Nucleocytoplasmic Transport Is Enhanced Concomitant with Nuclear Accumulation of Epidermal Growth Factor (EGF) Binding Activity In Both 3T3-1 and EGF Receptor Reconstituted NR-6 Fibroblasts

Lian-Wei Jiang and Melvin Schindler

Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824

Abstract. Measurements of nucleocytoplasmic transport of fluorescent-labeled macromolecules were performed in both an EGF-nonresponsive mutant fibroblast line (3T3-NR6) and in the same cell line reconstituted with active EGF receptors derived from rat hepatic membrane fraction. Immunolocalization studies of exogenously incorporated EGF receptors in reconstituted 3T3-NR6 fibroblasts demonstrated predominantly intracellular localization. The EGF receptor constructs also showed EGF-stimulated incorporation of [^{3}H]thymidine, providing biochemical evidence for functional integration of the exogenously supplied EGF receptors into the reconstituted fibroblasts. Additional support for the functional incorporation of receptor may be inferred from the enhanced cellular accumulation of ^{125}I-EGF in cells treated with chloroquine and leupeptin. ^{125}I-EGF binding and transnuclear macromolecular transport measurements in mutant and reconstituted cells, in conjunction with such measurements on nuclei isolated from these cells, provide data consistent with a growth factor/nuclear signaling mechanism dependent on the nuclear acquisition of EGF binding activity from the plasma membrane.

POLYPEPTIDE growth factors and hormones serve as regulatory molecules for cell differentiation and also as initiators of coordinated cellular responses to extracellular metabolic variation (15). Cell type–specific growth factor receptors in the plasma membrane of cells serve as the primary targets and ultimate discriminators for the large number of growth factors in the cellular environment (42, 43, 48). A question central to the mechanism of polypeptide growth factor signaling has been whether ligand-activated plasma membrane receptors (by themselves or in functional aggregates with other effector macromolecules [20, 22, 41]) produce a nuclear response uniquely as a result of either direct translocation to the nucleus (receptor translocation activation mechanism) (12, 17, 25, 27, 32, 33, 46, 47) or indirectly through the intracellular production of secondary messengers (14, 30) initiated by growth factor–mediated receptor kinase stimulation at the plasma membrane or a combination of multiple excitatory paths. An integrating control point for nuclear activation has been suggested to be the transnuclear transport channel defined by the nuclear pore complex (1, 34). It appears likely that growth factor–induced cellular changes would include modifications of bidirectional macromolecular transport through the nuclear pore complex. Previous work has demonstrated significant dose-dependent changes in nuclear macromolecular transport after addition of either EGF or insulin to 3T3 fibroblasts (16). That this type of activation could be related to nuclear receptor occupancy was suggested by demonstrations of enhanced EGF or insulin-induced macromolecular and RNA nuclear transport in isolated rat liver nuclei (2, 32, 36). Although these experiments and others are consistent with an occupancy-dependent effect of growth factor receptors on nuclear function, the question of whether the observed effects are a result of indigenous nuclear growth factor receptors or the migration of ligand-bound plasma membrane receptors to sites in the nucleus has been unclear (25, 46). To pursue this question of receptor localization and transport, we have used a technique developed by Bishayee et al. (3) in which EGF receptors are transferred in a biologically active state from donor hepatic membranes to receptor-deficient fibroblasts (3T3-NR6) (26), resulting in cells that are physically reconstituted with receptors. Such EGF receptor constructs were demonstrated to be responsive to EGF as measured by stimulation of DNA replication and cell division (3). Although molecular biological approaches have recently superseded many physical reconstitution protocols for receptor structure/function analyses (9, 11, 19), the motivation for choosing physical reconstitution approaches was based on the need to create a specific topologically defined placement of the receptor at the plasma membrane. Introduction of an EGF receptor gene into a receptor mutant results in expression and cellular localization at a number of intracellular sites (11), obscuring attempts to discriminate between indigenous nuclear localization of the EGF receptor or nuclear localization as a result of EGF-induced migration of these receptors from the plasma membrane to the nucleus. We now show, using the method of receptor reconstitution

in whole cells, that activation of transnuclear macromolecular transport by EGF is concomitant with plasma membrane–derived EGF binding activity appearing at the nucleus. In addition, nuclei isolated from EGF-nonresponsive cells (3T3-NR6) after coincubation with rat liver EGF receptor–enriched plasma membrane fraction become responsive to EGF-induced enhancement of dextran transport.

Materials and Methods

Reagents

Fluorescein-labeled dextrans (FITC-dextrans) (20,000 and 70,000 mol wt, designated 20K and 70K, respectively), chloroquine, and leupeptin were obtained from Sigma Chemical Co. (St. Louis, MO). Tritiated thymidine (10 Ci/mmol) was obtained from ICN Radiochemicals (Irvine, CA). EGF was from Gibco Laboratories (Grand Island, NY), while ^{125}I-EGF (152 μCi/μg) was obtained from New England Nuclear (Boston, MA). 3T3-1 (3T3 Swiss albino) cells were obtained from American Type Culture Collection (Rockville, MD), while the 3T3-NR6 EGF nonresponsive cell line isolated from murine Swiss albino 3T3 cells was a kind gift of Dr. H. R. Herschman, Laboratory of Biomedical and Environmental Sciences, University of California at Los Angeles, School of Medicine (Los Angeles, CA). Monoclonal anti-mouse EGF receptor antibody, clone 29.1 (mouse ascites fluid), and FITC-conjugated goat anti-mouse IgG were from Sigma Chemical Co.

