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Receptor-mediated Nuclear Translocation of Growth Hormone*

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Peter E. Lobie‡§, Hichem Mertani¶, Gerard Morel¶, Orlando Morales-Bustos‡, Gunnar Norstedt‡, and Michael J. Waters‖

From the ‡Centrum för Bioteknik, Karolinska Institutet, NOVUM, Huddinge 141 57, Sweden, ¶CNRS URA 1454, Neuroendocrinologie, Faculté de Medécine Lyon-Sud, 69600 Oullins, France, and the ‖Department of Physiology and Pharmacology, The University of Queensland, Brisbane, Queensland 4072, Australia

We have previously shown that the growth hormone (GH) receptor-binding protein is associated with the nucleus. We show here both by electron microscopy and nuclear isolation that GH is subject to rapid nuclear translocation. The intracellular fate of intravenously injected ^{125}I-bovine growth hormone (bGH) was examined in the rat hepatocyte by electron microscopic autoradiography. The hormone appeared rapidly at the plasma membrane, then sequentially in lysosomal and multivesicular bodies and/or the nuclear membrane before final translocation to the nuclear matrix. Maximal translocation to the nuclear matrix occurred within 30 min of injection. Nuclear translocation of ^{125}I-hGH was also studied by isolation of nuclei from cells stably transfected with cDNAs encoding the GH receptor, GH-binding protein, and a membrane bound but cytoplasmic domain-deficient receptor. Specific internalization and nuclear translocation of hormone only occurred in cells transfected with the full-length receptor. The translocation was rapid and became saturated within 1 h after addition of hormone to the culture media. SDS-polyacrylamide gel electrophoresis of isolated nuclei showed that GH is transported to the nucleus as the intact molecule. Pretreatment of cells with lysosomotropic agents (chloroquine, ammonium chloride, and bacitracin) decreased hormone degradation and increased nuclear translocation of GH. The nuclear translocation of GH was achieved independent of the cytoskeletal system (microtubular, microfilament, and intermediate filament networks). Thus, GH is subject to rapid receptor-dependent nuclear translocation via the endosomal pathway.

Growth hormone (GH)[1] is the major regulator of postnatal body growth (1) and is thought to initiate its biological actions by interaction with a specific plasma membrane bound receptor (2–6). A GH-binding protein (BP) derived by alternate splicing of the GH receptor gene exists in the rat (7) and mouse (8). We (9–11) and others (12) have recently shown that the growth hormone receptor-BP is associated with the nucleus. We proposed that the nuclear association of the GH receptor-BP may be one mechanism by which specific GH-dependent gene transcription is achieved. It has been reported that after binding to a cell surface receptor GH is internalized and is selected for lysosomal degradation (13–16) or exocytosis (17), presumably via the Golgi apparatus (18). However, there is evidence that at least some polypeptide ligands undergo nuclear translocation subsequent to internalization (for review, see Ref. 19). Such is the case for the homologous hormone prolactin (20, 21) as well as for insulin (22, 23), nerve growth factor (24, 25), EGF (26–28), thyrotropin releasing hormone (29, 30), somatostatin-28 (31), Neuropeptide Y (32), platelet-derived growth factor (28), atrial natriuretic peptide (33), gonadotropin releasing hormone (34), FGF (35), interleukin-1α (36), and β and δ interferons (37). However, an early ultrastructural study failed to demonstrate that GH is subject to nuclear translocation (13). In contrast, iodinated bGH administered *in vivo* has been shown to associate with a crude nuclear fraction derived from rat liver and kidney (38) and [^{3}H]hGH incubated with human liver slices was found to be preferentially incorporated into a nuclear fraction (39).

We have therefore been prompted to re-examine the intracellular movement of GH. To achieve this we used quantitive electron microscopic autoradiography and nuclear isolation from cDNA-transfected cells expressing the GH receptor or GHBP (5). We show rapid receptor but not BP-dependent nuclear translocation of growth hormone via the endocytotic lysosomal route.

MATERIALS AND METHODS

Iodination of Hormones—Recombinant hGH was a generous gift from Kabi-Pharmacia (Stockholm, Sweden). Recombinant bGH was a gift from American Cyanamid (St. Louis, MO). hGH was iodinated by the IODO-GEN method (40) and fractionated on a Sepharose G-25 column. bGH was iodinated by the lactoperoxidase method (41) and separated on a Sepharose G-100 column. Specific activity of hGH was 80–153 μCi/μg protein. Specific activity of bGH was 120 μCi/μg protein.

Animal Treatment, Tissue Fixation, and Autoradiography—Ten-week-old male GH-deficient Lewis dwarf rats (42) were housed at room temperature on a 12-h light-dark cycle. Animals were fed a diet of rat pellets and water *ad libitum*. Lewis dwarf rats were chosen as the paucity of endogenous GH would minimize competition with the administered radiolabeled hormone. Animals were anesthetized with ether and injected intracardially with 0.5 ml of PBS containing 4.4×10^7 cpm of ^{125}I-bGH. To test specificity of ^{125}I-bGH uptake, another group of animals were concomitantly injected with 100-fold excess of unlabeled bGH. Rats were killed by cervical dislocation at 2, 5, 15, 30, 60, and 120 min after injection. The upper lobe of the liver was excised, 1-mm^3 pieces were removed and immersion fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 1 h. After fixation the tissues were washed in PBS, postfixed in 1% OsO_4 for 1 h, dehydrated in ethanol, and embedded in Spurr's resin. Serum from the animals was also concomitantly removed and γ counted to determine the rate of removal of hormone from the circulation. For light microscopic autoradiography semi-thin sections were immersed in NTB_2 nuclear emulsion and exposed for 2–4 weeks. Slides were developed in D19 (Kodak) for 2 min and stained with toluidine blue.

For electron microscopic autoradiography ultrathin sections were coated with Ilford L4 emulsion using a loop to obtain a monolayer of

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§ To whom correspondence should be addressed: Karolinska Institutet, Institutionen för Medicinsk Näringslära, NOVUM, Huddinge 141 57, Sweden. Fax: 46-8-7745538.

[1] The abbreviations used are: GH, growth hormone: GHBP, growth hormone-binding protein; EGF, epidermal growth factor; FGF, fibroblast growth factor; hGH, human GH; bGH, bovine GH; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; IL, interleukin.

densely packed silver bromide crystals (43). Ultrathin sections were developed in D19 developer (2 min) and stained as previously described (29, 43, 44).

Quantitative Analysis of Electron Microscopic Autoradiography—The analysis of silver grain distribution was performed at the ultrastructural level. To achieve random selection in a given grid square every cell sectioned through the nucleus was photographed. The percentage of grains related to the organelles was evaluated on micrographs (magnification × 20,000) by the probability circle method. The circular area of 460 nm in diameter having the silver grain at its center encloses the radioactive source with a probability of 95% (45). If only one organelle was localized within the circle, the grain was classified as exclusive. According to the method of Nadler (45) the shared grains were assigned on the basis of probability to only one structure. The corrected number of grains for each organelle was the sum of the exclusive and adjusted shared grains and was expressed as a percentage of the total number of corrected grains. Since the error in random counting is proportional to the square root of the number of counts, up to 500 silver grains were counted for each time point. After obtaining the corrected grain counts the relative concentration of label in each organelle was calculated by dividing the percentage of silver grain by the percentage of area occupied by the organelles (Table I). A grain density greater than one is considered to represent specific labeling (46). Determination of background counts was performed on animals simultaneously injected with 100-fold excess unlabeled bGH. This background count was less than 10% of the grain frequency determined over labeled cells. Background was also estimated over an area of Spurr alone and was found to be less than 3%.

