Insulin Induces the Phosphorylation of DNA-Binding Nuclear Proteins Including Lamins in 3T3-F442A[†]

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ABSTRACT: Insulin binding to its plasma membrane receptor stimulates a cascade of protein kinases and phosphatases which ultimately affects multiple processes in the membrane, cytosol, and nucleus of the cell, including transcription of specific genes. To gain insight into the relationship between the kinase cascade and the mechanism of insulin-induced nuclear events, we have studied the effect of insulin on the phosphorylation of DNA-binding nuclear proteins in differentiated NIH-3T3-F442A adipocytes. Insulin induced the phosphorylation of seven DNA-binding proteins: pp34, pp40, pp48, pp62, pp64, pp66, and pp72. The half-maximal response was observed at 10–30 min and reached its maximum at 60 min. The insulin-induced phosphorylation of each of these proteins was dose-dependent with ED_{50}s of 2–10 nM. The phosphorylation of pp62, pp64, and pp72 took place on serine residues. On the basis of immunoprecipitation and immunoblotting experiments with anti-lamin antibodies, we found that the insulin-induced DNA-binding phosphoproteins pp62, pp64, pp66, and possibly pp48 were related to lamins A and C. The ED_{50} for insulin-stimulated lamin phosphorylation was ~10 nM, and phosphorylation was half-maximal at 30 min. The insulin-dependent phosphorylation of lamins and other DNA-binding proteins (pp34, pp40, and pp72) may play a mediatory role in the long-term effects of insulin.

Insulin regulates a vast variety of cellular functions including those involved in carbohydrate, lipid, and protein metabolism as well as cell growth and differentiation (Rosen, 1987; Kahn & White, 1988). Many of these effects result from activation of membrane transport systems and changes in the phosphorylation and activity state of cytoplasmic enzymes. In addition, insulin acts at the cell nucleus to regulate the expression of specific genes. These changes occur through modulation of gene transcription, mRNA translation, and mRNA stability. Although some evidence has been presented to show that insulin may act to increase nuclear protein and RNA transport (Schindler & Jiang, 1987; Purello et al., 1983), the signal transduction mechanism from its plasma membrane receptor to its potential nuclear sites of action remains unknown.

The binding of insulin to its membrane receptor activates the tyrosine kinase activity intrinsic to the receptor (Rosen, 1987; Kahn & White, 1988) and subsequently stimulates an elaborate cascade of protein kinases and phosphatases (Czech et al., 1988; Chan et al., 1988). Some of the participating enzymes, such as casein kinase II and protein phosphatase 1, have been identified in the nucleus as well as the cytoplasm of the cell (Pfaff & Anderer, 1988; Cohen, 1989). There is also a growing, but still very much incomplete, number of nuclear proteins which have been identified to change their phosphorylation status after the addition of insulin (Friedman & Ken, 1988; Feuerstein & Randazzo, 1991; Suzuki et al.,

1991).[1,2] The relationship of these phosphorylation events to insulin effects on gene expression is unclear. Recently, a number of DNA sequences have been identified as the sites required to confer insulin-induced changes in the transcription of the fos, phosphoenolpyruvate carboxykinase, glyceraldehyde-3-phosphate dehydrogenase, and glucagon genes (Stumpo et al., 1988; O'Brien et al., 1990; Nasrin et al., 1990; Philippe, 1991). These insulin response elements presumably bind one or more proteins which alter the rate of gene transcription. Although specific insulin-regulated transcription factors have not yet been identified, there are a large number of transcription factors, such as fos, jun, CREB, NFκB, serum response factor, and heat shock factor, which appear to change their transcriptional activity after being phosphorylated or dephosphorylated (Ofir et al., 1990; Boyle et al., 1991; Yamamoto et al., 1988; Lenardo & Baltimore, 1989; Prywes et al., 1988; Sorger & Pelham, 1988). Thus, it is reasonable to assume that insulin-induced changes in the level of phosphorylation of transcription factors and other nuclear proteins may play an important role in the transmission of the hormonal signal to the cell nucleus.

In the present report, we have examined insulin-induced phosphorylation and dephosphorylation of nuclear proteins in differentiated 3T3-F442A cells, a cell line which expresses a large number of insulin receptors and is insulin-responsive with respect to differentiation and gene expression (Reed et al., 1977). We have identified and characterized seven insulin-regulated nuclear phosphoproteins which bind to double-stranded DNA and may participate in the nuclear signal transduction of insulin action. At least two of these proteins are lamins A and C; the exact nature of the others remains to be determined.

[1] Peter Csermely and C. Ronald Kahn, unpublished results.
[2] Peter Csermely, Mark O. Olson, Tamás Schnaider, and C. Ronald Kahn, unpublished results.

Table I: Distribution of Biological Markers in Fractionated 3T3-F442A Cells[a]

marker	cell fraction	nuclear pellet (%)	supernatant (%)
lactate dehydrogenase	cytosol	1	99
5'-nucleotidase	plasma membrane	2	98
NADH–cytochrome c reductase	endoplasmic reticulum	5	95
Gal-1-P-uridyl transferase	Golgi	8	92
cytochrome oxidase	mitrochondria	9	91
DNA	nucleus	96	4

[a] The methods for each of the assays listed are as follows: lactate dehydrogenase (Cabaud & Wroblewski, 1958); 5'-nucleotide (Newby et al., 1975); NADH–cytochrome c reductase (Beaufay et al., 1974); Gal-1-P-uridyl transferase (Bretz & Straubi, 1977); cytochrome oxidase (Cooperstein & Lazarow, 1951); DNA (Labarca & Paigen, 1980).