Cell Growth, Fluorescent Dextran Incorporation, and Receptor Insertion

Cells were normally grown and maintained at 37°C in DME containing 10% FCS in a 10% CO_2 atmosphere, unless otherwise indicated. Cell synchronization and scrape-loading of fluorescein-derivatized dextrans (FITC-dextrans; 20,000 mol wt) was performed as previously described (16, 33). Scrape-loading is a technique used to incorporate FITC-dextrans into the cell cytoplasm to serve as neutral transport probes for the fluorescence photobleaching method of measuring transnuclear transport (16, 33). Cell growth and viability studies suggest that replated, scrape-loaded cells behave normally (16, 33, 40). EGF receptor–deficient 3T3-NR6 cells were replated on 35-mm tissue culture dishes immediately after scrape-loading and incubated in DME containing 2% calf serum at 37°C for 2.5 h. After reattachment and spreading, the cells were ready for EGF receptor insertion. The hepatic EGF receptor fraction used for insertion was isolated from fresh rat livers that were suspended in cold 1 mM $NaHCO_3$ buffer. Plasma membrane fraction was isolated as described by Emmelot et al. (8) with modifications. After sucrose gradient equilibrium centrifugation, the material at the interface and immediately below it was collected, pooled, and diluted approximately fivefold with 1 mM $NaHCO_3$ buffer. After centrifugation at 9,500 rpm for 20 min in a rotor (JA-1; Beckman Instruments, Inc., Fullerton, CA), the plasma membrane pellets were resuspended in buffer at an approximate protein concentration of 20 mg/ml and either stored at −20°C or processed for EGF receptor insertion. The specific EGF binding activity of this fraction was determined to be 0.5–1 pmol of ^{125}I-EGF bound/mg of total protein under standard incubation conditions (60-min incubation with 10 nM ^{125}I-EGF at 20°C). Similar approaches to EGF receptor isolation, showing approximately the same ^{125}I-EGF binding to isolated membranes as described here, have demonstrated that the receptor is fully functional as measured by binding kinetics and EGF-dependent autophosphorylation (18). The EGF receptor insertion experiments were performed as described by Bishayee et al. (3) for mouse hepatic EGF receptor fraction. For nuclear transport measurements, monolayers of 20K FITC-dextran–loaded 3T3-NR6 cells in 35-mm dishes were grown in DME plus 2% calf serum for 2.5 h, and then liver plasma membrane fraction containing ^{125}I-EGF binding activity was added at 0.5 mg membrane protein/ml and incubated for 6 h at 26°C. At the end of incubation, dishes were washed five times with HBSS and Hepes followed by five sequential washes with DME to remove unbound membranes. At maximal insertion (500 μg membrane protein/10^5 cells), $\sim 10^6$ EGF binding sites could be incorporated per 3T3-NR6 cell. The cells were then incubated in DME with 2% calf serum in the absence or presence of various combinations of leupeptin, chloroquine, and EGF. Subsequently, either these cells were assayed for cellular and nuclear EGF accumulation or nuclear transport measurements were performed using the FRAP technique (16, 33). For cell cycle experiments, cells were induced into the quiescent state (G_0) by washing three times with DME and then maintained in DME for 30–38 min. These cells were then scrape-loaded with 20K FITC-dextran and then replated on fibronectin-coated dishes in DME. After 2–3 h, cells were sufficiently spread for transport measurements.

^{125}I-EGF Binding Studies to Whole Cells and Isolated Nuclei

Cellular and nuclear accumulation of ^{125}I-EGF (152 μCi/μg; New England Nuclear) in 3T3-1 in the absence or presence of chloroquine or leupeptin were performed as described by Savion et al. (31) and Pruss and Herschman (26) with some modifications. Confluent 3T3-1 cell cultures ($\sim 5 \times 10^5$ cells/35-mm dish) had their media replaced by fresh DME plus 10% calf serum. Cultures containing lysosomotropic reagents were preincubated in the presence of chloroquine (12 μM) for 2 h or in the presence of leupeptin (100 μg/ml) for 16 h before the addition of ^{125}I-EGF (31). After incubation at 37°C with ^{125}I-EGF for 20 h, the cultures were washed eight times with ice-cold PBS-BSA (1 mg/ml BSA), and then the dishes were incubated on ice for 20–30 min in lysis buffer (15 mM Tris-HCl [pH 7.4], 2 mM EDTA, 0.5 mM EGTA, 60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM β-mercaptoethanol, 0.5% NP-40, and 1 mM PMSF [26, 31]). The cell lysate fraction was collected, and the bound ^{125}I-EGF was quantitated in a liquid scintillation counter (LS 100C; Beckman Instruments, Inc.). Nonspecific binding was determined in the presence of a 20-fold excess of unlabeled EGF. Such binding ranged from 5 to 12% of the total ^{125}I-EGF binding and was subtracted from all values. All assays were performed in duplicate, and the values reported represent the mean. Nuclei for binding studies were prepared in the following manner (31). After collecting the whole cell lysate described above, a monolayer of nuclei remained anchored to the cell culture dish. These nuclei were washed 8–10 times with PBS-BSA. 1 ml of double-distilled water was then added to each 35-mm dish, and the nuclei were removed by gentle pipetting. The resulting nuclei were then layered on an 0.5-ml sucrose cushion (0.5 M) and centrifuged for 15 min at 10,000 g at 4°C. The supernatant was discarded, and the radioactivity in the nuclear pellets was counted as described above for the cell lysate. Electron microscopic analysis of these fractions by Savion et al. (31) showed the absence of lysosomal vesicles, whether or not cells were pretreated with chloroquine, while an acid phosphatase assay demonstrated $\leq$1% of the total acid phosphatase activity in the isolated nuclear fraction. In the case of 3T3-NR6 cells, monolayers of NR6 cells in 35-mm dishes were treated with the membrane fraction for EGF receptor insertion (described above and in reference 3) and then incubated with ^{125}I-EGF as described for 3T3-1 cells.