Cellular Transfection of GH Receptor and GHBP cDNA—Rat GH receptor and GHBP cDNAs were cloned into an expression plasmid containing an SV40 enhancer and a metallothionin promoter. The cDNAs were transfected into CHO-K1 cells with lipofectin together with the pIPB-1 plasmid which contains a neomycin-resistance gene fused to the thymidine kinase promoter. Stable integrants were selected using 1000 μg/ml G418. The complete rat GH receptor cDNA (3) coding for amino acids 1–638 was expressed in CHO4–638 or CHOA-638 cells (5). A stop codon was created at amino acid 295 by *in vitro* mutagenesis of GH receptor cDNA to create a membrane bound but cytoplasmic domain-deficient receptor. This cDNA was expressed in CHO-294 cells (5). The rat GHBP cDNA consisted of the extracellular portion of the receptor cDNA (corresponding to amino acids (1–262) ligated to a cDNA sequence corresponding to the carboxyl-terminal 17 amino acids of the GHBP (7)). GHBP was expressed in CHO-BP2 cells (5). BRL cells (ATCC) were transfected with the complete rat GH receptor cDNA inserted in an expression vector containing the human cytomegalovirus enhancer and promoter (pcDNA1) (6).

Hormone Internalization, Degradation, and Nuclear Uptake in Transfected Cells—For hormone internalization and degradation assays CHO cells were grown to confluence in 6-well plates in Ham's F-12 medium supplemented with 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. For estimation of hormone nuclear uptake, cells were similarly grown to confluence in 100-mm diameter dishes. When hormone internalization was compared to hormone nuclear uptake both assays were performed in 100-mm diameter dishes. BRL cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. Confluent cells were incubated in serum free medium for 12 h before addition of ^{125}I-hGH (approximately 150,000 cpm/well). For BP2 cells the media was changed immediately before hormone addition to prevent interference from soluble GHBP in the media. Nonspecific or non-receptor-mediated uptake was estimated by the addition of 10 μg/ml unlabeled hGH to the media. For estimation of hormone internalization the media was removed, the cells were washed once with PBS, cell surface bound hormone was removed by washing the cells with 5 ml of PBS-HCl (pH 3) for 1 min (47), the cells were washed twice with 5 ml of PBS, solubilized with 0.1 M NaOH, 1% SDS, scraped, and transferred to tubes for γ spectrometry. Cell surface receptor was estimated by incubation of cells at 4 °C for 4 h with ^{125}I-hGH and nonspecific binding determined by the addition of 10 μg/ml unlabeled hGH. Degraded GH is released into the media as iodotyrosine (16). Hormone degradation was estimated by trichloroacetic acid precipitation (10%) of media and quantitation of radioactivity in the supernatant. Rapid isolation of nuclei was performed similarly as previously described (48). The nuclei isolated were free from membrane and cellular organelle contamination (Fig. 4, *a* and *b*). Cells were washed 3 times in PBS before removal with a rubber policeman. Cells were centrifuged at 800 × *g*, the supernatant removed, resuspended in a nuclear isolation buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM benzamidine hydrochloride, 3 mM aminoacetonitrile hydrochloride, and 0.5% Nonidet P-40. Extracts were vortexed for 10 s, placed on ice for 5 min, centrifuged at 400 × *g*, the supernatant removed and the process repeated twice more. Pellets were counted on an LKB γ spectrometer.

Electron Microscopy of Isolated Nuclei—To verify the purity of nuclei isolated by our protocol we subjected the nuclear fraction to electron microscopic analysis. Nuclei were resuspended and fixed in 0.1 M cacodylate buffer (pH 7.2) containing 1% glutaraldehyde, 3% paraformaldehyde, and 5% sucrose for 4 h at 4 °C. The nuclei were then pelleted by centrifugation and stored in 0.1 M cacodylate buffer at 4 °C. The pellets were postfixed with 1% osmonium tetroxide in veronal acetate buffer (pH 7) for 1 h, dehydrated in a graded ethanol series, and embedded in Araldite epoxy resin. Sections were stained with uranyl acetate and lead citrate.

SDS-Polyacrylamide Gel Electrophoresis of Nuclear Translocated ^{125}I-hGH—To determine the molecular mass of nuclear translocated ^{125}I-hGH we subjected isolated nuclei as prepared above to SDS-PAGE. Nuclei isolated from CHO4–638 and CHOA-638 cells and iodinated hormone were boiled in SDS sample buffer with dithiothreitol and electrophoresed on a 9% acrylamide gel. Gels were dried and autoradiographed with Kodak AR-5 film in an x-ray cassette with intensifying screens at −85 °C.

Drug Treatments—Colchicine, vinblastine, vincristine, demecolcine, acrylamide, cytochalasin B, cytochalasin D, chloroquine, ammonium chloride, and bacitracin were obtained from Sigma. Cytochalasin B and D were dissolved in ethanol. Cells were cultured and processed as described above and exposed to all drugs (except demecolcine) for 1 h prior to the addition of ^{125}I-hGH (approximately 150,000 cpm). Cells were exposed to demecolcine for 2 h (49) before nuclear uptake was commenced. Colchicine, vinblastine, vincristine, cytochalasin D, and

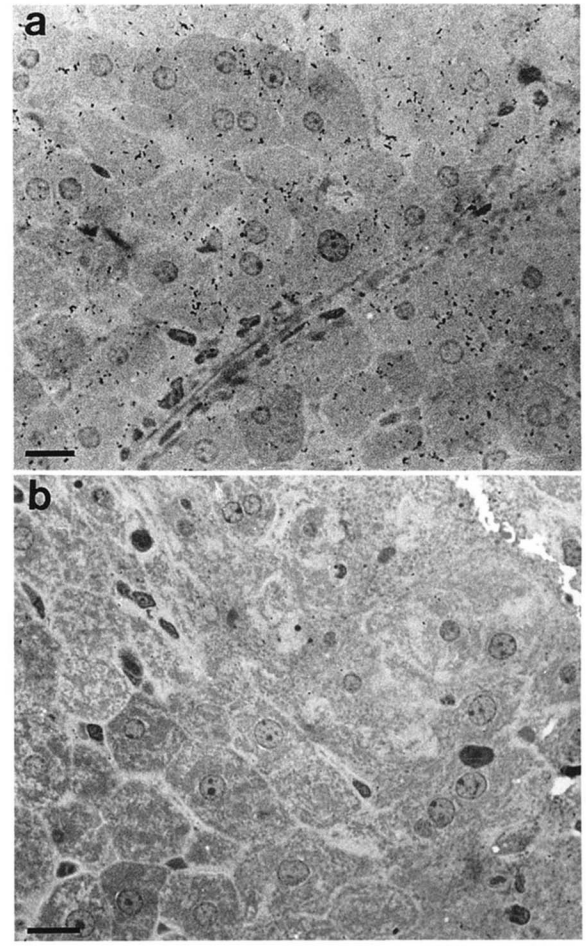

FIG. 1. **Specificity of cellular uptake of ^{125}I-bGH *in vivo*.** Light micrographs are derived from semithin sections of rat liver 50 min after injection of ^{125}I-bGH. Semithin sections were obtained and autoradiographed as described under "Materials and Methods." Sections were exposed for 30 days. *a*, liver from rat injected with ^{125}I-bGH; *b*, liver from rat injected concommitantly with ^{125}I-bGH and 100-fold excess of unlabeled bGH.

phalloidin were each used at 10 μM, demecolcine at 5 μM, cytochalasin B at 20 μM, acrylamide at 5 mM, chloroquine at 10 mM, ammonium chloride at 50 mM, and bacitracin at 5 mg/ml. The cells were in the continued presence of the drugs for the duration of the hormone processing experiments.

