THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 268, No. 13, Issue of May 5, pp. 9747-9752, 1993
Printed in U.S.A.

Insulin Induces the Phosphorylation of Nucleolin

A POSSIBLE MECHANISM OF INSULIN-INDUCED RNA EFFLUX FROM NUCLEI*

(Received for publication, July 6, 1992, and in revised form, December 3, 1993)

Peter Csermely‡, Tamás Schnaider§, Bentley Cheatham¶, Mark O. J. Olson‖, and C. Ronald Kahn‡‡**

From the Research Division, Joslin Diabetes Center, and Department of Medicine, Brigham and Women's Hospital, and Harvard Medical School, Boston, Massachusetts 02215 and the ‖Department of Biochemistry, the University of Mississippi Medical Center, Jackson, Mississippi 39216

Insulin induces the serine phosphorylation of the nucleolar protein nucleolin at subnanomolar concentrations in differentiated 3T3–442A cells. The stimulation is biphasic with phosphorylation reaching a maximum at 10 pM insulin and then declining to only 40% of basal levels at insulin concentrations of 1 μM. These changes are rapid, reaching half-maximal after 4 min and maximal after 15 min of incubation. The cell-permeable casein kinase II inhibitor 5,6-dichlorobenzimidazole-riboside prevents the insulin-stimulated phosphorylation of nucleolin suggesting that casein kinase II may mediate this effect of the hormone. Insulin-like growth factor 1 mimics the action of insulin on dephosphorylation of nucleolin at nanomolar concentrations suggesting that the latter effect may be mediated by insulin-like growth factor 1 receptors. Insulin treatment of 3T3–442A cells also results in a stimulation of RNA efflux from isolated, intact cell nuclei. The dose dependence of insulin-induced nucleolin phosphorylation and insulin-stimulated RNA efflux from intact cell nuclei are almost identical. Insulin induces an increase in the RNA efflux at subnanomolar concentrations in 3T3–442A adipocytes, while high (micromolar) concentrations of insulin inhibited the efflux of RNA. These data indicate that insulin regulates the phosphorylation/dephosphorylation of nucleolin, possibly via stimulation of casein kinase II, and this may play a role in regulation of the RNA efflux from nuclei.

Binding of insulin to its receptors in the plasma membrane induces a wide variety of cellular responses including short term changes in cellular metabolism and long term growth effects involving enhanced nuclear activity (1, 2). In spite of our rapidly increasing knowledge about the effects of insulin on the plasma membrane and cytosolic enzymes, the various mechanisms mediating insulin's nuclear function are less clear. Insulin alters the transcription rate of a number of genes (reviewed in Ref. 3) and has been shown to increase transport of proteins and RNA from nucleus to cytosol (4, 5). Some of the insulin-induced protein kinases and phosphatases, such as casein kinase II and protein phosphatase 1, have been localized in the nucleus (6, 7), although a role of insulin in regulation of these nuclear enzymes is lacking. There is also a growing, but still very much incomplete, number of nuclear proteins, such as lamins and numatrin (B23) (8, 9)[1] which have been shown to change their phosphorylation status after the addition of insulin.

In our recent studies we have identified and partially characterized several nuclear proteins which are able to bind to DNA and whose phosphorylation changes upon insulin stimulation. Four of the proteins were immunologically related to lamins while the identity of the others is not known yet.[1] One of the DNA-binding phosphoproteins, migrating at 94 kDa, was dephosphorylated after the addition of high (micromolar) concentrations of insulin. Nucleolin (C23) is an abundant nucleolar phosphoprotein which is thought to be involved in the synthesis, processing, and transport of preribosomal RNA (10–13). Nucleolin is able to bind to DNA and migrates from 92–110 kDa on SDS gels (11, 13, 14). This raised the possibility that the 94-kDa DNA-binding phosphoprotein observed in the previous experiments was related to nucleolin, and therefore, that insulin may induce changes in the phosphorylation of this protein. In the present report we have evaluated the effects of insulin on the phosphorylation of nucleolin and its relationship to insulin-induced changes in ribosomal RNA transport.

MATERIALS AND METHODS

Chemicals—Cell culture media were obtained from GIBCO. Pork insulin was purchased from Elanco Products Co. (Indianapolis, IN). Insulin-like growth factor 1 (IGF-1)[2] was from Eli Lilly Co. The chemicals used for polyacrylamide gel electrophoresis were from Bio-Rad. RNase was from Boehringer Mannheim. L-1-Tosyl-amido-2-phenylethyl chloromethyl ketone-trypsin was obtained from Worthington Biochemical Co. (Freehold, NJ). Protein-A acrylamide beads, constant boiling 6 N HCl, and Triton X-100 were obtained from Pierce Chemical Co. [^{32}P]PO_4 (carrier free) and [^{3}H]uridine were from Du Pont-New England Nuclear. Rabbit anti-nucleolin

* This work was supported by Research Grant DK 33201 from the National Institutes of Health, by NIH Grant DK 36836 to the Joslin Diabetes and Endocrinology Research Center, by a Pfizer Biomedical Research Award, and by the Marilyn M. Simpson Research Program in Diabetes. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of Fogarty International Research Fellowship Award 1F05 TW04319-01 B1-5. On leave from the Institute of Biochemistry I, Semmelweis University, Medical School, Budapest, Hungary.

§ On leave from the Institute of Biochemistry I., Semmelweis University, Medical School, Budapest, Hungary.

¶ Recipient of a Juvenile Diabetes Foundation Postdoctoral Fellowship.