EXPERIMENTAL PROCEDURES

Chemicals. Cell culture media and the NFκB-specific oligo-DNA affinity resin were obtained from Gibco (Grand Island, NY). Pork insulin was purchased from Elanco Products Co. (Indianapolis, IN). The chemicals used for polyacrylamide gel electrophoresis were from Bio-Rad (Richmond, CA). Poly-(I)–poly(C)–agarose was a product of Pharmacia (Uppsala, Sweden). TPCK-trypsin was obtained from Worthington Biochemical Co. (Freehold, NJ). Various batches of dsDNA-cellulose were purchased from Pharmacia, Worthington Biochemical Co., U.S. Biochemical Co. (Cleveland, OH), and Sigma Chemical Co. (St. Louis, MO). Protien A–acrylamide beads, constant-boiling 6 N HCl, and Triton X-100 were obtained from Pierce (Rockford, IL). [^{32}P]Orthophosphate (carrier-free) was from New England Nuclear (Wilmington, DE). Trans^{35}S-label and methionine/cysteine-free MEM were from ICN Biomedicals (Irvine, CA). Rabbit anti-lamin serum against lamins A, B, and C was kindly provided by Dr. Larry Gerace (Research Institute of Scripps Clinic, La Jolla, CA); LS-1 human autoantibody and IE$_4$ mouse monoclonal antibody against lamins A and C were a gift of Dr. Frank McKeon (Harvard Medical School, Boston, MA) (McKeon et al., 1983; Loewinger & McKeon, 1988). The anti-fos, anti-jun, and anti-NF-1 antibodies were a kind gift from Dr. Bruce J. Spiegelman (Dana Farber Cancer Research Institute, Boston, MA). Rabbit γ-globulin was a product of Jackson Immunolaboratories (West Grove, PA). The S6 kinase substrate peptide was obtained from Peninsula Laboratories (Belmont, CA). Nitrocellulose filters (0.45 μm) were purchased from Schleicher & Schuell (Keene, NH). All the other chemicals used were from Sigma Chemical Co.

Cell Culture. NIH-3T3-F442A cells were grown in DMEM medium with 10% calf serum in 5% humidified CO$_2$ atmosphere. Cells were differentiated in 10% CO$_2$ in DMEM medium supplemented with 10% fetal calf serum for 10–12 days. The insulin concentration of the calf and fetal calf sera was 8 and 10 microunits/mL (~0.4 ng/mL), respectively, as measured by conventional radioimmunoassay. The differentiation was enhanced by the addition of 5 μg/mL insulin for the first 6–8 days. By the end of this period, 90–95% of the cells acquired the characteristic adipocyte morphology, accumulating a large number of lipid droplets.

In Vivo ^{32}P Labeling. Cells were serum-starved for 18 h, then the cell culture medium was changed to phosphate-free DMEM, and 0.3 mCi/mL [^{32}P]phosphate was added for 2 h. After the addition of insulin or other stimulants specified in the individual experiments, the medium was removed, and the cells were scraped to an isolation buffer containing 20

mM Hepes, 1 mM ATP, 5 mM MgCl$_2$, 25 mM KCl, 2 mM PMSF, 0.1 mg/mL aprotinin, 1 mM sodium vanadate, 1 mM sodium molybdate, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, and 0.25 M sucrose, pH 7.4. The cells were disrupted by the method of Lee et al. (1988), passing them 4–5 times through a 1-mL tuberculin syringe with a Micro-Fine-IV needle. Cell nuclei were isolated according to the method of Blobel and Potter (1966). Briefly, samples were centrifuged at 3000g for 15 min at 4 °C, the supernatants were discarded, pellets were resuspended in 1 mL of isolation buffer, and 2 mL of isolation buffer supplemented with 1.6 M sucrose was layered under them. Nuclei were pelleted by centrifugation at 100000g for 35 min at 4 °C. Nuclei were resuspended in 0.5 mL of isolation buffer, and their protein concentration was determined according to Bradford (1976). The nuclear preparation was 90–95% pure as judged by marker DNA/enzyme analysis (Table I) and electron microscopy (data not shown).

Purification of dsDNA-Binding Proteins. DNA–cellulose chromatography was performed as described by Alberts and Herrick (1971). Proteins were extracted from aliquots of intact ^{32}P-labeled cell nuclei containing 0.5 mg of protein each by addition of NaCl and Triton X-100 at final concentrations of 0.6 M and 0.5%, respectively. Samples were rotated overnight and centrifuged in a microfuge, and the supernatants were removed and diluted 10 times with a buffer containing 10 mM Hepes, pH 7.4, 0.1% Triton X-100, and 10% glycerol. In some experiments, samples were precleared with the subsequent addition of 20 mg of cellulose and poly-(I)–poly(C)–agarose. Approximately 20 mg of dsDNA-cellulose was added, and samples were rotated for 2 h at 4 °C. After being washed 2 times with 1 mL of the same Hepes/Triton/glycerol buffer, 30 μL of Laemmli sample buffer (Laemmli, 1970) was added to each sample with 100 mM DTT, and the samples were analyzed with SDS–PAGE and consequent autoradiography. When we tested various batches of dsDNA-cellulose from Pharmacia, Worthington Biochemicals, U.S. Biochemicals, and Sigma, we found the Sigma dsDNA-cellulose gave the lowest background and best handling properties. Thus, we performed our experiments using the product of this supplier. In some experiments, dsDNA-binding proteins were eluted with 0.6 M NaCl.