Immunofluorescence for Cytoskeletal Integrity—Cells were grown on coverslips in 6-well plates and treated exactly as described above in terms of serum deprivation and drug treatment. Cells were fixed for 10 min in 4% paraformaldehyde, PBS (pH 7.4) at room temperature and washed with PBS (pH 7.4). The following reagents directed against cytoskeletal components were used: (*a*) a mouse anti-rat brain β-tubulin monoclonal antibody for demonstration of the microtubular network (Sigma; dilution 1:50); (*b*) mouse anti-pig eye lens vimentin for demonstration of the intermediate filament network (Sigma; dilution 1:50); and (*c*) rhodamine phalloidin for demonstration of the microfilament network (Sigma; 5 μg/ml). Cells were permeabilized with PBS, 0.1% Triton X-100 for 1 min and further washed with PBS, 1% bovine serum albumin. Nonspecific IgG binding was prevented by incubation with 10% normal sheep serum in PBS, 1% bovine serum albumin for 1 h. Cells were incubated for 12 h at 4 °C with the appropriate dilution of primary antibody, washed 4 times with PBS, and once with PBS, 1% bovine serum albumin, incubated 60 min with fluorescein isothiocyanate-conjugated second antibody (Amersham; sheep anti-mouse IgG; dilution 1:50) washed 3 times with PBS, and mounted in glycerol. For demonstration of the microfilament network, permeabilized cells were incubated with rhodamine phalloidin for 12 h at 4 °C and washed 3 times in PBS.

Statistics—All data are expressed as mean ± S.E. Data were analyzed using the two-tailed *t* test or ANOVA. Results were considered significant at the 5% level.

RESULTS

Cellular Uptake of ^{125}I-bGH into Rat Hepatocytes—To study the *in vivo* cellular uptake of GH we injected ^{125}I-bGH intravenously into male Lewis dwarf rats and examined the hepatocytic subcellular localization of the hormone by electron microscopic autoradiography at 2, 5, 15, 30, 60, and 120 min after injection. Somatogenic specificity was achieved by use of bGH as opposed to hGH which is both somatogenic and lactogenic (50). We also concommitantly followed the loss of GH from the circulation by measurement of serum radioactivity. A sharp decline was first observed between 2 and 5 min after injection followed by a slower loss to 120 min, presumably due to complex formation with the plasma GH-binding protein (51). Autoradiographic specificity was demonstrated by concommitant injection of the animal with 100-fold excess of unlabeled bGH. The specificity is demonstrated in Fig. 1, *a* and *b*, where the density of signal is significantly reduced by excess unlabeled hormone. Grain density (percentage of total grains/percentage of total cell area) over the different cellular compartments was calculated. The area occupied by each cellular compartment is given in Table I. The rough endoplasmic reticulum with the cytoplasmic matrix occupied the greatest area (37.2%), followed by the glycogen area (26.4%) and the mitochondrial area (19.5%). None of the other compartments constituted more than 8% of the total cellular area. The percentage of silver grains counted over various cell compartments are summarized in Table II. To evaluate the specific labeling pattern the grain density was determined for each compartment. Quantitive analysis revealed that seven compartments (plasma membrane, cytoplasmic matrix including the rough ER, Golgi apparatus, lysosomes, mitochondria, nuclear membrane, and nuclear matrix) displayed specific labeling (grain density > 1; Ref. 46). Glycogen and lipid droplet compartments were not specifically labeled (Table II). The different cellular compartments displayed differential kinetic association with ^{125}I-bGH. Silver grains were initially localized to the plasma membrane (Figs. 2*a* and 3*A*) (within 2 min after injection) and declined gradually until 60 min in this compartment with a slight recovery at 120 min. The lysosomal compartment (Figs. 2*c* and 3*B*) displayed biphasic labeling with maxima at 5 and 60 min after injection. Specific labeling of the lysosomal compartment was not observed at 2 and 15 min. Specific labeling of the Golgi apparatus was also observed (Fig. 2*b* and 3*D*). Labeling of the mitochondrial compartment was consistently high (around 25% of total silver grains) at all time points examined (Figs. 2*b* and 3*C*). The labeling of the nuclear membrane (Figs. 2*d* and 3*e*) was specific at 5 min, attained a maximum value at 30 min, then declined and remained constant 60 and 120 min after injection. Silver grains were also associated with the nuclear pore. Specific labeling of the nuclear matrix (Figs. 2*d* and 3*f*) was first observed 30 min after injection. Within the nucleus, silver grains were most frequently detected at the euchroma-

TABLE I
Relative percentage of area occupied by each cellular compartment in rat hepatocyte

Cellular compartment	Relative surface area
	%
Plasma membrane and cytoplasm near plasma membrane[a]	6.1
Golgi apparatus	0.3
Lysosome	0.8
Cytoplasmic matrix and rough endoplasmic reticulum	37.2
Glycogen area	26.4
Mitochondrion	19.5
Lipid droplets	0.2
Nuclear membrane	1.5
Nuclear matrix	7.2

[a] This cytoplasm area corresponds to an annulus equal in width to the radius of the probability circle.

TABLE II
Relative percentage of total grains counted and grains densities for cellular organelles of rat hepatocytes 2–120 min after injection of ^{125}I-bGH

Cellular compartment	2 min		5 min		15 min		30 min		60 min		120 min	
	%	GD[a]	%	GD	%	GD	%	GD	%	GD	%	GD
Plasma membrane and cytoplasm near plasma membrane	24.9	4.1	22.5	3.7	16.1	2.6	12.6	2.1	11.7	1.9	16.6	2.7
Golgi apparatus	0.0	0.0	0.2	0.7	0.4	1.3	0.7	2.3	1.1	3.7	0.2	0.7
Lysosome	0.0	0.0	2.6	3.3	0.8	1.0	1.4	1.8	3.0	3.8	1.4	1.8
Cytoplasmic matrixand endoplasmic reticulum	16.8	0.6	35.1	1.3	19.3	0.7	26.6	1.0	21.4	0.8	25.2	0.9
Glycogen area	23.8	0.9	11.1	0.4	24.5	0.9	18.9	0.7	18.8	0.7	18.6	0.7
Mitochondria	28.2	1.4	20.8	1.1	30.3	1.6	21.3	1.1	32.7	1.7	25.7	1.3
Lipid droplets	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	1.0	0.0	0.0
Nuclear membrane	0.7	0.5	2.6	1.7	2.4	1.6	4.9	3.3	2.2	1.5	2.3	1.5
Nuclear matrix	5.6	0.8	5.7	0.8	6.2	0.9	13.6	1.9	8.9	1.2	10.0	1.4
Number of silvergrains counted	446		423		466		486		462		429	

[a] GD, grain density: percentage of silver grain in one particular compartment/percentage of this cellular compartment area (see Table I).