[^{3}H]Thymidine Uptake Measurements in Whole Cells

Monolayers of cells were grown in 12-well clusters and incubated at 26°C for $\sim$6 h with 500 μg/ml of rat liver plasma membrane fraction in DME containing 2% calf serum. The cells were then washed free of unbound membrane and incubated at 37°C in DME and 2% calf serum for $\sim$24 h. 40 ng/ml of EGF was added to medium and incubated for 12 h. At the end of incubation, 15 μCi/ml of [^{3}H]thymidine was added to cells followed by an additional 12 h of incubation. Incorporated [^{3}H]thymidine was then measured by TCA precipitation. The data represents measurements with the standard deviation indicated by bars.

Fluorescence Measurements: FRAP and Immunolocalization

FRAP was performed as previously described for isolated nuclei and whole cells (16, 33) with the following modification for isolated nuclear measurements. 3T3 nuclei were isolated as described above for ^{125}I-EGF binding studies. Monolayers of nuclei remaining attached to the 35-mm tissue culture plate were equilibrated in 70K FITC-dextran solution for nuclear transport measurements. Repeated bleaching on the same sample was used to serve as an assay for photodamage. Fluorescence immunolocalization of EGF receptor on whole 3T3-NR6 and 3T3-1 parent cells was performed in the following manner. Cells were grown at 37°C in DME containing 10% calf serum in a 10% CO_2 atmosphere. They were then washed several times with PBS and treated for $\sim$1 min with methanol/acetone (3:7 [vol/vol]) at room temperature for fixation. Fixed cells were then air dried and rinsed in PBS. Dried cells were then incubated with the monoclonal

anti-mouse EGF receptor antibody in PBS plus 0.1% BSA (1:200 dilution) for 2 h at room temperature. The cells were then washed three times and incubated with FITC-conjugated goat anti-mouse IgG (1:50). After a thorough washing with PBS, fluorescence stain was observed using an epifluorescence photomicroscope (E. Leitz, Inc., Wetzlar, FRG). Mouse nonimmune serum was used as a control.

Results

^{125}I-EGF Binding to Whole Cells and Nuclei

Experiments to explore the mechanism and cellular pathway of EGF-induced activation of transnuclear transport were initiated by determining EGF binding activity on the cellular and nuclear level. The data presented in Fig. 1 demonstrate steady-state ^{125}I-EGF accumulation for whole 3T3-1 cells (Fig. 1, *A* and *C*) and for nuclei isolated from them (Fig. 1, *B* and *D*). Nuclear accumulation under these conditions was clearly saturable. It is particularly noteworthy that addition of chloroquine (12 μM; Sigma Chemical Co.) (Fig. 1, *A* and *B*) or leupeptin (100 μg/ml; Sigma Chemical Co.) (Fig. 1, *C* and *D*), agents affecting receptor recycling and degradation at different points in the endosomal pathway (6, 7, 13, 23), resulted in significantly enhanced whole cell (cell lysate) and nuclear ^{125}I-EGF accumulation. These results parallel observations by Savion et al. (31), demonstrating that inhibitors of lysosomal hydrolytic activity or endosomal-lysosomal fusion enhance cellular and nuclear accumulation of EGF in cultured bovine corneal endothelial and granulosa cells. A chloroquine-induced enhancement of nuclear localization for EGF receptor was also reported by Murthy et al. (24) using anti-EGF receptor and anti-EGF kinase domain antibodies as probes. Further analysis of EGF binding is presented in Fig. 2, demonstrating that chloroquine and leupeptin can enhance nuclear accumulation from a value of ~2% of total bound ^{125}I-EGF to ~8–10% (Fig. 2, *A* and *B*). Although somewhat difficult to assess due to the low level of bound radioactivity, half-maximal nuclear accumulation appeared to occur between 2 and 5 h. These experiments demonstrate not only that nuclear EGF accumulation occurred but that this activity could be considerably enhanced in the presence of inhibitors of endosomal-lysosomal fusion (chloroquine) (6, 7, 13, 23) or cathepsin B activity (leupeptin) (45).