** Supported by National Institutes of Health Grant GM28349.

‡‡ To whom correspondence should be addressed: Joslin Diabetes Center, One Joslin Place, Boston, MA 02215. Tel.: 617-732-2635; Fax: 617-732-259.

[1] P. Csermely and C. R. Kahn, manuscript in preparation.

[2] The abbreviations used are: IGF-1, insulin-like growth factor 1; DRB, 5,6-dichlorobenzimidazole-riboside; dsDNA, double-stranded DNA; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.

serum was produced as described by Olson *et al.* (15). Rabbit γ-globulin was a product of Jackson Immunolaboratories (West Grove, PA). Nitrocellulose filters (0.45 μm) were purchased from Schleicher & Schuell. All the other chemicals used were from Sigma.

Culture and Differentiation of 3T3–442A Cells—NIH-3T3–442A cells were grown in Dulbecco's modified Eagle's medium with 10% calf serum in 5% humidified CO_2 atmosphere. Cells were differentiated in 10% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum for 12 days or so otherwise indicated. The differentiation was accelerated by the addition of 5 μg/ml insulin for the first 8 days. By the end of this period, 90–95% of the cells acquired a characteristic adipocyte morphology accumulating a large number of lipid droplets. Insulin was removed from the culture media during the last 4 days of culture in an effort to enhance any insulin-induced nuclear signals.

^{32}P Labeling of 3T3–442A Cells—Cells were serum starved for 18 h, then the cell culture medium was changed to phosphate-free Dulbecco's modified Eagle's medium, and cells were incubated with 0.3 mCi/ml [^{32}P]phosphate for 2 h. After the addition of insulin or other stimulants at concentrations 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 phenylmethylsulfonyl fluoride, 0.1 mg/ml aprotinin, 50 μg/ml leupeptin, 1 mM sodium vanadate, 1 mM sodium molybdate, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 0.25 M sucrose, pH 7.4. In agreement with previous findings (16), preliminary experiments showed that the addition of leupeptin to the isolation buffer was necessary to prevent the excessive proteolysis of nucleolin.

Cells were disrupted by the method of Lee *et al.* (17) passing them four to five times through a 1-ml tuberculin-syringe with a microfine-IV needle. Cell nuclei were isolated by the method of Blobel and Potter (18). Samples were centrifuged at 3,000 × *g* for 15 min at 4 °C, and the supernatants were transferred to Eppendorf tubes. Fat was wiped from the walls of the centrifuge tubes with cotton-tipped applicators, 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. After a centrifugation at 100,000 × *g* for 35 min at 4 °C nuclei were resuspended in 0.5 ml of isolation buffer. The nuclear preparation was 90–95% pure, and the nuclei were more than 95% intact as judged by marker enzyme analysis and electronmicrography (data not shown).

Immunoprecipitations—Proteins were extracted from aliquots of both intact ^{32}P-labeled cell nuclei and the postnuclear supernatant by addition of 400 units of DNase I, 500 units of RNase, 0.6 M NaCl, and 0.5% (v/v) Triton X-100. 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, centrifuged in a microfuge, and the protein concentration of the supernatants was determined according to Bradford (19). Anti-nucleolin serum (5 μg) was added to aliquots of protein extracts containing 0.5 mg of protein, and the samples were incubated overnight at 4 °C. The immunocomplexes were adsorbed to 30 μl of protein A-beads by slow mixing 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 (20) containing 100 mM DTT. Samples were boiled for 3 min, centrifuged in a microfuge, and the supernatants were analyzed by SDS-PAGE and autoradiography. The results were quantified by a Molecular Dynamics (Sunnyvale, CA) 300A computing densitometer. The level of significance, *p*, was determined using the Student's *t* test.

Purification of Double-stranded DNA-binding Proteins—DNA-cellulose chromatography was performed as described by Alberts and Herrick (21). Briefly, the protein concentration of ^{32}P-labeled cell nuclei was determined according to Bradford (19). Nuclear proteins were then extracted from aliquots of nuclei containing 0.5 mg of protein each by addition of 0.6 M NaCl and 0.5% Triton X-100. Samples were rotated for 2 h at 4 °C, 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. Approximately 20 mg of double-stranded DNA-cellulose (dsDNA-cellulose) was added, and samples were rotated for 4 h at 4 °C. After washing two times with 1 ml of the same HEPES/Triton/glycerol buffer, 30 μl of Laemmli sample buffer with 100 mM DTT (19) was added to each sample, and the samples were analyzed with SDS-PAGE and autoradiography.

Phosphoamino Acid Analysis—Phosphoamino acid analysis was carried out by the method of Cooper *et al.* (22). The 94-kDa nucleolin phosphoprotein band was excised from the polyacrylamide gel, soaked in 20% methanol, digested with the addition of 2 × 100 μg of TPCK-trypsin in 50 mM ammonium carbonate for 24 h at 37 °C. The tryptic 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 thin layer chromatography plates at pH 3.5. Plates were dried, stained with ninhydrin, and analyzed by autoradiography.

Measurement of RNA Efflux from Isolated Nuclei—Release of RNA was measured according to the method of Agutter *et al.* (23). After differentiation, serum-starved 3T3–442A cells were incubated with 10 μCi/ml [^{3}H]uridine for 30 min. In the last 15 min of incubation, insulin was added to the dishes at various final concentrations. Cell nuclei were isolated as described above with the only difference that ATP was not included in the isolation buffer. After ultracentrifugation nuclei were suspended in 0.5 ml of RNA buffer containing 50 mM HEPES, pH 7.4, 25 mM KCl, 5 mM spermidine, 2 mM DTT, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.3 mM $MnCl_2$, 300 μg/ml yeast RNA, and 0.25 M sucrose. The protein concentration of the samples was determined according to Bradford (19).