The results were quantified by a Molecular Dynamics (Sunnyvale, CA) 300A computing densitometer. In most experiments, we scanned multiple autoradiograms after various times of storage at –70 °C (usually with an intensifying screen) to ensure that the exposure was in the linear range. The background values were subtracted, and in those few cases where the ^{32}P labeling of the samples was not uniform, values have been normalized to one of the constitutively phosphorylated dsDNA-binding proteins which did not show any change after the addition of insulin.

Phosphoamino Acid Analysis. Phosphoamino acid analysis was carried out by the method of Cooper et al. (1983). Phosphoprotein bands were excised from the acrylamide gel, soaked in 20% methanol, and digested with the addition of 2 × 100 μg of TPCK-trypsin in 50 mM ammonium carbonate for 24 h at 37 °C. The trypic digest was lyophilized and hydrolyzed in constant-boiling 6 N HCl at 110 °C for 70 min. The hydrolysates were washed with 2 × 1 mL of distilled water and subjected to electrophoresis on TLC plates at pH 3.5. Plates were dried, stained with ninhydrin, and analyzed by autoradiography.

Immunoprecipitations. Proteins were extracted from aliquots of intact ^{32}P-labeled cell nuclei by addition of 40 units

of DNase I (Sigma) NaCl and Triton X-100 at final concentrations of 0.6 M and 0.5% (v/v), respectively. The extracts were precleared by the simultaneous addition of 0.1 mg of rabbit γ-globulin and 50 μL of protein A–beads. Samples were rotated overnight at 4 °C and centrifuged in a microfuge, and the protein concentration of the supernatants was determined according to Bradford (1976). To aliquots of protein extracts containing 0.5 mg of protein each was added 5 μg of anti-lamin serum, and the samples were incubated overnight at 4 °C. The immunocomplexes were coupled to 30 μL of protein A–beads by rotating them for 2 h at 4 °C. Immunoprecipitates were washed successively with 1 mL each of buffers containing 50 mM Hepes, pH 7.4, supplemented with 0.1% (w/v) SDS, 1% (v/v) Triton X-100, and 0.1% (v/v) Triton X-100, respectively. The final pellets were eluted with Laemmli buffer (Laemmli, 1970) containing 100 mM DTT, samples were boiled for 3 min and centrifuged in a microfuge, and the supernatants were analyzed by SDS–PAGE and autoradiography.

Immunoblots. dsDNA-binding nuclear [^{32}P]phosphoproteins were purified and separated with SDS–PAGE as described above. The proteins were transferred to nitrocellulose filters according to the method of Towbin (Towbin et al., 1979), in a transfer buffer containing 25 mM Tris, 0.192 M glycine, and 20% (v/v) methanol. Filters were soaked in a 20 mM Tris, pH 7.4, 0.15 M NaCl, 0.5% (v/v) Tween 20, and 0.1% bovine serum albumin blocking buffer for 1 h at room temperature. After overnight incubation with a 1:200 dilution of rabbit anti-lamin serum or IE$_4$ mouse monoclonal anti-lamin antibody at 4 °C, the immunocomplexes were visualized by means of peroxidase-conjugated anti-rabbit or anti-mouse antibodies and subsequent treatment with 4-chloro-1-naphthol and H$_2$O$_2$. Radioactive bands were detected by autoradiography.

RESULTS

Insulin-Induced Phosphorylation of Double-Stranded DNA-Binding Proteins. Insulin treatment of ^{32}P-labeled differentiated 3T3-F442A cells induces the phosphorylation of several nuclear proteins capable of binding to double-stranded DNA (dsDNA) (Figure 1A). The highest level of phosphorylation was observed as a triplet migrated between 62 and 66 kDa (pp62, pp64, and pp66). Additional insulin-induced bands were seen at 48 and 34 kDa (pp48 and pp34, respectively). The stimulation of the proteins at 62–66 kDa was observed in all of the more than 20 experiments performed, while pp48 and pp34 were at the threshold of visibility in some of the experiments and thus could not be quantitated in all experiments. The pattern of ^{32}P-labeled phosphoproteins was similar if single-stranded or double-stranded DNA–cellulose was used for purification. The presence of divalent cations, such as Mg^{2+} or Zn^{2+}, did not cause any significant change in the amount of insulin-induced dsDNA-binding phosphoproteins. The majority of the binding was specific to the DNA; only about 10–20% of the triplet around 62–66 kDa bound to cellulose, the matrix of the DNA–cellulose affinity medium (data not shown). The changes in the intensity of ^{32}P-labeled protein bands appeared to reflect changes in their phosphorylation status rather than a shift in their affinity toward double- or single-stranded DNA, since there was no visible change in the amount of proteins following insulin treatment as judged by scanning of the corresponding Coomassie Blue stained gels or autoradiograms of ^{35}S-labeled dsDNA-binding nuclear extracts (data not shown).