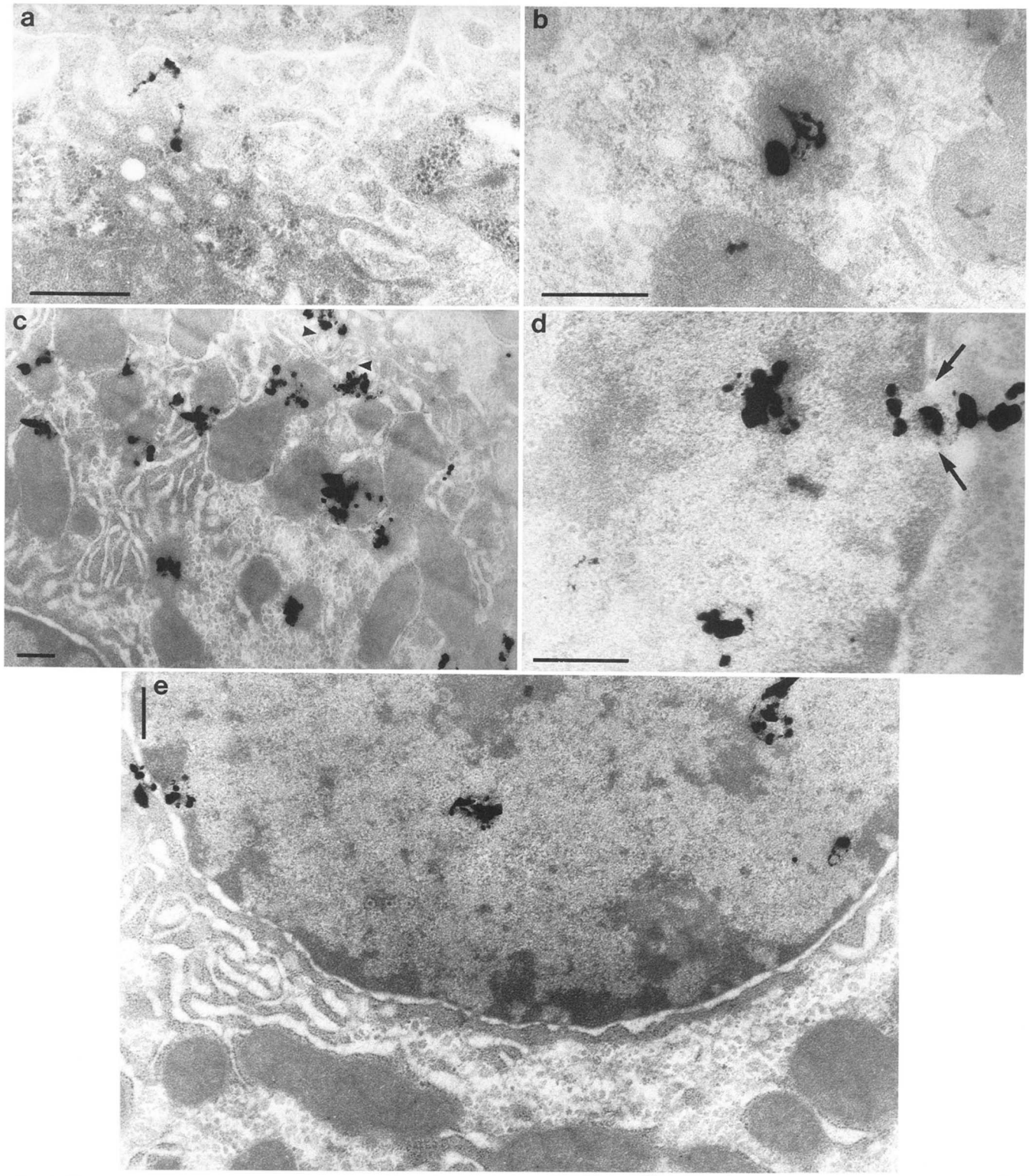

FIG. 2. **Electron microscopic autoradiograms of hepatocytic internalization of ^{125}I-bGH.** Autoradiograms were prepared as described under "Materials and Methods." *a*, silver grains localized over the plasma membrane 2 min after injection of ^{125}I-bGH. *b*, silver grains localized over lysosomal bodies 5 min after injection of ^{125}I-bGH. *c*, silver grains localized over the Golgi apparatus, cytoplasmic matrix, and mitochondria 15 min after injection of ^{125}I-bGH. *d*, silver grains observed over nuclear membrane particularly nuclear pore (*arrows*) and nuclear matrix. *e*, silver grains localized over the nuclear membrane and nuclear matrix 60 min after injection of ^{125}I-bGH. Magnification bar equals 0.5 μm.

tin-heterochromatin junction.

Internalization and Nuclear Uptake of ^{125}I-hGH in GH Receptor- and GHBP cDNA-transfected Cells—GH receptor-transfected CHO4–638 cells were previously shown to internalize hormone to a considerably greater extent than the untransfected parental cell line (52). We wished to determine if the receptor could also mediate hormone nuclear uptake (the purity of the isolated nuclei is demonstrated in Fig. 4, *a* and *b*). We therefore first examined the time course of internalization and compared this to nuclear uptake. Both internalization and nuclear uptake proceeded rapidly but the rate of accumulation of ^{125}I-hGH in the nucleus was slower than the rate of internalization (Fig. 5*A*). Also, the times at which saturation of internalization and nuclear uptake was achieved was different.

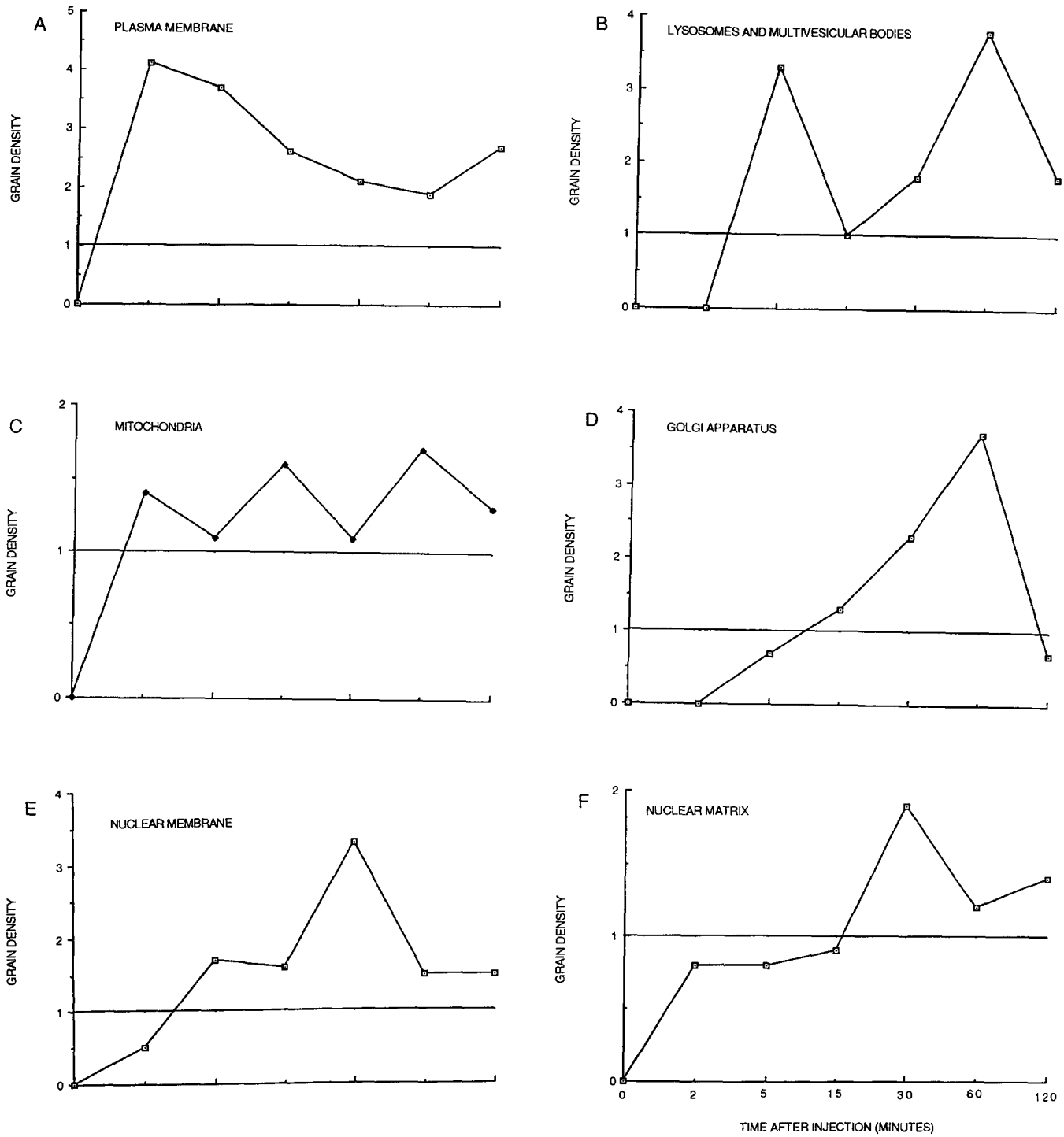

FIG. 3. **Kinetic analysis of the intracellular fate of internalized ^{125}I-bGH in the rat hepatocyte determined by electron microscopic autoradiography.** Grain density (percentage of total grains/percentage of total cell area) was determined as described under "Materials and Methods." Grain density values above one are considered significant. (*A*) plasma membrane; (*B*) lysosomal/multivesicular bodies; (*C*) mitochondria; (*D*) Golgi apparatus; (*E*) nuclear membrane, and (*F*) nuclear matrix.