The release of RNA was measured from aliquots of nuclei containing 0.5 mg of protein each in a final volume of 0.25 ml of RNA buffer supplemented with 2.5 mM ATP. Nuclei were incubated for 15 min at 37 °C. Preliminary experiments showed that the efflux of RNA was linear up to 30 min of incubation. The efflux was terminated by pelleting the nuclei in a microfuge. To the supernatants, 0.25 ml of 50% trichloroacetic acid was added, the precipitate was centrifuged, and the [^{3}H]uridine in RNA was measured by liquid scintillation counting. The level of significance, *p*, was determined using the Student's *t* test.

Immunoblots—Non-radioactive nuclei were isolated, nucleolin was immunoprecipitated from aliquots of the nuclear fraction, and the postnuclear supernatant and the immunocomplexes were purified and separated with SDS-PAGE as described above. The proteins were transferred to nitrocellulose filters according to the method of Towbin (24) 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, 0.1% bovine serum albumin blocking buffer for 1 h at room temperature. After overnight incubation with a 1:200 dilution of rabbit anti-nucleolin serum at 4 °C, the immunocomplexes were visualized by means of peroxidase-conjugated anti-rabbit antibodies and subsequent treatment with 4-chloro-1-naphthol and H_2O_2.

RESULTS

Concentration Dependence of Insulin-induced Phosphorylation and Dephosphorylation of Nucleolin—Insulin induces the phosphorylation of several nuclear proteins in 3T3–442A cells which are able to bind to double-stranded DNA-cellulose (dsDNA-cellulose).[1,2] In the experiment shown in Fig. 1, there is a marked insulin-induced phosphorylation of one 48-kDa and four 62–66-kDa proteins. There is a faint phosphorylation of a band at 34 and 40 kDa. The phosphorylation of these proteins shows little variation with the differentiation of 3T3–442A cells to adipocytes. Examination of the Coomassie Blue-stained gel reveals no identifiable abundant proteins of these molecular weights nor a significant change in the amount of protein migrating in these areas after insulin addition which suggests that the phenomenon observed is not simply due to a change in the affinity of some abundant protein toward dsDNA (data not shown). In separate experiments we obtained evidence that a significant component of the insulin-induced dsDNA-binding phosphoproteins around 62–66 kDa are isoforms of lamin C[1]. The phosphoprotein band at 40 kDa may correspond to numatrin (B23) which has been recently identified as an insulin-induced DNA-binding phosphoprotein (9, 25).

In nuclei of non-stimulated cells which have been allowed to differentiate for 4–8 days, there is a phosphoprotein of about 94 kDa which binds to dsDNA (Fig. 1*A*). Incubation of cells with insulin (1 μM) for 15 min induced a dephosphoryl-

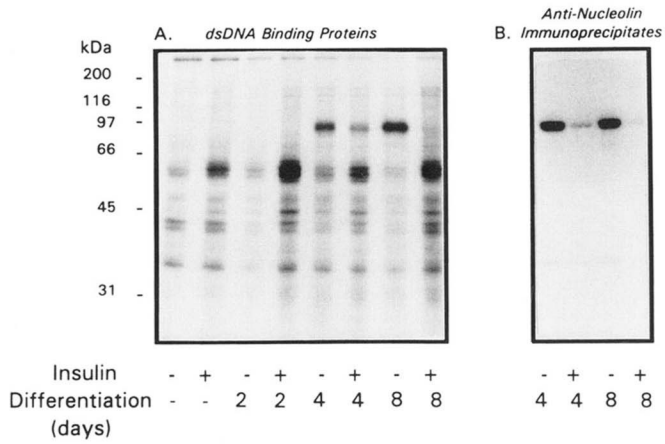

FIG. 1. **Insulin-induced phosphorylation of double-stranded DNA-binding nuclear proteins.** 3T3–442A cells were differentiated as described under "Materials and Methods." The differentiation of cells was enhanced by the addition of 5 μg/ml insulin for times indicated. After removal of insulin and serum starvation and labeling with [^{32}P]phosphate for 2 h, cells corresponding to even lanes (marked +) were treated with 1 μM insulin for 15 min. ^{32}P-labeled nuclei were isolated from each sample, and 0.5 mg of nuclear proteins were extracted with 0.6 M NaCl and 0.5% Triton X-100 (see "Materials and Methods" for details). Protein extracts were diluted 10 times with a buffer containing 10 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. In the experiment shown in *panel B*, proteins were eluted from dsDNA-cellulose with 0.6 M NaCl and immunoprecipitated with anti-nucleolin antibodies as described under "Materials and Methods." *Panel A*, dsDNA-binding nuclear phosphoproteins; *Panel B*, dsDNA-binding nuclear phosphoproteins immunoprecipitated with anti-nucleolin antibodies.

