

Immunological demonstration of the accumulation of insulin, but not insulin receptors, in nuclei of insulin-treated cells

(immunocytochemistry/immunoblotting/growth regulation/gene expression)

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ABSTRACT Although insulin is known to regulate nuclear-related processes, such as cell growth and gene transcription, the mechanisms involved are poorly understood. Previous studies suggested that translocation of insulin or its receptor to cell nuclei might be involved in some of these processes. The present investigation demonstrated that intact insulin, but not the insulin receptor, accumulated in nuclei of insulin-treated cells. Cell fractionation studies demonstrated that the nuclear accumulation of ^{125}I -labeled insulin was time-, temperature-, and insulin-concentration-dependent. Electron microscopic immunocytochemistry demonstrated that the insulin that accumulated in the nucleus was immunologically intact and associated with the heterochromatin. Only 1% of the ^{125}I -labeled insulin extracted from isolated nuclei was eluted from a Sephadex G-50 column as ^{125}I -labeled tyrosine. Plasma membrane insulin receptors were not detected in the nucleus by immunoelectron microscopy or when wheat germ agglutinin-purified extracts of the nuclei were subjected to PAGE, electrotransfer, and immunoblotting with anti-insulin receptor antibodies. These results suggested that internalized insulin dissociated from its receptor and accumulated in the nucleus without its membrane receptor. We propose that some of insulin's effects on nuclear function may be caused by the translocation of the intact and biologically active hormone to the nucleus and its binding to nuclear components in the heterochromatin.

Several laboratories have demonstrated that extracellular insulin affects nuclear processes including mRNA efflux (1), protooncogene expression (2), transcription of tyrosine aminotransferase (3) and phosphoenolpyruvate carboxykinase (4) genes, cell growth (5), and phosphate incorporation into the nuclear envelope (6) and nuclear lamins (7). The first step leading to these effects is the binding of insulin to receptors in the plasma membrane. The remaining events are not understood. In particular, it is not known whether insulin binding to receptors generates signals leading to these nuclear-related events or whether insulin is internalized and interacts directly with components of the nucleus. Miller showed (8) that microinjection of insulin into the cytoplasm of *Xenopus laevis* oocytes increased RNA and protein synthesis. Also, insulin added to isolated nuclei of oocytes stimulated RNA synthesis, suggesting a direct biological effect of insulin on nuclear function.

Although some earlier studies reported that insulin could bind to isolated nuclei (9–11) or accumulate in nuclei of intact IM-9 lymphocytes (12) and rat hepatocytes (13), other studies (14, 15) questioned those findings. This laboratory has provided (16) evidence that receptor-mediated internalization of insulin resulted in a time- and temperature-dependent nuclear accumulation of ^{125}I -labeled insulin and ferritin-labeled insulin

in 3T3-L1 adipocytes. The ferritin-labeled insulin was associated with the heterochromatin near nuclear pores. The presence of both ^{125}I and ferritin in the nucleus in that study suggested that intact insulin was present in the nucleus. A subsequent study using photoaffinity-labeled ^{125}I -labeled insulin suggested that the receptor might be translocated to hepatocyte nuclei along with insulin (17).

These two studies left unanswered two important questions. (i) Was the insulin in the nucleus intact and, therefore, potentially biologically active? (ii) Was the insulin-receptor complex translocated to the heterochromatin when the hormone was not covalently linked to the receptor and, therefore, might the receptor's activated tyrosine kinase be involved in the nuclear effects of insulin? The results of the present study indicated that intact insulin, but not the insulin receptor, accumulated in nuclei of insulin-treated cells.

MATERIALS AND METHODS

Cell Culture and Isolation of Intact Nuclei. H35 rat hepatoma cells and NIH 3T3 fibroblasts transfected with the human insulin receptor gene [HIR 3.5 cells (18)] were cultured as described (5, 18). Suspended cells were incubated with ^{125}I -labeled insulin as described (16) or under conditions described below. Cells were washed and resuspended in 6 ml of nuclear isolation buffer (NIB) containing 100 mM KF, 30 mM sodium phosphate, 25 mM Mops, 5 mM EDTA, 5 mM EGTA, 1 mM *N*-ethylmaleimide, 1 mM Na_3VO_4 , 1 mM phenylmethylsulfonyl fluoride, 2.5 μM pepstatin A, 100 μM leupeptin, and 1% Triton X-100. The cells were homogenized and centrifuged at $700 \times g$ for 10 min. The pellet was resuspended in 2 ml of NIB, diluted into 13 ml of 2.24 M sucrose in NIB, and centrifuged for 60 min at $50,000 \times g$. The pellet was resuspended in 1 ml of 0.25 M sucrose in 10 mM sodium phosphate (pH 7.4). ^{125}I -labeled insulin associated with the nuclear fraction was determined in a γ counter. Electron microscopy of nuclear fractions (data not shown) revealed that the pellet consisted entirely of intact nuclei without a nuclear envelope or membrane contamination.