Differentiation of 3T3-F442A cells to adipocytes resulted in some quantitative changes in the pattern of insulin-induced
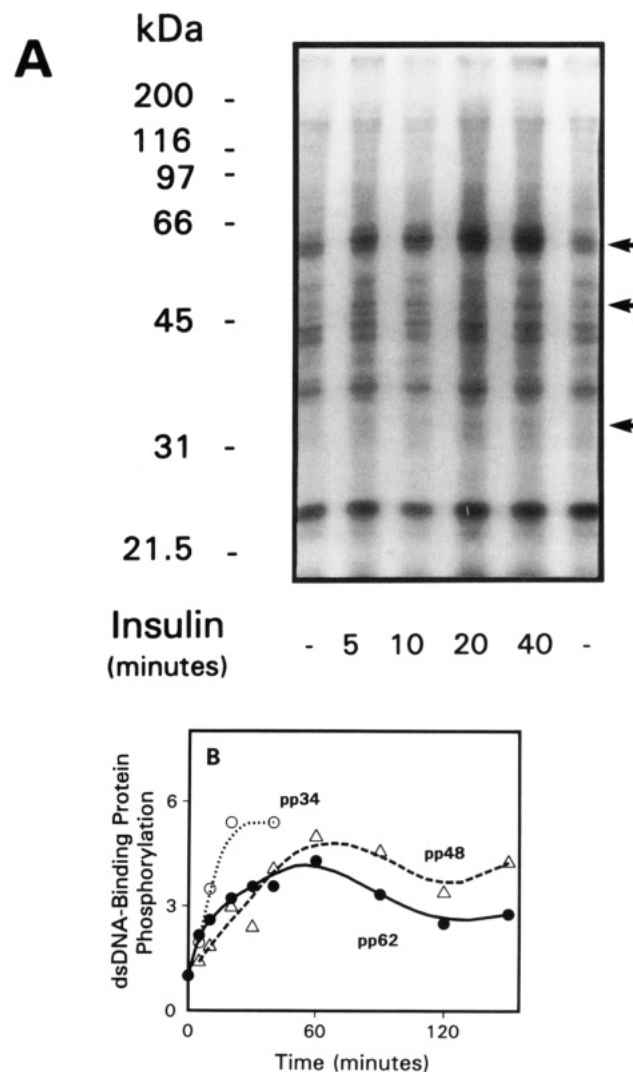
FIGURE 1: Time dependence of insulin-induced phosphorylation of dsDNA-binding nuclear proteins. Panel A: 3T3-442A cells were differentiated, serum-starved, and labeled with [^{32}P]phosphate as described under Experimental Procedures. Cells were treated with 1 μM insulin for the times indicated. ^{32}P-labeled nuclei were isolated from each sample, and 0.5 mg of nuclear proteins was extracted with 0.6 M NaCl and 0.5% Triton X-100 (see Experimental Procedures for details). Protein extracts were diluted 10 times with a buffer containing 100 mM Hepes, pH 7.4, 0.1% Triton X-100, and 10% glycerol. Approximately 20 mg of dsDNA–cellulose was added, and samples were rotated for 2 h at 4 °C. After extensive washing, samples were analyzed with SDS–PAGE and consequent autoradiography. Panel B: The results of the experiment shown in panel A and three other experiments were quantified by densitometry analysis as described under Experimental Procedures. Open circles, open triangles, and filled circles correspond to bands of pp34, pp48, and pp62, respectively. Data are means of two separate experiments. The changes in the phosphorylation of pp64 and pp66 were very similar with the time course of pp62 phosphorylation (data not shown). The phosphoprotein band corresponding to pp34 was too faint to quantify in the two experiments examining longer incubation with insulin.

dsDNA-binding phosphoproteins. For the 34-, 48-, 62-, 64-, and 66-kDa bands, there was only a small stimulation in insulin-treated nondifferentiated 3T3-F442A cells, and the magnitude of these insulin-induced phosphorylations increased by 2–4-fold as the cells progressively differentiated during the 8 days of insulin treatment. It is important to note that in all our studies, the insulin concentration in the culture medium was reduced from the 5 μg/mL level required for differentiation to the normal level in serum-supplemented

media (<1 ng/mL) for at least 2 days prior to the actual experiment. Failure to remove the high concentration of insulin resulted in a loss of the insulin-induced change in the ^{32}P-labeled dsDNA-binding proteins (data not shown). This is probably the result of down-regulation of insulin receptors and/or postreceptor desensitization due to continuous exposure of adipocytes to high levels of insulin (Reed et al., 1977). Serum starvation for 12 h also enhanced the insulin-induced changes; however, increasing the duration of the starvation in the range to 24 h caused little if any difference in the pattern of dsDNA-binding phosphoproteins.

Quantitation of the results from Figure 1A and other similar experiments by scanning densitometry revealed a time-dependent phosphorylation of dsDNA-binding proteins after the addition of insulin (Figure 1B). The half-maximal response was around 10 min for pp34, whereas pp48 and pp62 reached half-maximal phosphorylation at 30 min. The phosphorylations of all three of the major phosphoproteins was maximal by 60 min, after which it remained fairly stable.

Insulin Dose Dependence. The insulin-induced changes in the phosphorylation of dsDNA-binding proteins were concentration-dependent (Figure 2A). The insulin-induced phosphorylation of pp62 and pp64 displayed a half-maximal activation at 10 and 2 nM (60 and 12 ng/mL), respectively (Figure 2B). These values are close to the half-maximal binding of insulin to its receptor in 3T3-L1 adipocytes (5 nM or 30 ng/mL; Reed et al., 1977). The low level of pp48-associated radioactivity did not allow exact quantification of the dose response in these experiments. In this experiment, pp34 was not visible. However, there was a clear increase and then decrease in the phosphorylation of a dsDNA-binding protein around 97 kDa. In separate experiments, we obtained evidence that this protein is nucleolin (C23), one of the major constituents of the nucleolar RNA-processing machinery, and that its phosphorylation was highly correlated with the state of cell differentiation (P. Csermely et al., unpublished results).