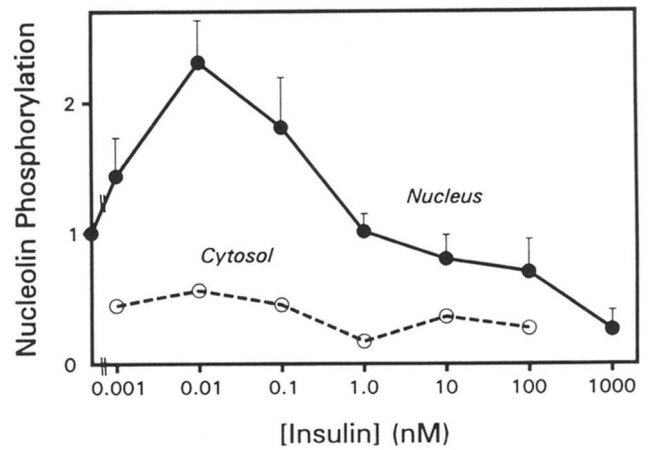

FIG. 2. **Concentration dependence of insulin-induced nucleolin phosphorylation.** 3T3–442A cells were differentiated, serum starved, and labeled with [^{32}P]phosphate as described under "Materials and Methods." Cells were treated with insulin at final concentrations indicated for 15 min. Proteins were extracted from both isolated cell nuclei and the postnuclear supernatant by addition of 400 units of DNase I, 500 units of RNase, 0.1 mg of rabbit γ-globulin, 50 μl of protein A-beads, NaCl, and Triton X-100 at final concentrations of 0.6 M and 0.5% (v/v), respectively. To aliquots of protein extracts containing 0.5 mg of protein each ,5 μg of anti-nucleolin serum was added, and the samples were incubated overnight at 4 °C. The immunocomplexes were adsorbed to 30 μl of protein A-beads by rotating them for 2 h at 4 °C. Immunoprecipitates were washed and analyzed by SDS-PAGE and autoradiography. The 94-kDa nucleolin bands were quantified by densitometry. In case of nuclear nucleolin data are mean ± S.D. of four separate experiments (*filled circles*); data representing the nucleolin band of the postnuclear supernatant (*open circles*) are representative of two separate experiments. In the other two experiments, the cytosolic nucleolin band was too faint to allow quantification.

ation of this protein. This phenomenon was strongly dependent on the status of differentiation of 3T3–442A cells, the 94-kDa protein being more prominent and the insulin effect more evident in cells only after several days of differentiation. Nucleolin (C23) is an abundant nuclear phosphoprotein which is able to bind to DNA and migrates approximately 92–110 kDa on SDS gels (11, 13, 14). When the dsDNA-binding phosphoproteins from the 4- and 8-day differentiated samples of Fig. 1*A* were eluted with 0.6 M NaCl, immunoprecipitated with anti-nucleolin antibodies and subjected to SDS-PAGE, most of the 94-kDa phosphoprotein band was recovered in the immunoprecipitates indicating that this band primarily represents phosphorylated nucleolin (Fig. 1*B*).

Detailed experiments showed that the concentration dependence of insulin-induced changes in nucleolin phosphorylation were biphasic (Fig. 2). At subnanomolar concentrations insulin induced a more than 2-fold increase in the phosphorylation of the 94-kDa immunoprecipitable nucleolin, while at high (micromolar) concentrations of insulin, a 70% decrease in the phosphorylation was observed (Fig. 2). Both the increase and decrease in the phosphorylation of nucleolin at 10^{-11} M and 10^{-6} M insulin concentrations, respectively, are significantly different from the control level at $p < 0.001$ as determined by Student's *t* test.

The amount of phosphorylated nucleolin in the postnuclear supernatant (*open circles* in Fig. 2) was relatively minor compared to that in nucleus and did not show significant changes after insulin addition. This low amount of phosphorylation was due to the low amount of non-nuclear nucleolin as judged by immunoblotting of non-nuclear protein extracts with anti-nucleolin antibodies (data not shown).

Nucleolin is very sensitive to proteolysis most of which can be prevented by the protease inhibitor leupeptin (16, 27, 28). If the isolation and extraction of nuclei were performed in the absence of leupeptin, only a minor portion of the 94-kDa phosphoprotein band could be detected. Even in the presence of leupeptin, we regularly observed an 80-, a 72-, and a 34-kDa phosphoprotein band in the immunoprecipitates which were minor, however, compared to the 94 kDa band. Since these phosphoproteins showed changes similar to those of the 94-kDa phosphoprotein after insulin addition they most likely represent proteolytic fragments of nucleolin (data not shown).

Time Course of Insulin-induced Nucleolin Phosphorylation—The time course of insulin-induced nucleolin phosphorylation using 0.01 nM insulin is shown in Fig. 3. The phosphorylation was rapid with half-maximal stimulation observed at 4 min and a maximum after 15 min of incubation with insulin. The insulin-induced nucleolin phosphorylation remained steady until 30 min, then declined after 60 min of incubation (Fig. 3).

Phosphoamino Acid Analysis of Nucleolin—Insulin induced the phosphorylation of nucleolin on serine residues (Fig. 4). When the 94 kDa band was cut from the gel and hydrolyzed, phosphoserine was the only detectable phosphoaminoacid in nucleolin immunoprecipitates from 3T3–442A cells (Fig. 4). This was true of both insulin-stimulated and control cells.

Effect of Insulin-like Growth Factor I and a Casein Kinase II Inhibitor on Insulin-induced Nucleolin Phosphorylation—Since 3T3–442A cells have IGF-1 receptors, and insulin is well-known to cross-react with these receptors at high, micromolar concentrations (28), we analyzed the effect of IGF-1 on the phosphorylation of nucleolin. In contrast to insulin, IGF-1 failed to stimulate the phosphorylation of nucleolin. However, IGF-1 did induce the dephosphorylation of nucleolin at

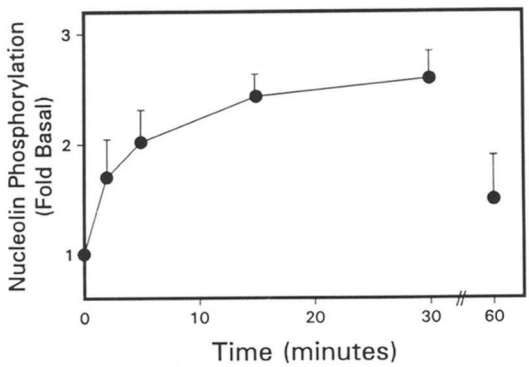

FIG. 3. **Time course of insulin-induced nucleolin phosphorylation.** 3T3–442A cells were differentiated, serum starved, and labeled with [^{32}P]phosphate as described under "Materials and Methods." Cells were treated with 0.01 nM insulin for times indicated. Cellular proteins were extracted and immunoprecipitated with anti-nucleolin antibodies as described in the legend of Fig. 2. The 94-kDa nucleolin bands were separated by SDS-PAGE, and the autoradiograms were subjected to densitometric analysis. Data are mean ± S.D. of three separate experiments.