The half-time for nuclear accumulation of ^{125}I-EGF in the presence of leupeptin (Fig. 2, *B* and *D*) is similar to that observed in the presence of chloroquine. No specific ^{125}I-EGF binding was observed to whole cells or nuclei isolated from them after incubation with EGF-nonresponsive 3T3-NR6 cells. These experiments help to eliminate the possibility of nonspecific trapping, other non-EGF receptor proteins that may associate with EGF, or functional internalization by fluid phase endocytosis. The observation that half-maximal nuclear accumulation of ^{125}I-EGF occurs after ~2–5 h may explain recently reported negative results by Carpentier et al. (4), who do not observe nuclear localization of EGF receptor after only 1 h of incubation. It is important to point out that under similar experimental conditions for measuring EGF binding to fibroblasts, Wiley et al. (45) showed that 92% of EGF associated with responsive human fibroblasts is located in an intracellular compartment. They further showed that ~60% of the internalized ^{125}I-EGF remained in a form that could still bind to EGF receptors.

Reconstitution of EGF Receptor into EGF-nonresponsive 3T3-NR6 Cells

Bishayee et al. (3) demonstrated that EGF receptors could

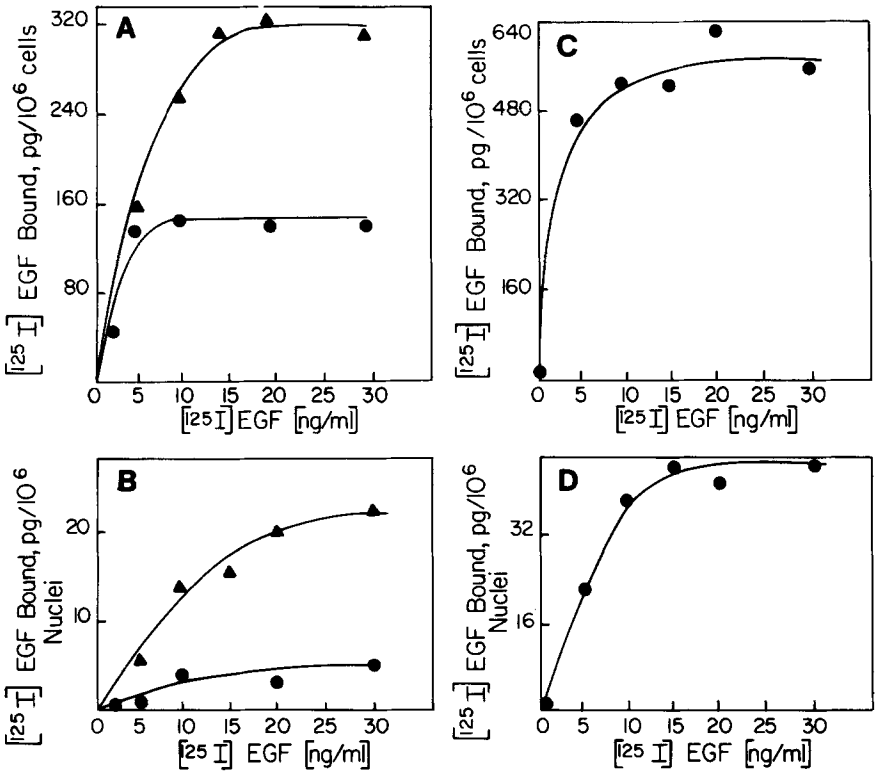

Figure 1. ^{125}I-EGF binding to whole cells and nuclei isolated from 3T3-1 fibroblasts. Cellular and nuclear accumulation studies of ^{125}I-EGF in 3T3-1 cells in the absence and presence of chloroquine or leupeptin were performed as described in Materials and Methods. Binding of ^{125}I-EGF to whole 3T3-1 cells (*A*) and their isolated nuclei (*B*) in the absence (●) and presence (▲) of chloroquine (12 μM). ^{125}I-EGF binding to whole 3T3-1 cells (*C*) and their isolated nuclei (*D*) in the presence (●) of leupeptin (100 μg/ml).