Immunoelectron Microscopy. HIR 3.5 cells or H35 hepatoma cells ($\approx 10^6$ cells per ml) were incubated at 37°C for 5 or 90 min with 100 ng of insulin per ml or for 90 min in the absence of insulin in Krebs-Ringer/Mops buffer (128 mM NaCl/5 mM KCl/5 mM NaH_2PO_4 /1.5 mM MgSO_4 /1.5 mM CaCl_2 /25 mM Mops) with 1% bovine serum albumin and 2 mM glucose (pH 7.4). The cells were washed, centrifuged at $150 \times g$ for 5 min and fixed in 4% (wt/vol) paraformaldehyde in 0.1 M sodium phosphate (pH 7.4). The cells were dehydrated in ethanol and embedded in LR White resin (London Resin). Colloidal gold was prepared as described (19). Polyclonal anti-insulin IgG (Chemicon) and anti-insulin receptor antibodies [A410 (20)] were coupled to 8-nm or 15-nm gold particles, respectively, and purified as described (21). Con-

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trol colloidal gold complexes were made from nonimmune IgG. Thin sections mounted on nickel grids were floated on drops of isotonic phosphate-buffered saline (pH 7.4), with 1% bovine serum albumin for 20 min and then incubated with the gold-labeled complexes for 4 hr at room temperature. After washing with 10 mM sodium phosphate (pH 8.2), the sections were counter-stained with aqueous uranyl acetate and observed with the electron microscope.

Extraction of Nuclei, Gel Filtration, PAGE, and Immunoblotting. HIR 3.5 cells or H35 hepatoma cells were incubated with 100 ng of insulin per ml for 90 min at 37°C. Isolated nuclei were digested with nucleases (22) and solubilized in 6.6 M urea/3 M acetic acid/0.2% Triton X-100. The extract was applied to a 1.6 × 43 cm Sephadex G-50 column and material was eluted with 1 M acetic acid. For PAGE, nuclease-treated (22) nuclei were solubilized in 1% SDS/50 mM Hepes, pH 7.4 and applied to a wheat germ agglutinin chromatography column (23) to partially purify insulin receptors, if present. Insulin receptors were purified from whole-cell extracts using an identical procedure. The glycoprotein-enriched eluates from cells or nuclei were subjected to SDS/PAGE (7.5% polyacrylamide gels) under reducing conditions (24). Proteins were electrotransferred to Immobilon membranes (Millipore) and immunostained with anti-receptor antibodies. Antibody binding was detected by ¹²⁵I-labeled protein A autoradiography on Kodak X-OMAT AR film.

RESULTS

Analysis of Insulin Accumulation in Nuclei. The nuclear accumulation was time-, temperature-, and insulin-concentration-dependent (Fig. 1). H35 hepatoma cells were incubated for up to 3 hr at 4°C or 37°C with ¹²⁵I-labeled insulin (100 ng/ml; Fig. 1a) or for 1 hr at 37°C with ¹²⁵I-labeled insulin (5–200 ng; Fig. 1b). Nuclear accumulation of ¹²⁵I-labeled insulin increased linearly with time at 37°C but at 4°C no significant nuclear accumulation was observed (Fig. 1a). Insulin accumulation was linear at concentrations of 5–50 ng/ml (Fig. 1b) but increased at concentrations >50 ng/ml even though total and intracellular insulin reached steady state at 50 ng of insulin per ml (data not shown). Similar time-, temperature-, and concentration-dependent results were obtained with HIR 3.5 fibroblasts (data not shown).