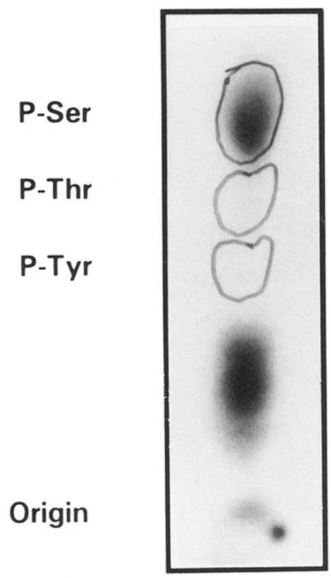

FIG. 4. **Phosphoamino acid analysis.** 3T3–442A cells were differentiated, serum starved, labeled with [^{32}P]phosphate, and treated with 0.01 nM insulin for 15 min. The insulin-induced 94-kDa nucleolin phosphoprotein band was cut from the SDS gel, and its phosphoamino acid composition was analyzed as described under "Materials and Methods." Data are representatives of two separate phosphoamino acid determinations.

nanomolar concentrations (Table I), at the same range where it stimulates glucose uptake in the closely related 3T3-L1 adipocytes (28). The extent of IGF-1-induced nucleolin dephosphorylation was similar to the amount of insulin-induced dephosphorylation at micromolar insulin concentrations. In addition, coincubation of cells with insulin and IGF-1 at concentrations of insulin (0.01 nM) which stimulate nucleolin phosphorylation and concentrations of IGF-1 (1 nM) which cause dephosphorylation results in an overall decrease in nucleolin phosphorylation. This effect is also observed when higher concentrations of both hormones are present.

As indicated above, IGF-1 and high concentrations of insulin induce the dephosphorylation of nucleolin. Since insulin is known to induce the activities of protein phosphatases 1 and 2A (7, 29, 30) we were interested in determining which protein phosphatase may be responsible for the dephosphorylation of nucleolin. However, we failed in our attempts to prevent the insulin-induced dephosphorylation of nucleolin by okadaic acid, a known inhibitor of these phosphatases (31). Trifluoperazine, an inhibitor of protein phosphatase 2B (31) was also without any effect (data not shown).

TABLE I

Effect of insulin-like growth factor I and a specific, cell-permeable inhibitor of casein kinase II on the insulin-induced nucleolin phosphorylation

3T3-442A cells were differentiated, serum starved, and labeled with [^{32}P]phosphate as described under "Materials and Methods." Cells were treated with various agents indicated in the table for 15 min. Cellular proteins were extracted and immunoprecipitated with anti-nucleolin antibodies as described in the legend of Fig. 2. The 94-kDa nucleolin bands were separated by SDS-PAGE and the autoradiograms were subjected to densitometric analysis. Data are mean ± S.D. of three separate experiments.

Treatment	Phosphorylation of nucleolin
	-fold of control
Control	1.0 ± 0.1
+ Insulin	
0.01 nM	2.4 ± 0.2
1 μM	0.4 ± 0.2
+ IGF-1	
0.1 nM	0.9 ± 0.1
1 nM	0.6 ± 0.2
10 nM	0.4 ± 0.2
+ Insulin/IGF-1	
0.01 nM, 1 nM	0.6 ± 0.1
1 nM, 10 nM	0.7 ± 0.1
+ DRB (5 mM)	0.8 ± 0.1
+ DRB/insulin (5 mM, 0.01 nM)	1.1 ± 0.2

Nucleolin is a good *in vitro* and *in vivo* substrate of casein kinase II (32, 33). Since insulin is known to induce this kinase in 3T3-L1 cells (30), a cell line closely related to 3T3–442A cells, we examined if a cell-permeable, specific inhibitor of casein kinase II, 5,6-dichlorobenzimidazole-riboside (DRB, 35) might be able to inhibit the insulin-induced phosphorylation of nucleolin. Treatment of 3T3–442A cells with DRB caused only a slight decrease in the phosphorylation of nucleolin in control cells (the level of significance, $p < 0.05$). More importantly, however, DRB efficiently prevented the insulin-induced increase of nucleolin phosphorylation ($p < 0.005$, Table I).

Insulin-induced RNA Efflux from Intact Nuclei—Since nucleolin is suspected to be involved in the transport of RNA through the nuclear membrane (12, 13) and insulin has been shown to induce the release of RNA from rat hepatocytes nuclei (5, 36), we investigated the possibility that insulin induced similar changes in RNA efflux from the nuclei of differentiated 3T3–442A cells. As is shown in Fig. 5, insulin induced a substantial increase in the efflux of RNA at subnanomolar concentrations (the level of significance, $p < 0.005$ at 10^{-11} M insulin concentration). The RNA efflux returned to the control levels with insulin in the nanomolar range and slightly declined at micromolar insulin concentrations (although this was not statistically significant). The RNA efflux is ATP dependent since only a minor fraction of the radioactive RNA observed in the trichloroacetic acid precipitates of the incubation medium is observed without ATP. The RNA released was not preferentially enriched in polyadenylated RNA and exhibited a very low level of binding to poly(U)-Sepharose (data not shown).