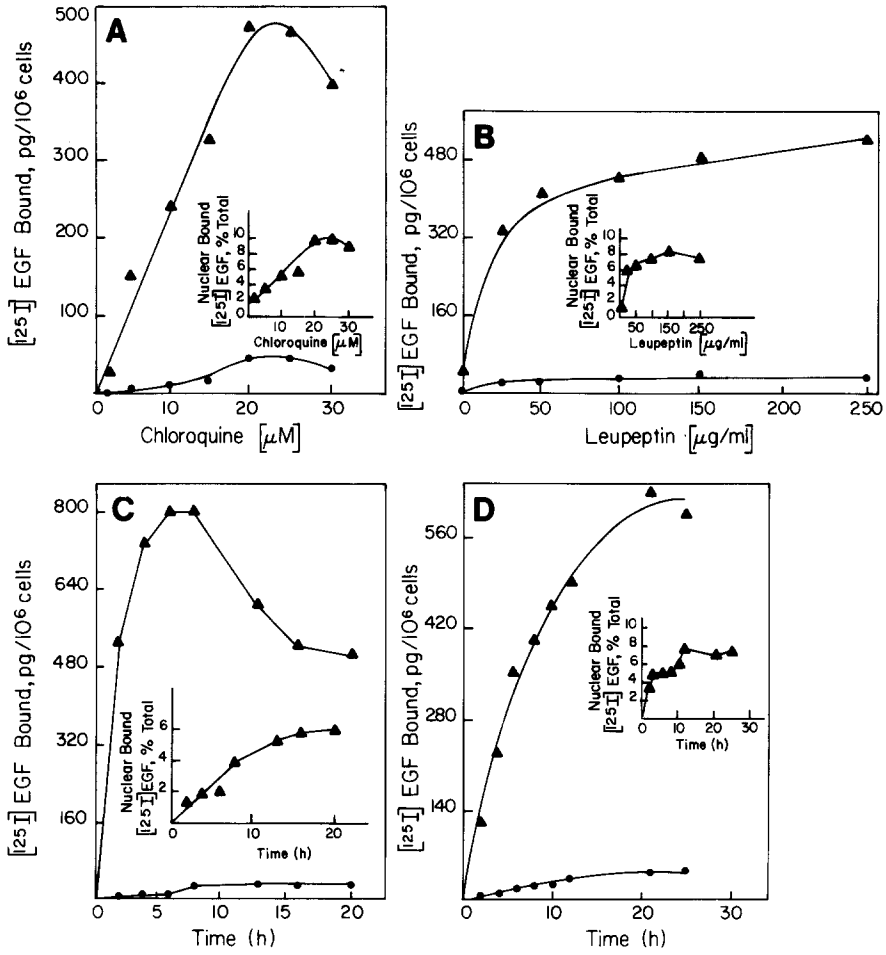

Figure 2. Effect of chloroquine and leupeptin on cellular and nuclear accumulation of ^{125}I-EGF in 3T3-1 fibroblasts. ^{125}I-EGF binding experiments were performed as described in Materials and Methods. ^{125}I-EGF binding in whole 3T3-1 cells (▲) and their nuclei (●) as a function of concentration of chloroqine (*A*) or leupeptin (*B*). Time course of ^{125}I-EGF binding to whole 3T3-1 cells (▲) and their nuclei (●) in the presence of 12 μM chloroquine (*C*) or 100 μg/ml leupeptin (*D*). ^{125}I-EGF concentration was 20 ng/ml. Insert in each panel shows the increase in nuclear accumulation of ^{125}I-EGF as a percentage of the total cell accumulation (whole cell lysate plus nuclear) as a function of chloroquine (*A*) or leupeptin (*B*) concentration and time (*C* and *D*).

be transferred in a biologically active state from donor hepatic membranes to receptor-deficient fibroblast cells (3T3-NR6). Enrichment for EGF receptor insertion when compared with bulk membrane protein from the donor hepatic membranes into the recipient cells was ~19-fold at 26°C (3). A Scatchard analysis of the donor EGF receptor demonstrated an apparent K_d of ~2.4 nM (3). Labeling the donor membrane with a photoreactive derivative of ^{125}I-EGF resulted in the specific radiolabeling of a 170,000-D polypeptide which was identical to the EGF receptor (3). The EGF receptor constructs prepared from the above-mentioned donor hepatic membranes acquired EGF responsiveness as measured by stimulation of DNA replication and cell division (3). Fig. 3 demonstrates the cellular localization of exogenously incorporated rat liver EGF receptor reconstituted as described (Materials and Methods) (Fig. 3, *A* and *B*). This is to be compared with the receptor distribution in parental 3T3-1 cells (Fig. 4, *A* and *B*). The higher levels of fluorescence observed for the receptor construct probably reflects the greater number of cellular receptors (approximately twofold; Table I). The fluorescence micrographs show a similar cellular distribution of EGF receptors when compared with results from other laboratories (11, 24). EGF-nonresponsive 3T3-NR6 demonstrates no cellular EGF receptor labeling (Fig. 3, *C* and *D*), consistent with its nonexpression in this mutant cell line (35) and lack of ^{125}I-EGF binding (Table I). Fig. 4, *C* and *D*, is the nonimmune control for the parental 3T3-1 cell line. The immunolocalization data provide evidence for physical integration of the rat liver EGF receptor and subsequent internalization into the 3T3-NR6 mutant. The results presented in Fig. 5 support the original observations of Bishayee et al. (3) that the exogenously incorporated receptor enables the 3T3-NR6 mutant to respond to EGF as reflected by [^{3}H]thymidine uptake experiments (see Materials and Methods). It can be observed in Fig. 5 *A*, column *2*, that the 3T3-1 parent cell line responds to EGF by enhanced uptake of [^{3}H]thymidine. Treatment of 3T3-1 cells with rat liver EGF receptor fraction in the manner used to reconstitute the 3T3-NR6 mutant did not significantly enhance [^{3}H]-thymidine uptake (Fig. 5 *A*, column *4*). As previously demonstrated, the 3T3-NR6 mutant did not respond to EGF (Fig. 5 *B*, column *2*). After incorporation of exogenously added rat liver EGF receptor fraction, the reconsituted 3T3-NR6 cells showed EGF-mediated enhancement of [^{3}H]thymidine uptake (Fig. 5 *B*, column *4*) to the extent observed for the parent 3T3-1 cell line (Fig. 5 *A*, column *2*). The data presented in Fig. 3 and Table I support the idea that exogenously incorporated rat liver EGF receptor fraction can successfully integrate into the plasma membrane and then undergo internalization with apparent redistribution to intracellular compartments. The sensitivity to chloroquine and leupeptin (Table I) provides further evidence that the incorporation and