Immunoelectron Microscopic Analysis. Accumulation of immunologically intact insulin or insulin receptors in the nucleus was detected using gold-labeled antibodies. HIR 3.5 cells or H35 hepatoma cells were incubated in the absence of insulin for 90 min (control) or in the presence of insulin (100 ng/ml) for 5 or 90 min at 37°C. Gold-labeled anti-insulin IgG was used to detect insulin in thin sections of the cells. The complex detected insulin in insulin-treated cells but failed to react with cells incubated in the absence of insulin (Table 1). Colloidal gold complexed to nonimmune IgG did not bind to cells. Fig. 2 shows representative electron micrographs of HIR 3.5 cells incubated with 100 ng of insulin per ml for 5 or 90 min, respectively. When cells were incubated with insulin for 5 min, gold-labeled anti-insulin IgG particles were found on the plasma membrane and in endosomes, but no insulin was detected in the nucleus. In contrast, there were numerous gold-labeled anti-insulin IgG particles associated with the nuclear heterochromatin of cells incubated with insulin for 90 min. Similar observations (data not shown) were made with H35 hepatoma cells. Quantitative analysis of both cell types revealed there were few gold-labeled anti-insulin IgG particles associated with nuclei of cells incubated in the absence of insulin or with 100 ng of insulin per ml for 5 min (Table 1). In contrast, insulin was readily detected in the nuclei of both cell types after 90 min of incubation with insulin. The number of gold-labeled anti-insulin IgG particles found in HIR 3.5 was higher than in H35 cells due to the greater number of

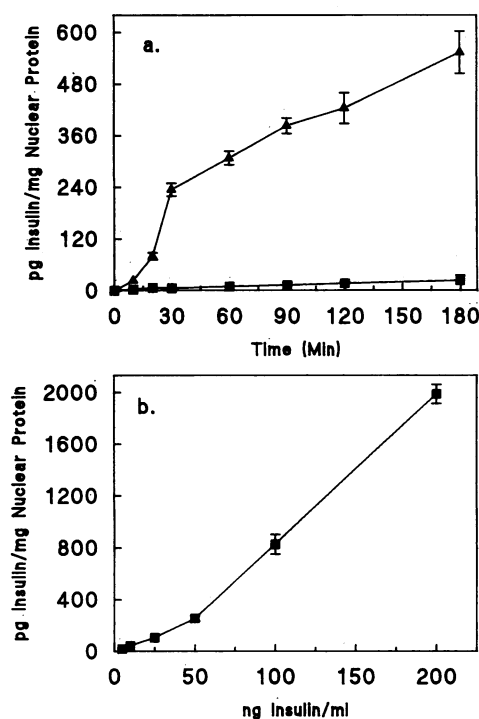


FIG. 1. Time-, temperature-, and concentration-dependent accumulation of ¹²⁵I-labeled insulin in H35 hepatoma cells. Cells (1.2×10^7 cells per ml in Krebs-Ringer/Mops buffer with 1% bovine serum albumin and 2 mM glucose) were incubated with 100 ng of ¹²⁵I-labeled insulin per ml ($\approx 5 \times 10^5$ cpm/ml) for 0–180 min at 4°C (■) or 37°C (▲) (a) or at 37°C for 60 min with 5–200 ng of ¹²⁵I-labeled insulin per ml (b) in a total volume of 30–35 ml. The cells were washed, nuclei were isolated, and ¹²⁵I associated with the nuclear pellet was determined. Results are the mean \pm SD from at least four experiments for each condition.

insulin receptors and greater amount of insulin bound in the HIR 3.5 cells (18).

Gold-labeled anti-insulin receptor antibody A410 (20) (Au-A410 antibody) was used to detect insulin receptors. The total number of Au-A410 antibody particles observed on the plasma membrane and microvilli and in endosomes in 50 thin-sectioned HIR 3.5 cells or H35 hepatoma cells was 286 and 92, respectively. This difference is consistent with the difference in the number of insulin receptors in the two cell types. Nontransfected fibroblasts bound negligible amounts of Au-A410 antibody compared to the cells transfected with the human insulin receptor (data not shown). In addition, the specificity of Au-A410 antibody for the insulin receptor was further demonstrated by incubating sections with unlabeled

Table 1. Immunoelectron microscopic detection of insulin or insulin receptors associated with cell nuclei

Cell type	Incubation conditions		Particles per 50 thin-sectioned nuclei	
	Insulin, ng/ml	Time, min	Au-IAb	Au-A410
HIR 3.5	0	90	14	13
	100	5	21	11
	100	90	321	9
H35 hepatoma	0	90	11	9
	100	5	12	10
	100	90	107	8

Au-IAb, colloidal gold-labeled anti-insulin IgG complex; Au-A410, colloidal gold-labeled anti-insulin receptor IgG complex. These data are results of five and four experiments for each condition using HIR 3.5 cells and H35 hepatoma cells, respectively.

