonboiled (lanes 3-6) extracts that had been processed with 3 µl nonimmune serum (lanes 3 and 7), 3 µl antiserum (lanes 4 and 8), 3 µl antiserum plus 100 ng rabbit skeletal muscle I-2 (lanes 5 and 9), or affinity purified antibody (20 µg) (lane 6). In lanes 1 and 2, 2.5 µl of nonboiled and boiled extract, respectively, were added.

Figure 2 Synergistic phosphorylation of adipocyte I-2 by casein kinase II and F_A/GSK-3. Adipocytes were homogenized in buffer containing reduced EDTA (1 mM). Samples (70 µl) of heat-treated extract were incubated for 1 h with 0.1 mM [γ-^{32}P]ATP and 5 mM magnesium acetate, either without added kinases or with the following protein kinases: catalytic subunit of cAdPK (2 µg/ml), casein kinase II (CK-II) (10 µg/ml), F_A/GSK-3 (4 µg/ml), or the combination of casein kinase II plus F_A/GSK-3. I-2 was then immunoprecipitated and subjected to SDS/PAGE. The relative amounts of ^{32}P in I-2 was determined from optical density scans of an autoradiogram, and are presented in arbitrary units of mV/min, reflecting the output of the scanning densitometer.

Incubating ^{32}P-labeled adipocytes with insulin caused an increase of approximately 3-fold in ^{32}P associated with an extract species which had the same electrophoretic mobility as rabbit muscle I-2 (results not shown). However, most of this insulin-stimulated ^{32}P-labeling was absent in boiled extracts, indicating that it did not occur in I-2, which is heat-stable. Insulin did increase I-2 phosphorylation. When cells were incubated for 5 min with 100 microunits/ml insulin, the amount of [^{32}P]I-2 immunoprecipitated was increased by 18 $\pm$ 6 % (mean $\pm$ S.E. from 10 experiments; $p < 0.02$,



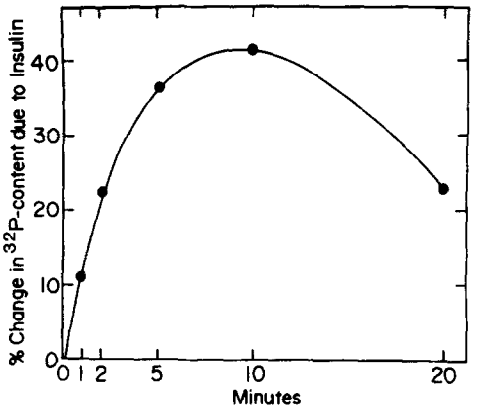


Figure 3 Inhibition of low Mr phosphoprotein phosphatase by immunoprecipitated species. Immunoprecipitations were performed from an extract (50 μl) of cells that had been incubated in the absence of $^{32}P_i$. The effects of increasing amounts of the immunoprecipitated samples on phosphatase catalytic subunit activity were determined using [^{32}P]phosphorylase a (2 mg/ml, 2.9 x 10^7 cpm/ml) as substrate. Ten μl of the sample represents inhibitory activity immunoprecipitated from 0.5 mg adipocytes. The values presented are the cpm of $^{32}P_i$ released.

Figure 4 Stimulation of I-2 phosphorylation by insulin. I-2 was immunoprecipitated from extracts by using an excess of antiserum. The results are expressed as percent changes in the ^{32}P-content of I-2 produced by 500 microunits/ml insulin and are mean values from 2 experiments.

paired comparisons). The hormonal effect reached a maximum within 10 min (Fig. 4), and was less pronounced at incubation times longer than 10 min.

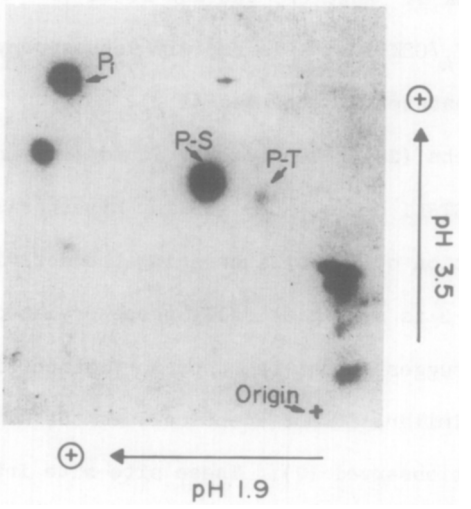


Figure 5 Phosphoamino acid analysis of [^{32}P]I-2. ^{32}P-Labeled fat cells were incubated with 500 microunits/ml insulin for 10 min. [^{32}P]I-2 was immunoprecipitated, subjected to SDS/PAGE, sliced from the gel, and hydrolyzed with HCl (5.7 N for 2 h at 110°). Phosphoaminoacids were resolved by 2 dimensional high voltage electrophoresis at pH 1.9 and 3.5.

Both ^{32}PS and ^{32}PT were detected following partial acid hydrolysis of immunoprecipitated I-2, although the level of PT was less than 5% of the PS (Fig. 5). Insulin did not detectably change the amount of PT present. However, because these analyses are not quantitative, a 20% increase in PT would probably not have been detected. It does seem clear that the increased ^{32}P-labeling of the I-2 in response to insulin was not restricted to PT. If this had been the case, a 20% increase in phosphorylation in response to insulin would have resulted in a severalfold increase in ^{32}PT.

DISCUSSION

The present results indicate that phosphorylation of I-2 is increased by insulin in rat fat cells. The increase was of the same order as the insulin-stimulated decrease in phosphorylation of I-1 previously reported (4). Phosphoamino acid analysis of I-2 revealed mostly ^{32}PS, although a small amount of ^{32}PT was also present. A comparable amount of ^{32}PT was detected in I-2 from ^{32}P-labeled mouse diaphragm muscles (19). The ^{32}PT content did not appear to be changed by insulin, suggesting that I-2 from insulin-treated cells contained little, if any, more phosphate in the site phosphorylated by F_A/GSK-3. However, the low level of ^{32}PT was not unexpected, since the F_A/GSK-3 site is rapidly dephosphorylated when the Mg:ATP-dependent phosphatase is activated (1,2).

Sommercorn and Krebs (20) have recently found that insulin increases casein kinase II activity in 3T3L1 cells. Our working hypothesis is that insulin stimulates phosphorylation of I-2 by increasing the activity of this kinase. The finding that fat I-2 is synergistically phosphorylated by casein kinase II and F_A/GSK-3 (Fig. 2) suggests that the phosphorylation sites of the kinases in adipocyte I-2 are similar, if not identical, to the sites in muscle I-2 where the synergism was first observed (7). These site-site interactions provide a mechanism for indirectly controlling the phosphorylation of I-2 by F_A/GSK-3.

Insulin activates glycogen synthase by causing dephosphorylation of the sites selectively phosphorylated by F_A/GSK-3 (21,22). Phosphorylation of

synthase in site 5 by casein kinase II has no effect on synthase activity, but enhances the phosphorylation and inactivation of the enzyme by F_A/GSK-3 (16). Therefore, increasing the activity of casein kinase II might be expected to lead to synthase inactivation. However, there is evidence that site 5 is nearly filled in synthase from control cells (22), so that any additional phosphorylation that might result from increased casein kinase II activity should have only a small effect on phosphorylation of synthase by F_A/GSK-3. Therefore, casein kinase II activity increased by insulin might selectively target I-2 for increased phosphorylation by F_A/GSK-3.

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