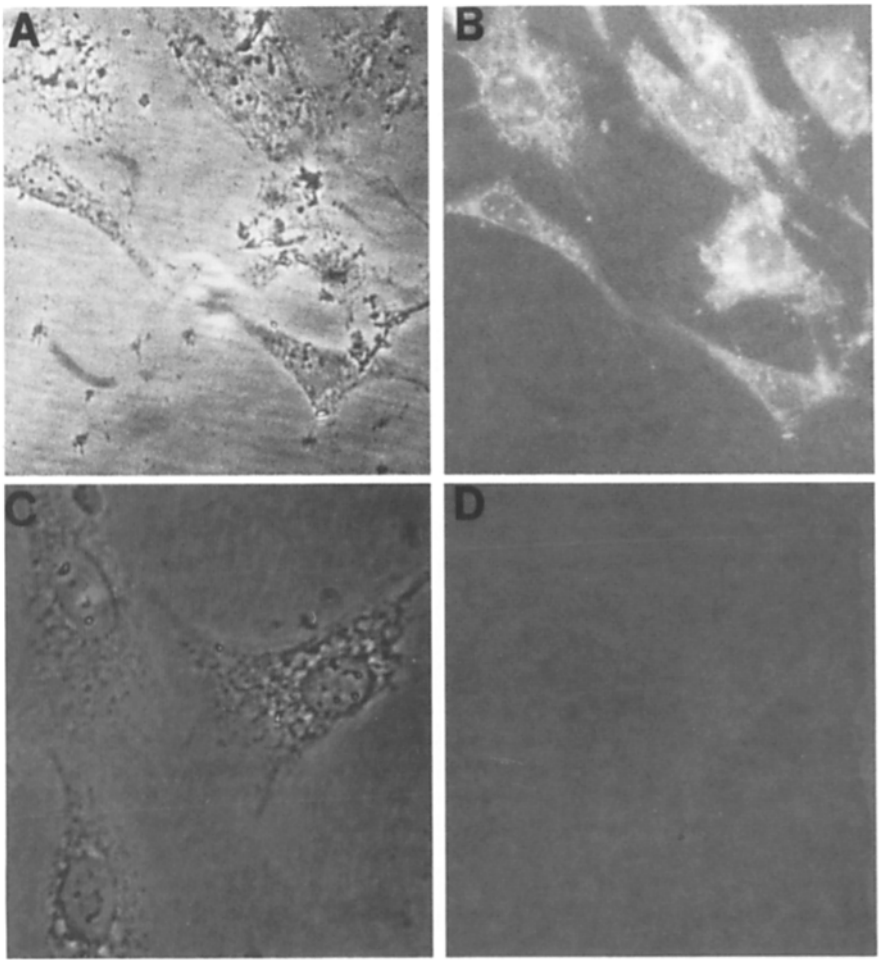

Figure 3. Localization of EGF receptor in 3T3-NR6 EGF receptor constructs as determined by indirect immunofluorescence. Cells grown in monolayer were fixed, processed, and stained for indirect immunofluorescent analysis as described in Materials and Methods. Phase (*A*) and fluorescent (*B*) views of reconstituted cells. Phase (*C*) and fluorescent (*D*) views of non-reconstituted mutant NR6 cells stained as above.

intracellular processing of the exogenously incorporated receptor mimics that of other cell lines under similar conditions of endosomal and lysosomal alkalinization (23, 24, 45).

Comparative Analysis of EGF-mediated Enhancement of Transnuclear Transport in Whole Cells and Nuclei from 3T3-1, 3T3-NR6, 3T3-NR6 EGF Receptor Constructs

Nuclear Transport Measured in Whole Cells. To examine the correlation between the ^{125}I-EGF binding capacity of whole cells, nuclei isolated from them, and nuclear transport, we initiated a series of transnuclear transport experiments to pursue a direct comparison between nuclear accumulation of EGF binding activity and transnuclear transport for 3T3-1, 3T3-NR6, and 3T3-NR6 EGF receptor constructs and nuclei isolated from these cells. A compilation of these results is presented in Tables I and II. In view of our binding assay in which unbound or processed ^{125}I-EGF should be solubilized (38, 45), it is considered likely that EGF binding activity reflects the presence of either functional EGF receptors or EGF receptor in complex with other activated macromolecules (20, 22, 41). An initial view of the data in Table I suggests that nuclear occupancy of ~420 receptors per nucleus or 2.8% of the total cellular bound ^{125}I-EGF in the parental 3T3-1 line is sufficient to maximally enhance nucleocytoplasmic transport in whole cells (approximately threefold). Addition of chloroquine (15 μM) increases nuclear EGF accumulation to a much greater extent (eightfold) than the enhancement observed for whole cell binding. A similar enhancement in binding was observed in the presence of leupeptin (data not shown). No greater increase, however, for transnuclear transport in whole cells is observed after chloroquine treatment. This is consistent with the observation that transport rates in the absence of chloroquine appear to be the maximal rates obtainable in both whole cells under conditions of EGF stimulation or transformation (16). 3T3-NR6 EGF receptor constructs in the presence of EGF demonstrate nuclear EGF binding activity and a significantly enhanced rate for nuclear transport (approximately twofold) in whole cells (Table I). The 3T3-NR6 mutant without EGF receptor displays neither ^{125}I-EGF binding activity for whole cells nor enhancement of nuclear transport in whole cells as a result of EGF addition (Table I). After exposure to chloroquine, the 3T3-NR6 EGF receptor constructs show enhanced nuclear accumulation in a manner similar to the parent 3T3-1 (Table I).