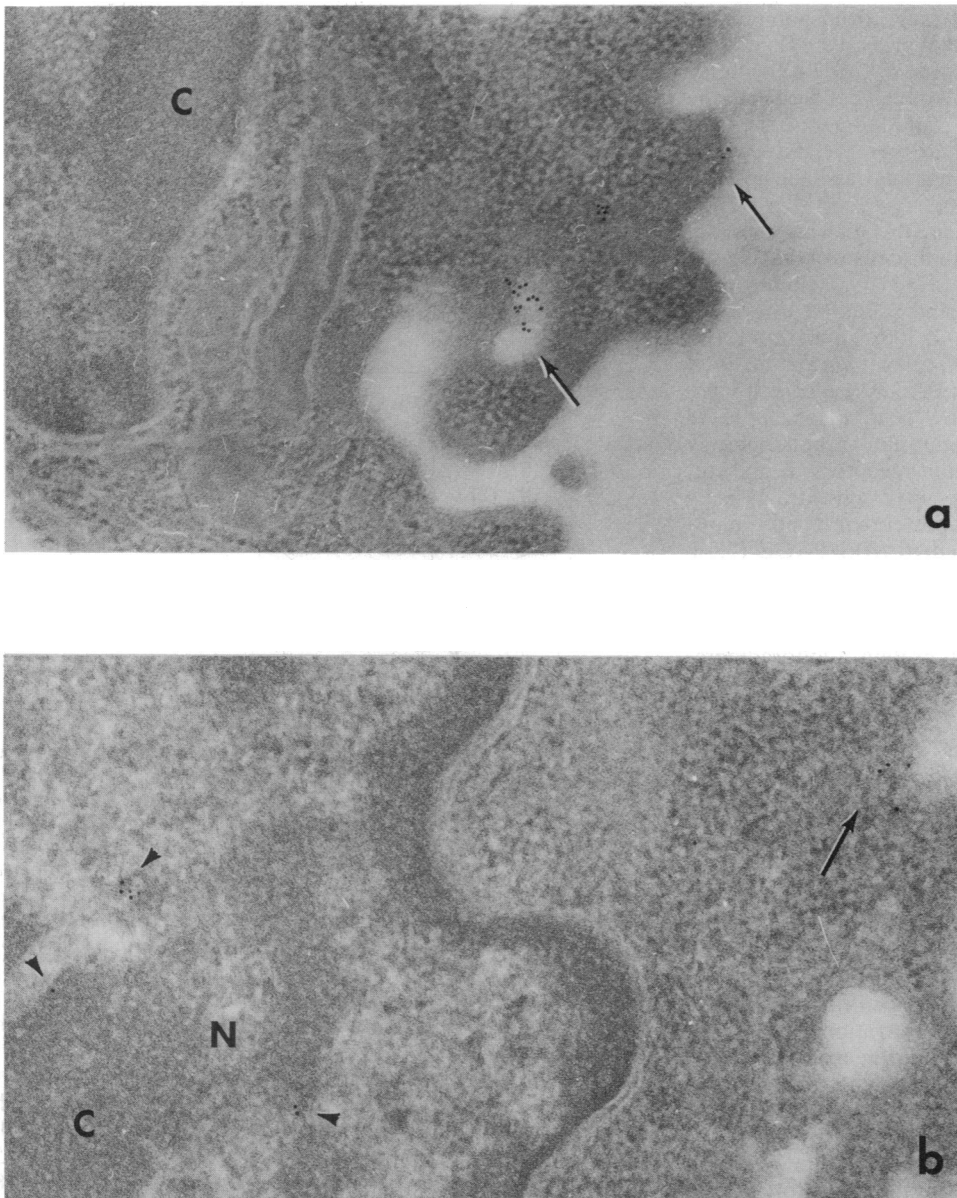


FIG. 2. Immunoelectron microscopic localization of insulin in HIR 3.5 cells. Cells (10^6 cells per ml) were incubated at 37°C for 5 min (a) or 90 min (b) with 100 ng of insulin per ml and prepared for electron microscopy. (a) After a 5-min incubation, insulin (arrows) was detected on the plasma membranes and in endosomes but not in the nucleus. (b) After a 90-min incubation, large numbers of gold-labeled anti-insulin antibody particles (arrowheads) were observed in the nucleus (N), primarily associated with the heterochromatin (C). ($\times 68,000$.)

anti-human insulin receptor antibody (RPN.538, Amersham) prior to incubating the sections with Au-A410 antibodies. Anti-human insulin receptor antibody prevented $>98\%$ of the binding of Au-A410 antibody to insulin receptors on HIR 3.5 cells. In contrast, the anti-human insulin receptor antibody, which does not bind to the rat insulin receptor, did not affect the binding of Au-A410 antibody to the H35 hepatoma cell. Colloidal gold complexes prepared with nonimmune IgG did not bind to the cells.