Maximum internalization of hormone was achieved at 2 h, whereas hormone accumulation in the nucleus was saturated within 1 h (Fig. 5*A*). Maximum nuclear uptake of hormone represented approximately 20% of total internalized hormone. With the continued presence of ^{125}I-hGH there was a slight decrease in both total hormone internalized and hormone accumulation in the nucleus with time (to 8 h). It has previously been reported that internalization of hormone is temperature dependent and no internalization occurs at 4 °C (Ref. 52 and references therein). Similarly, when cells were incubated with ^{125}I-hGH at 4 °C no specific nuclear uptake of hormone was observed despite ample cell surface receptor binding. Thus hormone uptake to the nucleus is a specific receptor-mediated phenomenom. We verified this observation by use of 2 more cell lines stably transfected with cDNA for the full-length receptor. Results for CHOA-638 cells were similar but greater in magnitude to that obtained for CHO4–638 cells under the same conditions (Fig. 5, *B* and *C*). These observations were not due to membrane contamination as no nuclear uptake (when normalized to total cellular binding) was observed in cells transfected

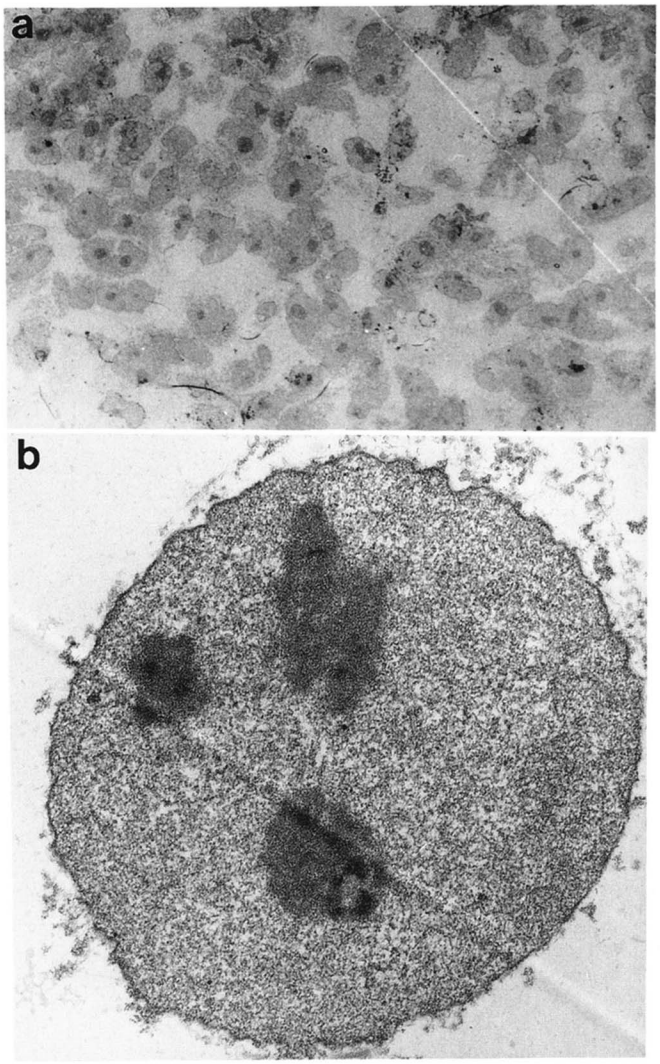

FIG. 4. **Electron micrograph of nuclei purified from CHO4–638 cells as described under "Materials and Methods".** *a*, low magnification photomicrograph of the nuclear preparation. Many nuclei are morphologically distorted and show spillage of nuclear content. No other cellular organelles were present in the preparation. *b*, high magnification photomicrograph of the nuclear preparation to demonstrate lack of membranous contamination.

with a membrane bound but cytoplasmic domain-deficient receptor (CHO-294: Ref. 5) (data not shown). This cell line has previously been demonstrated to bind GH without internalization (52). Since the electron microscopic observations were performed in rat liver we also examined a rat liver cell line transfected with the full-length receptor (BRL4–638). Again similar results were obtained with approximately 20% of internalized hormone associated with the nucleus at 1 h (data not shown). We also wished to determine if the GHBP could mediate either internalization or nuclear uptake of the hormone. Thus experiments were performed with CHO cells transfected with the GHBP containing the hydrophilic carboxyl terminus (BP2). Neither internalization nor nuclear uptake (quantity or rate) was altered in comparison to the parental cell line (Fig. 5, *B* and *C*). Thus the BP in isolation is not involved in these processes. No significant differences in trichloroacetic acid soluble radioactivity in the media was observed between the different CHO cell lines.

Molecular Mass of Nuclear Translocated hGH—It has previously been demonstrated that GH is susceptible to partial proteolysis by endosomal fractions *in vitro* (53). We wished to determine whether intact GH or a GH fragment was translocated to the nucleus. We therefore isolated nuclei from CHO4–638 and CHOA-638 cells incubated in the presence of ^{125}I-hGH for 90 min as described above, solubilized, and subjected the nuclei to SDS-PAGE. As is observed in Fig. 6, ^{125}I-hGH of the same molecular mass (22,000) as the added hormone was also extractable from the nuclear fraction. Radioactive degradation products running at the gel front were observed in all samples analyzed including the added hormone. Thus hGH is transported to the nucleus as the intact molecule.

Effect of Lysosomotropic Agents on Hormone Nuclear Uptake—One of the known target organelles for GH within the cell is the lysosome, where it undergoes energy-dependent degradation (13). When ^{125}I-GH is used the degraded hormone is released into the media as trichloroacetic acid soluble iodotyrosine (16). Lysosomotropic agents (chloroquine and ammonium chloride) have previously been demonstrated to decrease the degradation of ^{125}I-GH (14). Since the majority of early internalized hormone is directed either to the lysosomal compartment or to the nucleus we wished to determine the effect of prevention of degradative processing on nuclear uptake of GH. Both 10 mM chloroquine and 50 mM ammonium chloride were effective in decreasing hormone degradation in both receptor-transfected cell lines (CHO4 and CHOA) (Fig. 7*A*). This decrease in degradation led to an apparent increase in the amount of internalized hormone as described previously (Refs. 14 and 17, and references therein). Chloroquine produced a small decrease in cell surface receptor expression. Nuclear uptake of hormone was significantly increased by treatment of the cells with these two lysosomotropic agents (Fig. 7*A*). Thus diversion of the hormone from degradative processing allows further nuclear uptake of hormone. In support of this we also found that bacitracin (a known inhibitor of insulin degrading activity (54)) decreased degradative processing of GH and concommitantly resulted in increased nuclear uptake (Fig. 7*B*). This effect was observed in both receptor-transfected CHO cell clones.