DISCUSSION

Nucleolin (C23) is a 92–110-kDa nucleolar protein thought to be involved in the regulation of polymerase I transcription, binding, packaging, and transport of ribosomal RNA (11–13).

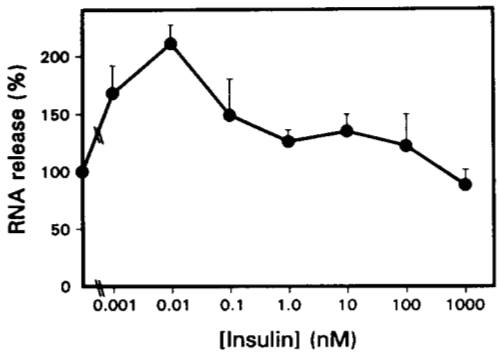

FIG. 5. **Concentration dependence of insulin-induced nuclear RNA efflux.** 3T3–442A cells were differentiated, serum starved, and labeled with [^{3}H]uridine as described under "Materials and Methods." Cells were incubated with insulin at final concentrations indicated for 15 min. RNA efflux was measured from isolated cell nuclei in 0.5 ml of RNA buffer containing 50 mM HEPES, pH 7.4, 25 mM KCl, 5 mM spermidine, 2 mM DTT, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.3 mM $MnCl_2$, 2.5 mM ATP, 300 μg/ml yeast RNA, and 0.25 M sucrose for 15 min at 37 °C. Nuclei were pelleted by rapid centrifugation, [^{3}H]uridine-labeled RNA was precipitated with trichloroacetic acid from the supernatant, and its radioactivity was measured by liquid scintillation counting. Basal RNA efflux was 12 ± 3% of total nuclear radioactivity. Data are mean ± S.D. of three separate experiments.

A number of studies indicate that the phosphorylation and dephosphorylation of nucleolin may play a role in the regulation of these processes (12, 16, 26). In the present study we report that insulin, which induces the activation of a cascade of protein kinases and phosphatases (6, 7), elevates the serine phosphorylation of nucleolin at subnanomolar concentrations, while it effectively promotes the dephosphorylation of nucleolin at the micromolar concentration range. The dose-response of the phosphorylation and dephosphorylation of nucleolin is almost identical with the insulin-induced effects on the RNA efflux from isolated nuclei suggesting that insulin-induced phosphorylation and dephosphorylation of nucleolin may be a regulator of (ribosomal) RNA transport through the nuclear membrane.

Insulin promotes the phosphorylation of a 94-kDa nuclear protein in differentiated 3T3–442A cells which is recognized by anti-nucleolin antibodies, binds to dsDNA, and in the absence of leupeptin has proteolytic products of similar mobility on SDS gels as purified nucleolin. These observations strongly suggest that the insulin-induced 94-kDa phosphoprotein is identical with nucleolin (C23). During the preparation of this article, Suzuki *et al.* (37) reported that insulin, epidermal growth factor, and dexamethasone synergistically induce the phosphorylation of nucleolin in rat hepatocytes. However, in these experiments, insulin was always present at high concentrations (100 nM) and in the presence of epidermal growth factor and/or dexamethasone. In addition, these changes in nucleolin phosphorylation were observed only following long term (8 h) incubations. In the present study we have shown that insulin alone has dramatic short term effects.

The dose-response of insulin-induced nucleolin phosphorylation and dephosphorylation is rather unusual. Half-maximal phosphorylation of nucleolin is achieved at concentrations of insulin below 0.01 nM. The dissociation constant of insulin binding to its receptors in the plasma membrane is 4 nM in the closely related differentiated 3T3-L1 cells, and insulin induces half-maximal uptake of deoxyglucose at similar concentrations (28, 38). The mechanism of this marked amplification of insulin action on the phosphorylation of this nuclear protein is not known; however, insulin also induces many of its other nuclear effects, including stimulation of RNA efflux and nucleoside triphosphatase activity and alterations of specific genes such as PEPCK, in the picomolar concentration range (5, 39, 40).

Micromolar concentrations of insulin induce the dephosphorylation of nucleolin. Since IGF-1 has a similar effect at nanomolar concentrations, insulin may be acting via IGF-1 receptors at these high concentrations. When cells are treated with both hormones at concentrations of insulin which stimulate nucleolin phosphorylation and low nanomolar concentrations of IGF-1, there is a net decrease in nucleolin phosphorylation. While insulin and IGF-1 appear to have distinct differences with respect to phosphorylation of nucleolin, both hormones have similar effects on the phosphorylation and dephosphorylation of pp160/insulin receptor substrate-1, a primary cytosolic substrate of these receptors in 3T3-L1 adipocytes (41, 51).

Insulin induces the activation of a cascade of protein kinases including casein kinase II (6, 34). Casein kinase II preferentially phosphorylates nucleolin both *in vitro* and *in vivo* on serine residues (32, 33). Our observation that the cell-permeable casein kinase II inhibitor, 5,6-dichlorobenzimidazole-riboside (DRB, 35) prevents the insulin-induced serine-phosphorylation of nucleolin suggests that this effect may be mediated by casein kinase II. Recently, the phosphorylation of nucleolin by cdc2 kinase was also reported (42, 43). However, cdc2 kinase phosphorylates nucleolin on threonine residues, and nucleolin does not seem to be a preferential substrate of cdc2 kinase in whole nuclear extracts of NIH 3T3 fibroblasts overexpressing the insulin receptor (44). Furthermore, the cdc2 kinase is not involved in the insulin-induced phosphorylation of another closely related nucleolar protein, numatrin (9). These observations suggest that the effect of cdc2 kinase may be restricted to mitosis, and during interphase the phosphorylation of nucleolin is mediated by casein kinase II.