Nuclear Transport Measured in Isolated Nuclei. To examine whether the whole cell measurements of nucleocytoplasmic transport in 3T3-1 and 3T3-NR6 EGF receptor constructs are a reflection of modified transport activity in nuclei isolated from them, dextran transport experiments were per-

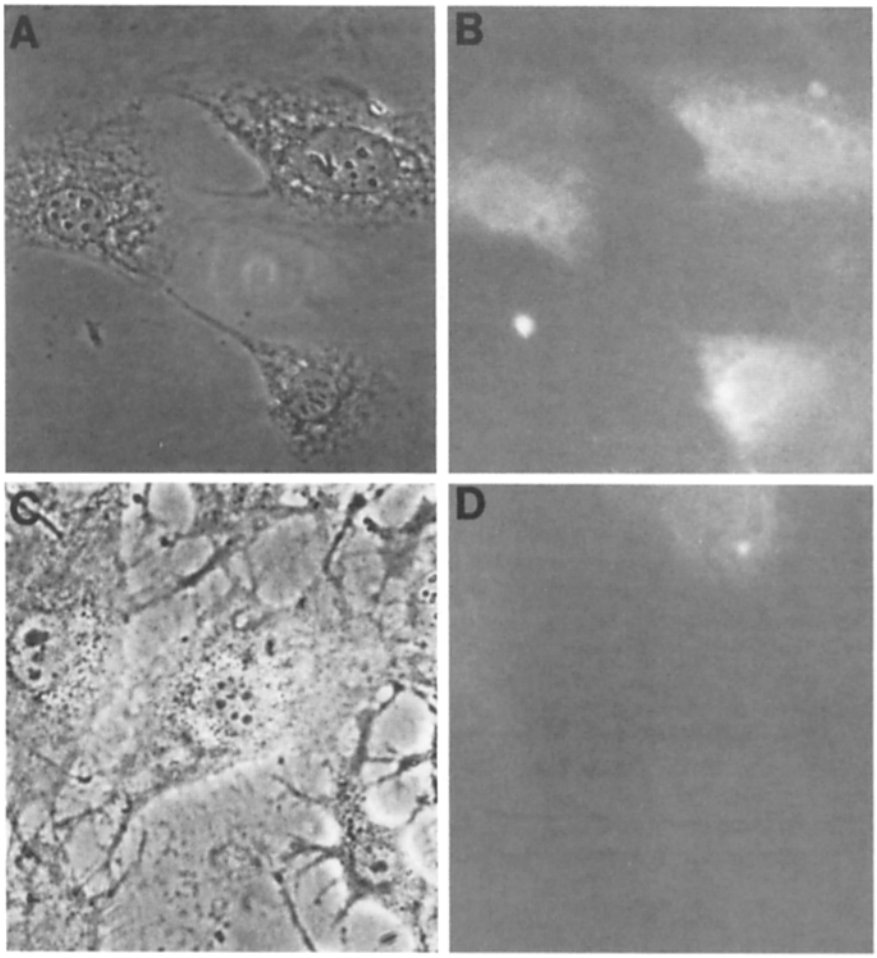

Figure 4. Localization of EGF receptor in 3T3-1 cells as determined by indirect immunofluorescence. Fixing and staining were performed as described in Fig. 3 and Materials and Methods. Phase (*A*) and fluorescent (*B*) views of 3T3-1 cells. Phase (*C*) and fluorescent (*D*) views of 3T3-1 cells probed with nonimmune serum.

Table I. Correlation of Transnuclear Dextran Transport Rate in Whole Cells with Cellular and Nuclear EGF Accumulation

Cell line/treatment	Cellular accumulation of EGF*	Nuclear bound EGF‡	Dextran transport rate§	Change from control
	EGF molecules per cell‖	*EGF molecules per nucleus*‖	$\times 10^3 s^{-1}$‖	%
3T3-1 (parent) (control)	–	–	6.4 ± 1.6 (8) ¶	–
3T3-1 + EGF	15,000 ± 1,600	420 ± 160	19.7 ± 3.0 (19)	+208
3T3-1 + chloroquine (15 μM) + EGF	47,000 ± 5,000	3,400 ± 1,300	18.9 ± 2.7 (13)	+195
3T3-NR6	–	–	7.1 ± 1.0 (7)	+ 11
3T3-NR6 + EGF	ND	ND	7.4 ± 1.5 (12)	+ 16
3T3-NR6 EGF receptor construct	–	–	6.1 ± 1.8 (7)	− 5
3T3-NR6 EGF receptor construct + EGF	24,000 ± 2,600	350 ± 200	12.1 ± 3.2 (9)	+ 89
3T3-NR6 EGF receptor construct + chloroquine (30 μM) + EGF	50,000 ± 7,300	7,600 ± 500	13.6 ± 3.5 (11)	+113

* Cells were incubated with ^{125}I-EGF for 20 h at a concentration of 20 ng/ml and then lysed in lysis buffer (Materials and Methods), and the ^{125}I-EGF activity of lysate was counted as described in Materials and Methods.