Effect of Cytoskeletal Disrupting Agents on Nuclear Uptake of GH—To examine whether the nuclear uptake of GH was mediated by the cytoskeletal system we selectively disrupted the three major components with specific pharmacologic agents (49, 55–59). Thus the microtubular system was disrupted with colchicine, vinblastine, and vincristine, the microfilament system with cytochalasin B, cytochalsin D, and phalloidin and the intermediate filament system with demecolcine and acrylamide. These drugs have well documented effects on the cytoskeletal network (55, 59). We also concurrently measured cell surface receptor and hormone internalization and degradation for control purposes. None of the three microtubular disrupting drugs affected the level of cell surface receptor, hormone degradation, or nuclear uptake, although a significant increase in hormone internalization was observed with colchicine, vinblastine, and vincristine (Fig. 8*A*). That these drugs were pharmacologically active at the utilized concentration is evidenced by morphological changes (cell rounding), the disappearance of the microtubular network with colchicine, and the disappearance of the microtubule network and formation of tubulin paracrystals with vincristine and vinblastine (data not shown). Even higher concentrations (40 μM) of the microtubular disrupting drugs were without effect on the nuclear uptake of GH. Drugs affecting the microfilament system (cytochalasin B, cytochalasin D, and phalloidin) did not significantly affect any of the indices of cellular hormonal processing (Fig. 8*C*) despite gross morphological changes including bulging of the nucleus and complete disruption of the microfilament system (data not shown). Again higher concentrations (100 μM) of the microfilament disrupting drugs were without effect on nuclear uptake of

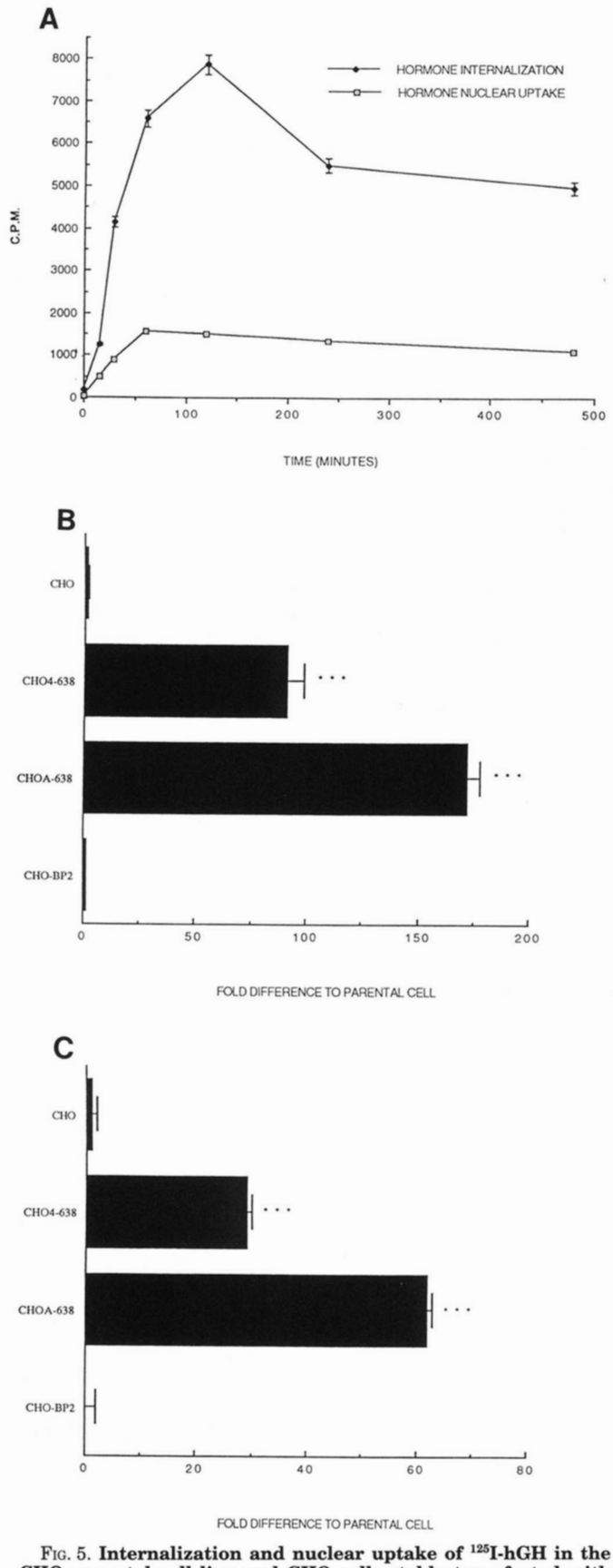

FIG. 5. **Internalization and nuclear uptake of ^{125}I-hGH in the CHO parental cell line and CHO cells stably transfected with the GH receptor and GHBP cDNA.** Estimation of internalized hormone and hormone nuclear uptake was performed as described under "Materials and Methods." Each point is the mean ± S.E. of triplicate estimations. The results shown are representative of three experiments. *A*, time course of ^{125}I-hGH internalization and nuclear uptake in receptor-transfected cells (CHO4–638). Cells were prepared as described under "Materials and Methods." Total added radioactivity was 150,000 cpm per plate. Nonspecific uptake was estimated in the presence of 10 μg/ml unlabeled hGH. *B*, hormone internalization in cells expressing the GH receptor (CHO4–638, CHOA-638), the GHBP (CHO-BP2), and the parental cell line (CHO). Cells were prepared as described under "Materials and Methods." Total added radioactivity was 150,000 cpm per well/plate. Nonspecific uptake was estimated in the presence of 10 μg/ml unlabeled hGH. ***, $p < 0.001$ when compared to parental cell line. *C*, hormone nuclear uptake in cells expressing the GH receptor (CHO4–638, CHOA-638), the GHBP (CHO-BP2), and the parental cell line (CHO). Cells were prepared as described under "Materials and Methods." Total added radioactivity was 150,000 cpm per well/plate. Nonspecific uptake was estimated in the presence of 10 μg/ml unlabeled hGH. ***, $p < 0.001$ when compared to parental cell line.

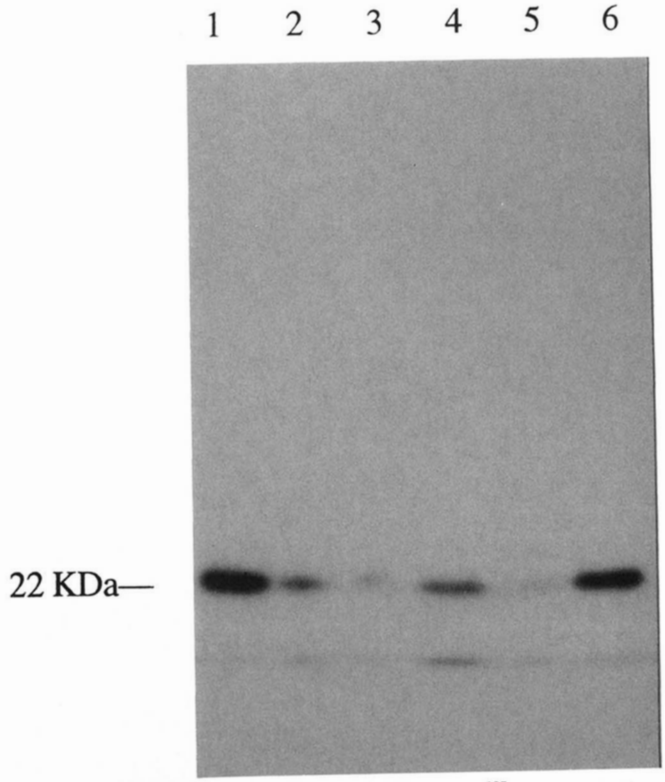

FIG. 6. **SDS-PAGE of nuclear translocated ^{125}I-hGH.** Nuclear translocation was performed as described under "Materials and Methods." Nuclei were isolated from cells (CHO4–638 and CHOA-638) incubated for 90 min at 37 °C in the presence of ^{125}I-hGH (approximately 200,000 cpm) ± excess (10 μg/ml) hGH and subjected to SDS-PAGE. *Lane 1*, ^{125}I-hGH. *Lane 2*, CHO4–638 cell nuclei. *Lane 3*, CHO4–638 cell nuclei when nuclear uptake was performed in the presence of excess unlabeled hGH. *Lane 4*, CHOA-638 cell nuclei. *Lane 5*, CHOA-638 cell nuclei when nuclear uptake was performed in the presence of excess unlabeled hGH. *Lane 6*, ^{125}I-hGH.

hormone. Drugs affecting the intermediate filaments (demecolcine and acrylamide) significantly increased the level of internalized hormone but were without effect on cell surface receptor, hormone degradation, or hormone nuclear uptake (Fig. 8*C*). The pharmacological activity of these drugs was evidenced by disruption of the intermediate filament network (as demonstrated by immunofluorescence for vimentin as previously described by Perrot-Applanat *et al.* (49); data not shown). Drug combinations (demecolcine, 2 μM, and cytochalasin B, 20 μM) resulting in the disruption of all three cytoskeletal networks were without effect on hormone nuclear uptake.