Insulin-Induced Phosphorylation of Lamins. Lamins are abundant nuclear phosphoproteins migrating in the range of 60–74 kDa in SDS gels (Krohne & Benavente, 1986). The observation that insulin induces the phosphorylation of lamins A and C in BHK-21 fibroblasts (Friedman & Ken, 1988) and other data indicating that lamins may be able to bind to DNA (Yuan et al., 1991; Hakes & Berezney, 1991) raised the possibility that some of the insulin-induced dsDNA-binding phosphoproteins were lamins. As a first step to address this question, we examined if insulin induces the phosphorylation of lamins in differentiated 3T3-F442A adipocytes using anti-lamin antibodies for immunoprecipitation.

As shown in Figure 3A, insulin induces a dose- and time-dependent phosphorylation of lamins in 3T3-F442A cells. These migrated as a closely spaced triplet at 62–66 kDa. A faint phosphoprotein band was also observed at 45–60 kDa in these immunoprecipitates which may correspond to proteolytic fragments of lamins A and C (Tökés & Clawson, 1989). Half-maximal stimulation of lamin phosphorylation was observed at 10 nM insulin concentration and reached its maximum around 100 nM insulin (Figure 3B). The time course of lamin phosphorylation was rather slow, reaching half-maximal after 20 min and maximal after 1 h of stimulation.

Relationship between the dsDNA-Binding Phosphoproteins and Lamins. In hopes to better define the DNA-binding proteins in the nuclear extract by diminishing the nonspecific binding to the affinity resin and to determine the specificity of the lamin–DNA interaction, we performed a series of experiments in which we precleared the high-salt ^{32}P-nuclear
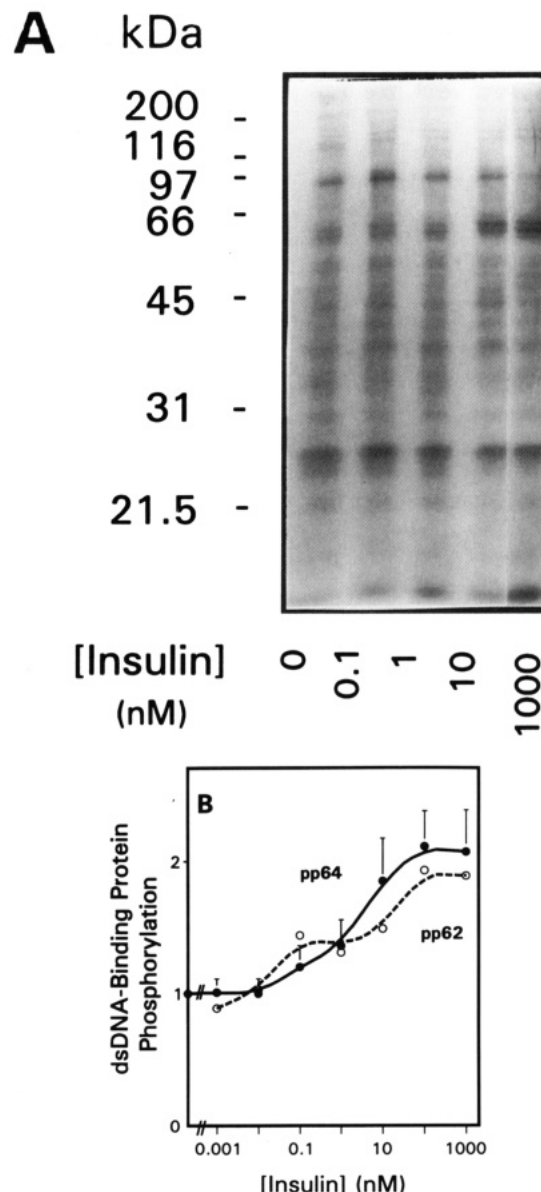

FIGURE 2: Dose response of insulin-induced phosphorylation of dsDNA-binding proteins. Panel A: Differentiated, ^{32}P-labeled 3T3-442A cells were treated with insulin for 15 min at the final concentrations indicated. Nuclear proteins have been extracted and bound to dsDNA–cellulose as described under Experimental Procedures. Samples have been analyzed by SDS–PAGE and autoradiography. Panel B: Bands from the autoradiograms of panel A and three other experiments corresponding to pp62 (open circles) and pp64 (filled circles) were quantified by densitometry as detailed under Experimental Procedures. Data represent means ± SDs of at least three separate experiments. To convert nanomolar to nanograms per milliliter, multiply by 6.

extracts with both free cellulose and the nonspecific polynucleotide poly(I)–poly(C)–agarose prior to adsorption to dsDNA–cellulose. After these preclearing steps, the insulin-induced pp34, pp48, and the triplet around 62–66 kDa remain clearly visible.