The distribution of insulin receptors was similar in both nontreated (Fig. 3a) and insulin-treated (Fig. 3b) HIR 3.5 cells. Insulin receptors were detected on the plasma membrane and microvilli and in endosomes, but no Au-A410 antibody was found over the nuclei. Quantitative analysis of the Au-A410 antibody particles in the nuclei of HIR 3.5 cells and H35 hepatoma cells showed there was no difference between control or insulin-treated cells nor between cells incubated with insulin for 5 or 90 min (Table 1). The low number of Au-A410 antibody particles observed in the nucleus regardless of incubation condition or cell type suggested that these particles represented a low-level background association of the gold complex with the nucleus rather than an insulin-induced translocation of receptors from the plasma membrane to the nucleus.

Analysis of Nuclear Extracts by Sephadex G-50 Chromatography and Immunoblotting. Nuclear extracts were analyzed by Sephadex G-50 chromatography and immunoblotting in an attempt to detect intact insulin or an insulin-induced translocation of insulin receptors to the nucleus, respectively. As shown in Fig. 4, only about 1% of the recovered ^{125}I in the extracts of the isolated nuclear fraction was degraded insulin (peak III). The ^{125}I eluting from the column was almost equally divided between two peaks corresponding to intact insulin (peak II) and a high molecular weight peak (peak I) that eluted at the void volume.

Anti-insulin receptor antibodies recognizing either the β [AbP5 (25)] or both α and β [L1-6 (26)] subunits were used for immunodetection of electroblotted proteins. The ability of the anti-receptor antibodies to bind to potential insulin receptors in nuclear extracts after the extraction and solubilization procedures was assessed. Lectin-purified insulin receptors (23) from HIR 3.5 and H35 whole cell extracts were subjected to the same extraction protocols as the isolated nuclei. As shown in Fig. 5, two antibodies, AbP5 (lanes 1–4) and L1-6 (lanes 5 and 6), recognized receptor subunits prepared from whole-cell extracts of H35 hepatoma (lane 1) or HIR 3.5 cells (lanes 3 and 5). The detection of the receptors purified from the whole-cell extracts demonstrated that the