DISCUSSION

We have examined the intracellular movement of GH and found that the hormone is translocated to the nucleus in a receptor-dependent manner. This nuclear translocation was demonstrated both *in vivo* at the ultrastructural level and by

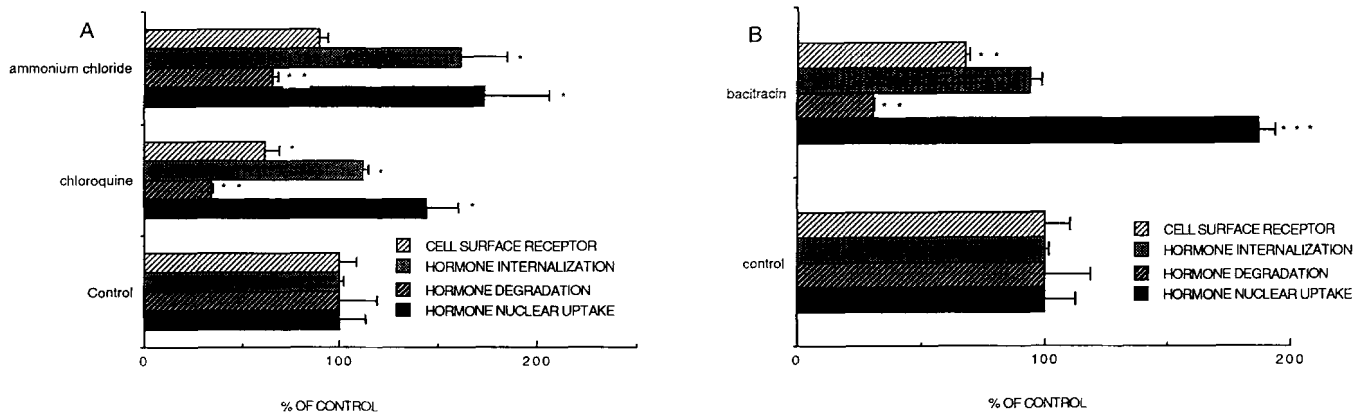

FIG. 7. *A*, effect of lysosomotropic agents on cell surface receptor, hormone internalization, hormone degradation, and hormone nuclear uptake in CHO4–638 cells. All four parameters were measured as described under "Materials and Methods" after exposure to the respective drug (chloroquine, 10 mM, and ammonium chloride, 50 mM). Results are expressed as the percentage of control (untreated CHO4–638) and represent the mean of triplicate determinations with S.D. indicated. Total added radioactivity was approximately 150,000 cpm. *, $p < 0.05$; **, $p < 0.005$, when compared to control values. *B*, effect of bacitracin on cell surface receptor, hormone internalization, hormone degradation, and hormone nuclear uptake in CHO4–638 cells. All four parameters were measured as described under "Materials and Methods" after exposure to bacitracin at 5 mg/ml. Results are expressed as the percentage of control (untreated CHO4–638) and represent the mean of triplicate determinations with S.D. indicated. Total added radioactivity was approximately 150,000 cpm. **, $p < 0.005$; ***, $p < 0.001$, when compared to control values.

use of receptor-transfected cells. The specificity of this translocation is demonstrated by the fact that: (*a*) concommitant injection of excess unlabeled ligand reduces grain density at the ultrastructural level to background; (*b*) co-incubation of GH receptor-transfected cells with excess unlabeled ligand but not with unrelated ligands prevents labeled hormone uptake to the nuclear fraction; (*c*) there are differential kinetics between internalization and nuclear translocation both at the ultrastructural level and with receptor-transfected cells; (*d*) there is saturation of nuclear translocation *in vitro* before saturation of internalization; (*e*) there is no significant specific nuclear uptake in receptor-transfected cells incubated at 4 °C; (*f*) there is no significant specific nuclear uptake in the untransfected parental cell line nor in a cell line expressing a membrane bound but cytoplasmic domain (and internalization) deficient receptor. Thus one intracellular destination of GH after internalization is the nucleus.

The internalization and nuclear translocation pathway utilized by GH is likely to be shared by other polypeptide growth factors. Thus we describe here a receptor-mediated endosomal pathway for the nuclear translocation of GH. This is further substantiated by the fact that lysosomotropic agents increase nuclear uptake of GH in the GH receptor cDNA-transfected cell lines. Moreover, *in vivo* the hormone appears rapidly at the plasma membrane and in endosomal bodies before translocation to the nuclear membrane and finally the nuclear matrix. That the actual translocation is achieved by passage of the hormone via the nuclear pore or by membranovesicular fusion (60) or both is possible. The EM analysis described here suggests that before nuclear translocation, the hormone binds rapidly to the nuclear membrane before slower translocation to the nuclear matrix, presumably through nuclear pores since label was observed to be associated with these structures *in vivo*. It does appear, however, that at least insulin (61), interleukin-1α (36), EGF (26, 62), and prolactin (21) also utilize this endosomal route for nuclear translocation of the hormone. Thus inhibitors of lysosomal hydrolytic activity enhance nuclear accumulation of EGF (27, 62) and IL-1α (36) as is the case here for GH. In this study we observed a rapid nuclear translocation of hormone both *in vivo* at the ultrastructural level and *in vitro*. Translocation of GH in this study occurred in a similar time frame to that previously reported for insulin (22, 23, 63), gonadotropin releasing hormone (34), FGF (35), nerve growth factor (28), atrial natriuretic peptide (33), and gonadotropin-releasing factor (44). However, the time taken for nuclear translocation of other polypeptide ligands varies enormously. The nuclear translocation of EGF continues for 24–48 h after addition of hormone (28, 62) and maximal IL-2 stimulated nuclear translocation of prolactin is attained between 18 and 24 h (21). Similarly somatostatin-28 requires 18 h to enter the nuclear compartment (31).

Nuclear translocation of hormone was observed in GH receptor but not GHBP-transfected cells. Thus nuclear translocation of GH is receptor dependent. This conclusion is concordant with receptor-dependent translocation to the nucleus of epidermal growth factor and nerve growth factor (28). However, whether the receptor is required only for internalization of GH and not intracellular movement of the hormone could not be determined. It is possible that once internalized the hormone-receptor complex could dissociate and the hormone translocate by a receptor-independent pathway, perhaps complexed to the GHBP. It has previously been demonstrated that such a situation exists for insulin (64) and prolactin (65), whereby the hormone-receptor complex is transported to the nuclear periphery but only the ligand is subject to nuclear translocation. Furthermore, mutation of the nuclear localization sequences in the IL-1 receptor do not affect the functional response to hormone (66) and IL-1 is still nuclear localized with most of the cytoplasmic portion of the receptor deleted (67). Some polypeptide ligands (platelet-derived growth factor, FGF, and IL-1) also nuclear translocate due to the presence of a nuclear localization signal in their primary sequence (68–70).