Casein kinase II may be also involved in basal phosphorylation of nucleolin (32, 33). This possibility is not refuted by the observation that the casein kinase II inhibitor, DRB caused only a slight decrease in phosphorylation of nucleolin in non-stimulated 3T3–442A cells (Table I), since the 15 min of DRB incubation was much shorter than the 2 h of ^{32}P labeling. Casein kinase II is induced during differentiation of 3T3-L1 cells (45). This induction may explain the increase of phosphorylation of nucleolin during differentiation of the closely related 3T3–442A cells (see Fig. 1). This assumption is further supported by the fact that the level and activity of casein kinase II are the limiting factors in the phosphorylation of nucleolin (46) and casein kinase II activity changes parallel with phosphorylation of nucleolin in growth of human cell cultures and HeLa cells (47).

The mechanism of IGF-1 and high concentrations of insulin to induce the dephosphorylation of nucleolin are less clear. We did not observe any significant recovery of the phospho-nucleolin in the presence of okadaic acid or trifluoperoxide, inhibitors of protein phosphatases 1, 2A, and 2B, respectively. These results do not necessarily mean that the IGF-1- and insulin-induced dephosphorylation of nucleolin is mediated via different protein phosphatases since the high fat content of adipocytes may significantly diminish the effective concentration of these lipophilic inhibitors (29) and the *in vitro* dephosphorylation of nucleolin is rather difficult with any of protein phosphatases 1, 2A, 2B, or 2C (48). We might observe a decrease in the nucleolin-associated ^{32}P label if high concentrations of insulin and IGF-1 induced the proteolytic cleavage

of a small, phosphorylated fragment of the protein from its COOH or NH_2 termini. This explanation seems rather unlikely, however, since nucleolin is phosphorylated on multiple sites both *in vitro* and *in vivo* (32, 33, 49).

The dose-response curves of insulin-induced nucleolin phosphorylation and nuclear RNA efflux are almost identical (*cf.* Figs. 2 and 5). This suggests that insulin may regulate the nuclear RNA efflux via changing the phosphorylation status of nucleolin which is thought to participate in packaging and transport of ribosomal RNA (11–13). The RNA released is not polyadenylated and does not bind to poly(U)-Sepharose, suggesting that it contains a significant amount of ribosomal RNA. Picomolar concentrations of insulin have been shown to induce the release of messenger RNA from the nucleus, an effect which appears to be mediated by the dephosphorylation and activation of the nuclear envelope nucleoside triphosphatase-mRNA carrier complex (5, 39, 50). Thus, it is possible that insulin induces the release of mRNA and rRNA via different mechanisms, the former by dephosphorylation of the mRNA carrier and the latter by phosphorylation of the "rRNA carrier" nucleolin. Alternatively, the similarity in the nucleolin phosphorylation and RNA efflux curves simply reflect a correlation between nucleolin phosphorylation and the rate of ribosome assembly, including preribosomal RNA transcription. It is likely that the RNA exiting the nucleus is in the form of ribosome subunits. Thus, it is possible that insulin-regulated nucleolin phosphorylation is involved with the earlier stages of ribosome assembly rather than the later, transport stages.

In summary, the data of this study provide direct evidence for effects of insulin at the cell nucleus which occur through a propagated cascade of phosphorylation and dephosphorylation. Further studies may reveal a strict subnuclear compartmentalization of insulin-induced nuclear protein kinases and phosphatases and may provide insight as to the role of these phosphorylation events in the effects of insulin on cell growth, differentiation, and gene expression.

Acknowledgments—We thank Drs. Bruce M. Spiegelman and Robert J. Distel (Dana Farber Cancer Research Institute, Boston MA) for the 3T3-442A cell line. P. C. is thankful to Drs. Anna Faragó and János Somogyi (Semmelweis University, School of Medicine, Budapest, Hungary) for helpful discussions.