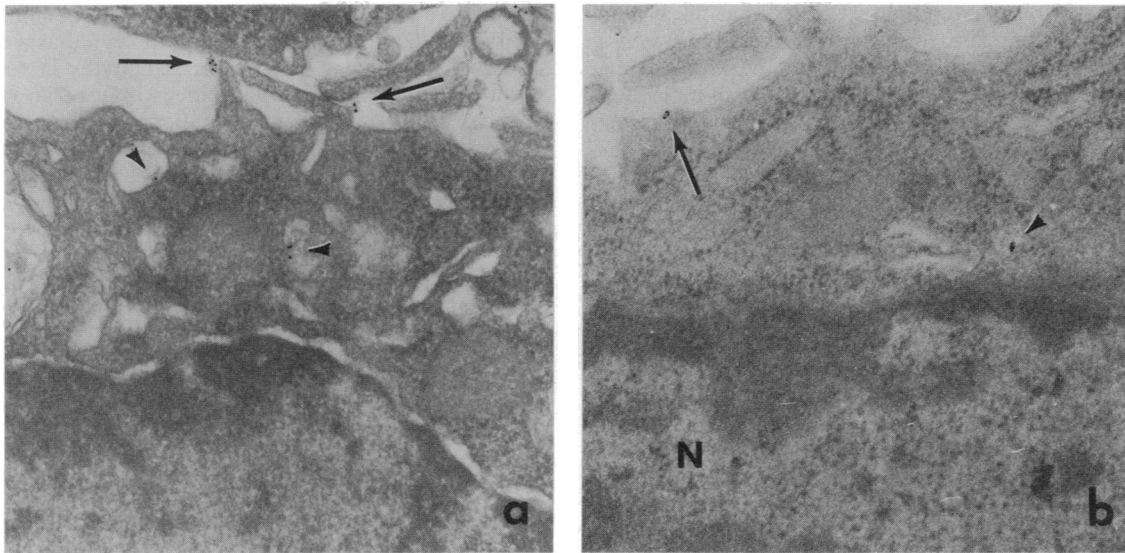


FIG. 3. Immunoelectron microscopic localization of insulin receptors in HIR 3.5 cells. Cells were incubated at 37°C for 90 min in the absence (a) or presence (b) of 100 ng of insulin per ml and prepared for electron microscopy. Gold-labeled anti-insulin receptor (Au-A410) antibody particles were observed on the plasma membrane (arrows) and in endosomes (arrowheads). In the micrographs shown no gold particles were found in the nucleus (N) (see Table 1 for quantitative analysis). The distribution of the gold particles was similar in both control and insulin-treated cells. ($\times 34,000$.)

extraction procedure did not adversely affect the immunogenicity of the receptor subunits. Insulin receptors were not detected in extracts of nuclei from H35 hepatoma (lane 2) or HIR 3.5 cells (lanes 4 and 6) incubated for 90 min with 100 ng of insulin per ml.

These negative findings were not caused by analyzing an insufficient amount of nuclear extract, since we had first determined the minimum detectable concentration of receptors based on the total number of receptors in whole-cell extracts of H35 or HIR 3.5 cells. The potential number of receptors in the nuclear extract was then estimated from the amount of ^{125}I -labeled insulin associated with the nuclear extract assuming a 1:1 molar ratio of insulin to receptor. Sufficient wheat germ eluate from the nuclear extracts was used so that $<10\%$ of the expected receptor concentration would have been detected. These data corroborate the immunoelectron microscopic findings that the insulin receptor does not accumulate in the nucleus.

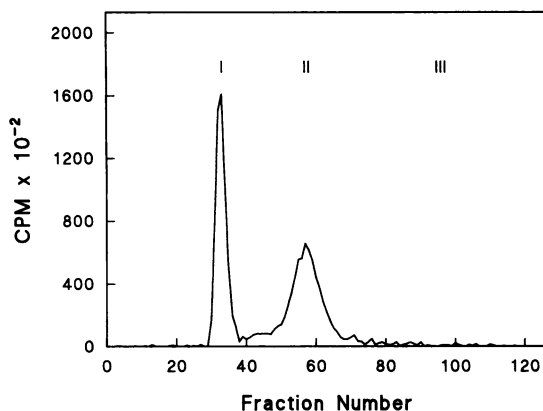


FIG. 4. Sephadex G-50 chromatography of ^{125}I -labeled insulin extracted from isolated nuclei. Cells were incubated for 60 min with ^{125}I -labeled insulin (100 ng/ml) as described in Fig. 1. Isolated nuclei were prepared, extracted, and chromatographed. Radioactivity was determined in 0.9-ml fractions of the eluate. These data are from a single experiment; similar results were observed in four additional experiments. I, void volume; II, intact insulin; III, iodotyrosine.

DISCUSSION

The purpose of this study was to address two unresolved questions. (i) Do intact, and potentially biologically active, insulin accumulate in the nucleus? (ii) Is the insulin-receptor complex, with a potentially activated tyrosine kinase, translocated to nuclei? The present study provided evidence that intact immunologically reactive insulin accumulated in the nuclei of target cells. The gold-labeled anti-insulin antibody was not found in significant numbers over cells not treated with insulin, indicating the specificity of the gold-labeled complex for insulin. Although it is possible, but highly unlikely, that the polyclonal anti-insulin antibody might bind to epitopes of partially degraded insulin molecules, Sephadex G-50 chromatography demonstrated that the ^{125}I -labeled insulin associated with isolated nuclei was not degraded. These data provide strong evidence that the insulin translocated to and associated with the nucleus was intact.