The functional significance of a nuclear GH receptor (9) and nuclear translocation of GH has not yet been demonstrated. Functional activity of other nuclear translocated polypeptide ligands has been clearly demonstrated (*e.g.* Refs. 68 and 71). For example, acidic FGF lacking a putative nuclear localization sequence fails to induce DNA synthesis and cell proliferation at concentrations sufficient to induce intracellular receptor-mediated tyrosine phosphorylation and c-*fos* expression. However, attachment of the nuclear localization sequence from yeast histone 2B to the amino terminus of acidic FGF results in restoration of mitogenic activity (68). A similar phenomenon was observed for prolactin in which enhancement of IL-2 induced proliferation was observed when a chimeric prolactin molecule containing the SV40 nuclear localization sequence was used (71). No canonical nuclear localization sequences (72) are present in GH (73) and thus it is likely that GH arrives in the

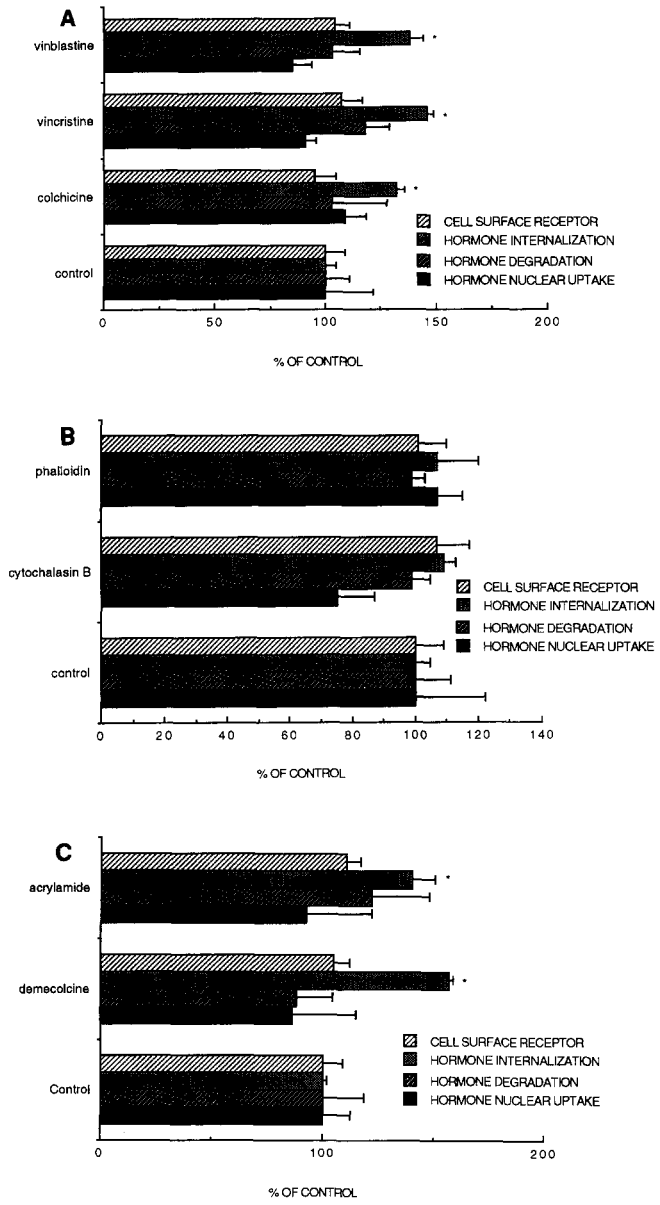

FIG. 8. *A*, effect of microtubular disrupting agents on cell surface receptor, hormone internalization, hormone degradation, and hormone nuclear uptake in CHO4–638 cells. All four parameters were measured as described under "Materials and Methods" after exposure to the respective drug (colchicine, vincristine, and vinblastine at 10 μM). Results are expressed as the percentage of control (untreated CHO4–638) and represent the mean of triplicate determinations with S.D. indicated. Total added radioactivity was approximately 150,000 cpm. *, $p < 0.005$. *B*, effect of microfilament disrupting agents on cell surface receptor, hormone internalization, hormone degradation, and hormone nuclear uptake in CHO4–638 cells. All four parameters were measured as described under "Materials and Methods" after exposure to the respective drug (cytochalasin B at 20 μM and phalloidin at 10 μM). Results are expressed as the percentage of control (untreated CHO4–638) and represent the mean of triplicate determinations with S.D. indicated. Total added radioactivity was approximately 150,000 cpm. *C*, effect of intermediate filament disrupting agents on cell surface receptor, hormone internalization, hormone degradation, and hormone nuclear uptake in CHO4–638 cells. All four parameters were measured as described under "Materials and Methods" after exposure to the respective drug (demecolcine at 5 μM and acrylamide at 5 mM). Results are expressed as the percentage of control (untreated CHO4–638) and represent the mean of triplicate determinations with S.D. indicated. Total added radioactivity was approximately 150,000 cpm. *, $p < 0.05$.

nucleus in conjunction with an associated molecule, most probably the receptor. Indeed, in preliminary experiments we have shown that the GH receptor is capable of rapid ligand-dependent nuclear translocation.[2] Whether the nuclear hormone-receptor complex is involved in transcriptional events or cell cycle regulation or both remains to be determined. It is possible that immediate and common events in signal transduction such as early response gene stimulation are dependent on protein phosphorylation, whereas ligand-dependent gene transcription and cell proliferation may require nuclear translocation of hormone and/or receptor. We have so far obtained no evidence that GH or a GH-GH receptor-BP complex is able to bind to DNA (Spi 2.1 promotor) either directly or indirectly.[3] We are presently investigating whether chimeric GH molecules containing the SV40 nuclear localization sequence display enhanced biological activity.

We did not observe any effect of the disruption of the cytoskeletal system on the nuclear translocation of GH. These results suggest that the cell has access to a nuclear translocation mechanism for GH that does not operate through any of the three main cytoskeletal networks. This is interesting considering the well known interaction between the cytoskeleton and endosomes (*e.g.* Ref. 74). However, early endosomal function does not require an intact microtubular network (75). Since the hormone arrives at the nuclear membrane and the lysosomal compartment simultaneously, both destinations may be mediated by the microtubular independent endosomal pathway. Such cytoskeletal system independent nuclear translocation of proteins has also been reported for the adenovirus E1A protein (59), the 72- (59) and 90-kDa (76) heat shock proteins, and the progesterone receptor (49). Moreover, the GH receptor[4] is not incorporated into vinblastine-induced paracrystals (76) indicative that the receptor is not associated at least with the microtubular network. Nevertheless, we cannot exclude the possibility that the GH receptor may associate with some cytoskeletal component, since the receptor is ubiquinated (77) and ubiquitin is a component of the microtubular network (78). Indeed, the EGF receptor is an actin-binding protein (79) and is known to associate with the cytoskeletal network. Furthermore, transport of the GH receptor from the Golgi apparatus to the plasma membrane has been reported to be dependent on both the microfilamentous and microtubular network (47).

In conclusion, we have shown a receptor-dependent nuclear translocation of GH. At present the functional significance of such an intracellular fate for GH is not known. That nuclear targeting of other polypeptide ligands confers a complete biological response (at least in terms of mitogenesis) lends credence to functionality of intranuclear GH. Whether the intranuclear ligand is only required for cell cycle events or ligand-dependent transcriptional activation needs to be determined. It is possible that the liganded receptor (or the activated unliganded receptor) participates in a transcription complex although we have no evidence to support this contention. Transcription factor p91 (80) has previously been shown to associate directly with the EGF receptor (81). Such direct receptor-transcription factor associations would provide the required transcriptional specificity necessary from shared kinase activation.

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[4] P. E. Lobie and G. Norstedt, unpublished data.

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