The immunoblots of these DNA-binding proteins with mouse anti-lamin A and C antibodies show the presence of at least four lamin isoforms around 62–66 kDa and some minor bands migrating at 48 kDa (Figure 4, lanes c and d). When we analyzed the blots with the rabbit anti-lamin antiserum which recognizes lamin B besides lamins A and C, two additional bands in the 62–66-kDa region were observed which probably correspond to isoforms of lamin B (data not shown). Comparing the pattern of dsDNA-binding phos-
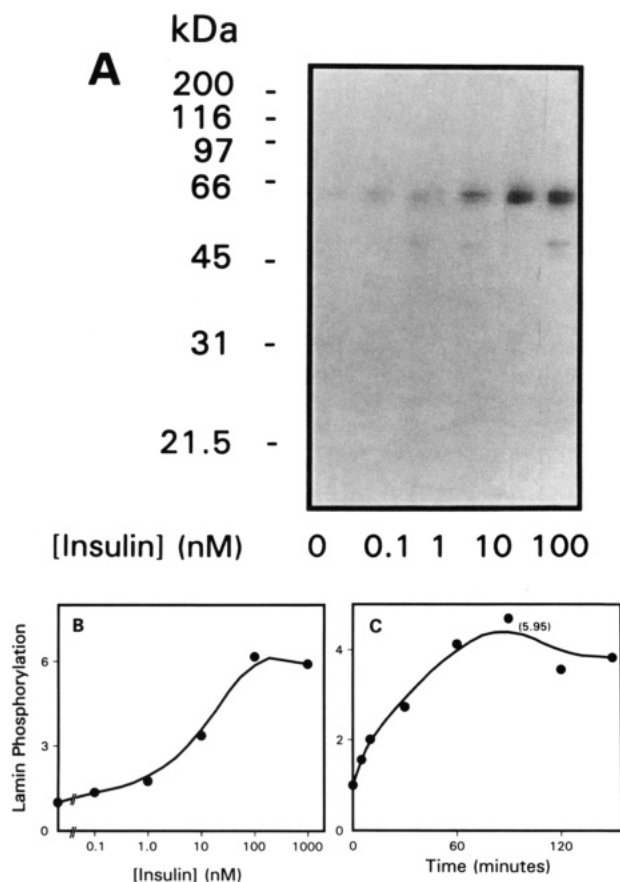
kDa

A

200	-
116	-
97	-
66	-
45	-
31	-
21.5	-

[Insulin] (nM)   0   0.1   1   10   100

B

C (5.95)

FIGURE 3: Insulin-induced phosphorylation of lamins. 3T3-F442A cells were differentiated to adipocytes and labeled with [^{32}P]phosphate as described under Experimental Procedures. Cells were treated with insulin for 15 min at the final concentrations indicated (panels A and B), or the insulin concentration was fixed at 1 μM and the time of incubation was varied (Panel C). Nuclear proteins were extracted and immunoprecipitated with rabbit anti-lamin antiserum. The immunoprecipitates were adsorbed to protein A–beads, washed extensively, and analyzed by SDS–PAGE and autoradiography. Panel A: Autoradiogram of a dose response experiment. The autoradiogram is representative of three separate experiments. Panel B: Concentration dependence of insulin-induced lamin phosphorylation. The radioactivity associated with the lamin bands was quantified by densitometric analysis. Data are means of two experiments. Panel C: Time dependence of insulin-induced lamin phosphorylation. Data are means of the densitometric analysis of two separate experiments. The off-scale value corresponding to 90-min insulin treatment is given in parentheses.

phoproteins with anti-lamin immunoblots (Figure 4, lanes a,b and c,d), we concluded that the triplet of pp62, pp64, and pp66, as well as pp48, is at least partly recognized by antibodies against lamins A and C. This (partial) identity was also confirmed when we dissolved the dsDNA-binding proteins from dsDNA–cellulose by high-salt treatment and successively immunoprecipitated the proteins with anti-lamin antibodies (Figure 4, lanes a,b and e,f). In addition, the gel background was significantly reduced and two new insulin-induced phosphoproteins at 40 and 72 kDa could be observed (Figure 4, lanes a and b).

In our attempts to identify the remaining insulin-induced dsDNA-binding nuclear phosphoproteins, we performed immunoprecipitation after an elution from dsDNA–cellulose with 0.4 M NaCl using antibodies against several candidate proteins. Specifically, we could not show any significant immunoprecipitation with anti-fos, anti-jun, or anti-NF-1 antibodies of the insulin-induced, dsDNA-binding nuclear phosphoproteins (data not shown). Also there was no significant difference in the ^{32}P-labeled proteins bound to an