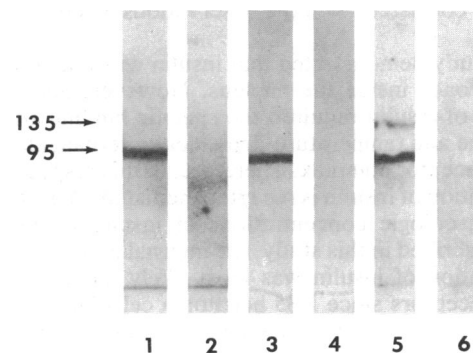


FIG. 5. Immunoblot analysis of insulin receptors in HIR 3.5 and H35 cell and nuclear extracts. HIR 3.5 cells or H35 hepatoma cells were incubated with 100 ng of insulin per ml for 90 min at 37°C and the nuclei were isolated, extracted, and processed for immunoblotting. Lanes: 1-4, anti-insulin receptor antibody AbP5; 5 and 6, anti-insulin receptor antibody L1-6; 1 and 2, whole-cell and nuclear extracts, respectively, of H35 hepatoma cells; 3 and 5, whole-cell extracts of HIR 3.5 cells; 4 and 6, nuclear extracts from HIR 3.5 cells. These results are from a single experiment representative of four experiments.

Sephadex G-50 chromatography revealed that the insulin associated with the nucleus was equally divided between a peak that was eluted as intact insulin and a peak containing a high molecular weight complex. A similar high molecular weight peak has been reported (27) by many investigators after insulin incubation of cells or plasma membranes and has usually been attributed to insulin-receptor complexes. However, since we failed to detect internalized plasma membrane receptors in the nuclei by two techniques, immunoelectron microscopy and immunoblotting, the high molecular weight peak may represent ^{125}I -labeled insulin complexed with components of the nuclear heterochromatin.

In this study we did not detect plasma membrane-like or -derived insulin receptors in nuclei of insulin-treated cells by either immunoelectron microscopy or immunoblotting. The absence of the insulin receptor in the nucleus suggests that insulin must dissociate from the receptor prior to entering the nucleus. This conclusion differs from that obtained using photoaffinity-labeled insulin (17), which suggested that the insulin-receptor complex was translocated to the nucleus. These differences could be attributed to differences in the processing of crosslinked and receptor-bound insulin. (i) In the studies using the photoaffinity-labeled insulin, the insulin-receptor complex was generally restricted to the periphery of the nucleus. Although those observations were consistent with those made using ferritin-labeled insulin (16), the limited resolution of autoradiography made it difficult to determine whether the insulin-receptor complex was in fact in the nuclear heterochromatin or associated with the nuclear envelope or other structures outside the nucleus. (ii) In the present study, nuclear accumulation of ^{125}I -labeled insulin was readily detectable after 30 min of incubation and continued in a linear fashion for at least 3 hr. Nuclear association of the photoaffinity-labeled ^{125}I -labeled insulin was also detected at 30 min; however, maximum and steady-state levels were reached by 60 min (17). The different time courses may be due to cell-specific differences. However, we now believe that, in the studies using the photoaffinity analog, the hormone-receptor complex may have been detected at an intracellular processing site where insulin dissociation and subsequent translocation of the hormone to the nucleus and recycling of the receptor to the cell surface should have occurred. Crosslinking insulin to the receptor would have prevented the dissociation of insulin but not the recycling of the receptor (28) thereby resulting in an apparent steady state at the dissociation site outside the nucleus. This scenario would be consistent with the observations made in these two studies.

This study demonstrated that insulin was not bound to its receptor once inside the nucleus. However, nuclear accumulation of insulin required the specific binding and subsequent time and temperature-dependent internalization of the insulin-receptor complex. Therefore, in this respect, nuclear accumulation of insulin is receptor-mediated. Despite the use of pharmacologic concentrations of insulin in the experiments described in this study, the internalization and nuclear accumulation of insulin was most likely mediated through insulin receptors since H35 hepatoma cells lack insulin-like growth factor I receptors (29) and the number of insulin-like growth factor I receptors on the transfected HIR 3.5 cell is orders of magnitude lower than the number of insulin receptors (18).

The association of the gold-labeled anti-insulin antibody with the nuclear heterochromatin in the present study agreed with the localization of ferritin-labeled insulin reported (16). This observation is particularly interesting since the edges of the condensed chromatin are reportedly the most active sites of gene transcription (30) and this is one of the nuclear

processes affected by insulin (3, 4). The demonstration by Miller (8) that insulin injected into the cytoplasm or added to isolated nuclei of *Xenopus laevis* oocytes increased RNA synthesis suggests that the nucleus may be an intracellular site of insulin action. These studies raise the exciting possibility that receptor-mediated internalization of insulin and the translocation of insulin to the nucleus and its association with components of the heterochromatin may directly affect gene transcription and functions related to cell growth.

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