REFERENCES

1. Rosen, O. M. (1987) *Science* **237,** 1452–1458
2. Kahn, C. R., and White, M. F. (1988) *J. Clin. Invest.* **82,** 1151–1156
3. O'Brien, R. M., and Granner, D. K. (1991) *Biochem. J.* **278,** 609–619
4. Schindler, M., and Jiang, L. (1987) *J. Cell Biol.* **104,** 849–853
5. Purrello, F., Burnham, D. B., and Goldfine, I. D. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 1189–1193
6. Czech, M. P., Klarlund, J. K., Yagaloff, K. A., Bradford, A. P., and Lewis, R. E. (1988) *J. Biol. Chem.* **263,** 11017–11020
7. Chan, C. P., McNall, S. J., Krebs, E. G., and Fischer, E. H. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 6257–6261
8. Friedman, D. L., and Ken, R. (1988) *J. Biol. Chem.* **263,** 1103–1106
9. Feuerstein, N., and Randazzo, P. A. (1991) *Exp. Cell Res.* **194,** 289–296
10. Olson, M. O. J., Orrick, L. R., Jones, C., and Busch, H. (1974) *J. Biol. Chem.* **249,** 2823–2827
11. Herrera, A. H., and Olson, M. O. J. (1986) *Biochemistry* **25,** 6258–6264
12. Jordan, G. (1987) *Nature* **329,** 489–490
13. Borer, R. A., Lehner, C. F., Eppenberger, H. M., and Nigg, E. A. (1989) *Cell* **56,** 379–390
14. Olson, M. O. J., Rivers, Z. M., Thompson, B. A., Kao, W.-K., and Case, S. T. (1983) *Biochemistry* **22,** 3345–3351
15. Olson, M. J. O., Guetzkow, K., and Busch, H. (1981) *Exp. Cell Res.* **135,** 259–265
16. Bouche, G., Caizergues-Ferrer, M., Bugler, B., and Amalric, F. (1984) *Nucl. Acids Res.* **12,** 3025–3035
17. Lee, K. A. W., Bindereif, A., and Green, M. R. (1988) *Gene Anal. Techn.* **5,** 22–31
18. Blobel, G., and Potter, V. R. (1966) *Science* **154,** 1662–1664
19. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
20. Laemmli, U. K. (1970) *Nature* **227,** 680–685
21. Alberts, B., and Herrick, G. (1971) *Meth. Enzymol.* **20,** 198–217
22. Cooper, J. A., Sefton, B. M., and Hunter, T. (1983) *Meth. Enzymol.* **99,** 387–402
23. Agutter, P. S., McCaldin, B., and McArdle, H. J. (1979) *J. Biochem.* **182,** 811–819
24. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
25. Feuerstein, N., Mond, J. J., Kinchington, P. R., Hickey, R., Karjalainen-Lindsberg, M.-L., Hay, I., and Ruyechan, W. T. (1990) *Biochim. Biophys. Acta* **1087,** 127–136
26. Bourbon, H., Bugler, B., Caizergues-Ferrer, M., and Amalric, F. (1983) *FEBS Letters* **155,** 218–222
27. Chen, C.-M., Chiang, S.-Y., and Yeh, N.-H. (1991) *J. Biol. Chem.* **266,** 7754–7758
28. Kohanski, R. A., Frost, S. C., and Lane, M. D. (1986) *J. Biol. Chem.* **261,** 12272–12281
29. Haystead, T. A. J., Weiel, J. E., Litchfield, D. W., Tsukitani, Y., Fischer, E. H., and Krebs, E. G. (1990) *J. Biol. Chem.* **265,** 16571–16580
30. Klarlund, J. K., Jaspers, S. R., Khalaf, N., Bradford, A. P., Miller, T. B., and Czech, M. P. (1991) *J. Biol. Chem.* **266,** 4052–4055
31. Cohen, P. (1989) *Annu. Rev. Biochem.* **58,** 453–508
32. Caizergues-Ferrer, M., Belenguer, P., Lapeyre, B., Amalric, F., Wallace, M. O., and Olson, M. O. J. (1987) *Biochemistry* **26,** 7876–7883
33. Schneider, H. R., and Issinger, O.-G. (1988) *Biochem. Biophys. Res. Commun.* **156,** 1390–1397
34. Sommercorn, J., Mulligan, J. A., Lozeman, F. J., and Krebs, E. G. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 8834–8838
35. Zandomeni, R., and Weinmann, R. (1984) *J. Biol. Chem.* **259,** 14804–14811
36. Schumm, D. E., and Webb, T. E. (1981) *Arch. Biochem. Biophys.* **210,** 275–279
37. Suzuki, N., Kobayashi, M., Sakata, K., Suzuki, T., and Hosoya, T. (1991) *Biochim. Biophys. Acta* **1092,** 367–375
38. Rosen, O. M., Smith, C. J., Hirsch, A., Lai, E., and Rubin, C. S. (1979) *Recent Progr. Hormone Res.* **35,** 477–499
39. Purrello, F., Vigneri, R., Clawson, G. A., and Goldfine, I. D. (1982) *Science* **216,** 1005–1007
40. Andreone, T. L., Beale, E. G., Bar, R. S., and Granner, D. K. (1982) *J. Biol. Chem.* **257,** 35–38
41. Madoff, D. H., Martensen, T. M., and Lane, M. D. (1988) *Biochem. J.* **252,** 7–15
42. Peter, M., Nakagawa, J., Dorée, M., Labbé, J. C., and Nigg, E. A. (1990) *Cell* **60,** 791–801
43. Belenguer, P., Caizergues-Ferrer, M., Labbé, J. C., Dorée, M., and Amalric, F. (1990) *Mol. Cell. Biol.* **10,** 3607–3618
44. Feuerstein, N. (1991) *J. Biol. Chem.* **266,** 16200–16206
45. Sommercorn, J., and Krebs, E. G. (1987) *J. Biol. Chem.* **262,** 3839–3843
46. Belenguer, P., Baldin, V., Mathieu, C., Prats, H., Bensaid, M., Bouche, G., and Amalric, F. (1989) *Nucl. Acid Res.* **17,** 6625–6636
47. Schneider, H. R., and Issinger, O.-G. (1989) *Biochim. Biophys. Acta* **1014,** 98–100
48. Schneider, H. R., Mieskes, G., and Issinger, O.-G. (1989) *Eur. J. Biochem.* **180,** 449–455
49. Marmack, M. D., Olson, M. O. J., Busch, H. (1979) *Biochemistry* **18,** 3381–3386
50. Schröder, H. C., Wenger, R., Ugarkovic, D., Friese, K., Bachmann, M., and Müller, W. E. G. (1990) *Biochemistry* **29,** 2368–2378
51. Sun, X. J., Rothenberg, P., Kahn, C. R., Backer, J. M., Araki, E., Wilden, P. A., Cahill, D. A., Goldstein, B. J., and White, M. F. (1991) *Nature* **352,** 73–77