kDa

200	—
116	—
97	=
66	—
45	—
31	—

a b c d e f

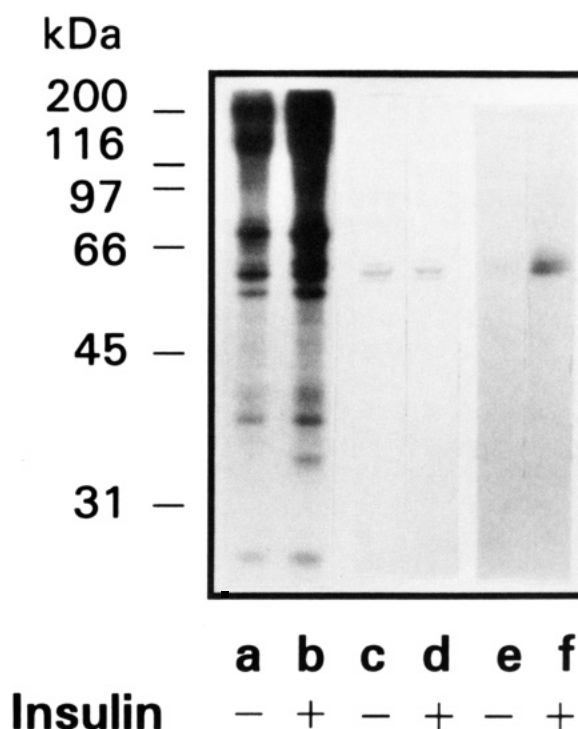**Insulin**   —   +   —   +   —   +

FIGURE 4: Comparison of insulin-induced dsDNA-binding phosphoproteins and lamins. Differentiated, serum-starved, ^{32}P-labeled 3T3-442A cells were incubated in the presence (lanes b, d, and f) or absence (lanes a, c, and e) of 1 μM insulin for 15 min. Nuclear proteins were extracted with high-salt/Triton X-100 treatment as detailed under Experimental Procedures. Nuclear extracts were precleared with the successive addition of cellulose and poly(I)–poly(C)–agarose. The extracts were adsorbed to dsDNA–cellulose and washed extensively. Samples were subjected to SDS–PAGE; ^{32}P-labeled proteins were blotted to nitrocellulose filters and analyzed by autoradiography (lanes a and b). On the same blots, lamins were visualized with IE$_4$ anti-lamin antibody as detailed under Experimental Procedures (lanes c and d). Separate samples were dissolved from the dsDNA–cellulose column by high-salt treatment, immunoprecipitated with IE$_4$ anti-lamin antibody, and then analyzed with SDS–PAGE and autoradiography (lanes e and f). The autoradiogram and immunoblot are representatives of three separate experiments.

NKκB-affinity oligo-DNA column from control and insulin-treated 3T3-F442A cells (data not shown).

Phosphoamino Acid Analysis of dsDNA-Binding Phosphoproteins. Phosphoamino acid analysis of various bands from the SDS–PAGE gel of Figure 4A is shown in Figure 5. The insulin-induced phosphorylation of pp72, pp64, and pp62 was almost exclusively on serine residues. In the case of pp72, there was a small amount of labeled phosphothreonine; however, this was also present in the control samples which had not been treated with insulin (data not shown). Unfortunately, the amount of radioactivity associated with pp34, pp40, and pp48 was too small to analyze their phosphoamino acid composition.

DISCUSSION

After insulin binds to its receptor on the cell surface, there is an elaborate cascade of signal transduction steps induced in the plasma membrane and cytosol. As a result of intensive research, many of the initial events which are linked to the acute metabolic effects of insulin have been characterized (Rosen, 1987; Kahn & White, 1988). On the other hand, some of the growth effects of insulin, as well as some of the metabolic effects, require that the insulin signal must reach the cell nucleus. Although there are reports showing the association of insulin and/or insulin receptors with the cell
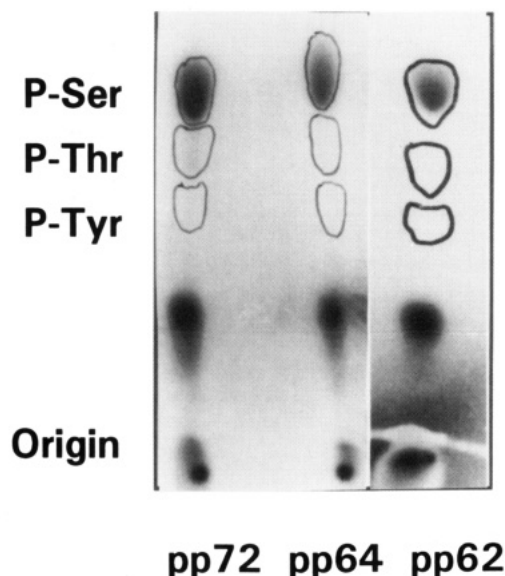
FIGURE 5: Phosphoamino acid analysis. Bands corresponding to pp72, pp64, and pp62 were cut from the gel the autoradiogram of which is shown in Figure 4, lane b. The phosphoamino acid composition of the proteins was analyzed as described under Experimental Procedures. Data are representatives of two separate phosphoamino acid determinations.

nuclei (Goldfine & Smith, 1976; Goldfine et al., 1977; Smith & Jarett, 1987) and an insulin-induced increase in nuclear RNA and protein transport (Schindler & Jiang, 1987; Purello et al., 1983), the mechanisms coupling insulin receptor binding to insulin action at the nuclear level are largely unknown.

In the present study, we report that insulin induces the phosphorylation of seven dsDNA-binding nuclear proteins. The insulin-induced phosphorylation of five nuclear proteins, pp34, pp48, pp62, pp64, and pp66, can be detected directly by their ability to bind to double-stranded DNA–cellulose. Preclearing of the high-salt nuclear extracts with cellulose and the nonspecific nucleotide resin poly(I)–poly(C)–agarose allows detection of two additional insulin-induced phosphoproteins, pp40 and pp72, which bind to the DNA affinity column. The reason why pp40 and pp72 are able to bind to the dsDNA–cellulose only after the preclearing steps is unknown, but could relate to enhancement of the dissociation of protein complexes in the nuclear extract by these additional steps of purification, or simply to enhanced sensitivity afforded by this methodologic modification.

From immunoblotting experiments, we gained evidence that three of the seven insulin-induced dsDNA-binding phosphoproteins (a triplet at 62–66 kDa) comigrate with the isoforms of lamins A and C. The time course and insulin concentration dependence of pp62, pp64, and pp66 phosphorylation and lamin phosphorylation are almost identical (compare Figures 1B and 3B and 2B and 3C). When the dsDNA-binding phosphoproteins are dissolved with high-salt treatment, anti-lamin antibodies are able to recognize a doublet around 64 kDa. These experiments further support the conclusion that pp62, pp64, and pp66 are at least partially identical with the lamins of differentiated 3T3-F442A cells.

Lamins are a large family of nuclear skeletal proteins. Recently, several isoforms of the three major lamins, A, B, and C, have been identified (Krohne & Benavente, 1986; Lehner et al., 1986). At present, no detailed characterization of mouse adipocyte lamins has been made; thus, it is difficult to assess exactly which lamins correspond to the three insulin-induced dsDNA-binding phosphoproteins. When the immunoblotting experiments with the rabbit antibodies (which recognize lamins A, B, and C) are compared with the experiments using mouse antibodies (which recognize only lamins A and C), one additional band is observed. This band in the 66-kDa region probably represents lamin B and was not identified in the ^{32}P-labeled nuclear extracts with immunoprecipitation using the two antibodies (data not shown). These data suggest that insulin does not induce the phosphorylation of lamin B in 3T3-F442A cells and the lamin species which become phosphorylated correspond to isoforms of lamin C or isoforms of lamins A and C. This conclusion is in agreement with the results of Friedman and Ken (1988), who found that insulin induces the phosphorylation of lamins A and C but not lamin B in quiescent baby hamster kidney fibroblasts (BHK-21 cells). The insulin dose response of lamin phosphorylation is very similar in the two cells, but the maximal phosphorylation of lamins is achieved at 15 min in BHK-21 cells while differentiated 3T3-F442A cells display a slower response, peaking at 60 min. Our conclusion that the insulin-induced dsDNA-binding phosphoproteins pp62, pp64, and pp66 are at least partially identical with isoforms of lamin C and lamin A is in agreement with the recent findings that lamins A and C, but not B, are able to bind to DNA (Yuan et al., 1991; Hakes & Berezney, 1991).

There is also a group of minor phosphoproteins around 48 kDa which is recognized by anti-lamin antibodies in immunoblots and immunoprecipitation experiments. These lamin-related dsDNA-binding phosphoproteins are migrating close to the reported molecular weight of the 46-kDa nucleoside triphosphatase which is thought to participate in nucleocytoplasmic transport of RNA and which is generated via the self-digestion of lamins A and C (Yuan et al., 1991). This finding raises the possibility that the insulin-induced phosphorylation of the 46/48-kDa lamin fragment may participate in the induction of RNA efflux from the nuclei after insulin treatment, as suggested by Purello et al. (1983).

Since the lamins are phosphorylated on serine residues, it is unlikely that they are direct substrates of the insulin receptor or another insulin-induced tyrosine kinase. Recently, several kinase such as protein kinase C, the S6 kinase, and cdc2 kinase have been shown to participate in the phosphorylation of various lamins (Hornbeck et al., 1988; Fields et al., 1988; Ward & Kirschner, 19990; Peter et al., 1990). In other experiments, we have found that phorbol ester (TPA) treatment induces the phosphorylation of the same set of lamins as insulin and that insulin increases the nuclear S6 kinase activity in differentiated 3T3-F442A cells, suggesting that protein kinase C and nuclear S6 kinase are good candidates for this role (data not shown). The exact identification of the protein kinases which are participating in the insulin-induced phosphorylation of lamins A and C will require further research.

The identity of the other insulin-induced dsDNA-binding phosphoproteins, pp34, pp40, and pp72, is not known. Recently, insulin has been reported to induce phosphorylation of the 40-kDa numatrin (B23) (Feuerstein & Randazzo, 1991). Numatrin is known to be able to bind to both ssDNA and dsDNA (Feuerstein et al., 1990), and this further enhances the possibility that pp40 may be identical with this protein.

There are several transcription factors such as fos, jun, NFκBl, nuclear factor 1 (NF-1), and serum response factor which are known to be regulated by phosphorylation and which would migrate close to one of the insulin-induced dsDNA-binding proteins (Ofir et al., 1990; Boyle et al., 1991; Yamamoto et al., 1988; Lenardo & Baltimore, 1989; Prywes

et al., 1988; Sorger & Pelham, 1988). In this study, however, we failed to detect any insulin-induced phosphorylation of ^{32}P-labeled fos, jun, or NF-1 immunoprecipitates or in NFκB-affinity oligo-DNA-purified ^{32}P-labeled nuclear extracts. Experiments with the serum response factor antibodies were in progress at the submission of this paper.

In summary, insulin induces the phosphorylation of lamin C (and A) isoforms and several other DNA-binding proteins in 3T3-F442A cells. Further studies are to determine if the insulin-induced lamin phosphorylation participates in the $G_0 \rightarrow G_1$ reorganization of nuclear structure, in analogy with cdc2 kinase-induced lamin disassembly in mitosis (Ward & Kirschner, 1990; Peter et al., 1990; Ottaviano & Gerace, 1985; Heald & McKeon, 1990), and to identify the remaining members of this insulin-induced, nuclear protein group. Whether these might represent new transcription factors or other nuclear signaling proteins remains to be determined.

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