‡ Cells were incubated with ^{125}I-EGF for 20 h at a concentration of 20 ng/ml, the nuclei were subsequently isolated, and ^{125}I-EGF activity was counted (Materials and Methods).

§ Transport rate constant of 20K FITC-dextrans (Sigma Chemical Co.) in whole living 3T3 cells. In the case of EGF (LR-EGF; Collaborative Research, Inc., Bedford, MA) stimulation, the transport measurements were performed after adding EGF (50 ng/ml) to 20K FITC-dextran-loaded culture cells and incubating at 37°C for 3-6 h. FRAP experiments were performed as described (16, 33).

‖ Mean ± SD.

¶ Number of experiments (in parentheses).

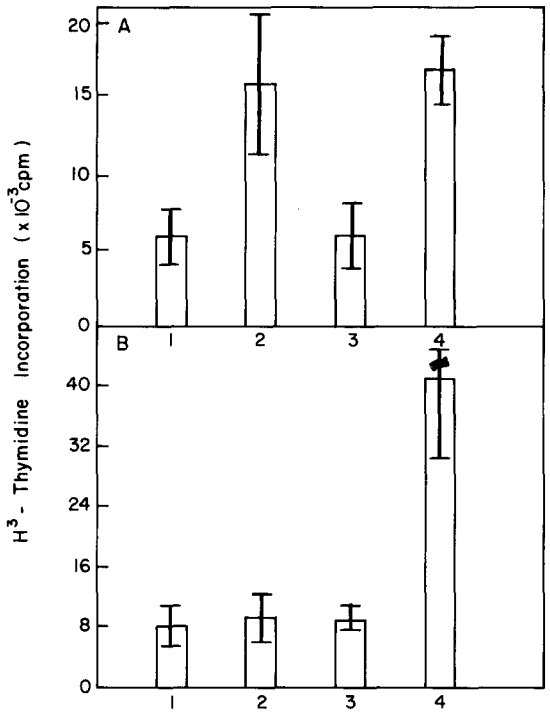

Figure 5. EGF-induced stimulation of DNA synthesis in 3T3-NR6 EGF receptor constructs and the parent 3T3-1 cell line. *A* represents [^{3}H]thymidine incorporation for the 3T3-1 parent cells, while *B* represents the [^{3}H]thymidine uptake in the 3T3-NR6 EGF receptor constructs. The measurement conditions involved are (columns *1–4*, respectively) no rat hepatocyte receptor fraction pretreatment nor EGF addition, no rat hepatocyte receptor fraction pretreatment and 40 ng/ml EGF, rat hepatocyte receptor fraction reconstitution and no EGF, and rat hepatocyte receptor fraction reconstitution and 40 ng/ml EGF. Error bars represent the standard deviation for three measurements. Incubation and reconstitution procedures are as described in Materials and Methods.

formed on isolated nuclei (isolated from variously treated cells as described in Materials and Methods). A comparison of nuclear transport results for 3T3-1 nuclei isolated from cells grown for 20 h in media containing 2% calf serum but no EGF (unprimed) with nuclei isolated from cells grown in media containing EGF (20 ng/ml; primed) demonstrates that only in the nuclei isolated from primed cells can EGF (50 ng/ml added to the transport assay) enhance macromolecular transport (+136%). Measurements of transport with nuclei isolated from primed cells containing no EGF in the transport assay demonstrated no significant enhancement (Table II). The enhancement observed for nuclei isolated from primed cells in the presence of EGF was found to be larger when nuclei were isolated from primed cells that had also been treated with chloroquine (15 μM) (as described in Materials and Methods). These results would support the idea that an induction in the cell of EGF binding activity and a subsequent nuclear accumulation of EGF binding capacity is required to successfully modulate nuclear transport (Tables I and II). Nuclei isolated from the EGF-nonresponsive mutant (3T3-NR6) demonstrated no enhanced nuclear transport after isolation from primed or unprimed cells in the presence or absence of EGF (50 ng/ml) in the transport assay. The

Table II. Transnuclear Dextran Transport Rate in Isolated Fibroblast Nuclei

Nuclei and cell treatment	Dextran transport rate*	Change from control
	$\times 10^3 s^{-1}$‡	%
Nuclei from parent cell line		
3T3-1 (parent) (control) grown without EGF and containing no EGF in the nuclear transport assay	3.3 ± 1.0 (5)§	–
3T3-1 grown without EGF but containing EGF (50 ng/ml) in the nuclear transport assay	3.8 ± 1.4 (9)	+ 15
3T3-1 grown in presence of EGF (20 ng/ml) but containing no EGF in the nuclear transport assay	3.8 ± 0.5 (3)	+ 15
3T3-1 grown in presence of EGF (20 ng/ml) and containing EGF (50 ng/ml) in the nuclear transport assay	7.8 ± 1.2 (8)	+136
3T3-1 pretreated with chloroquine (15 μM) (Materials and Methods), grown in the presence of EGF (20 ng/ml), and containing EGF (50 ng/ml) in the nuclear transport assay	9.6 ± 1.5 (6)	+191
Nuclei from EGF-nonresponsive cell line		
3T3-NR6 grown without EGF and containing no EGF in the nuclear transport assay	2.4 ± 0.8 (6)	–
3T3-NR6 grown without EGF but containing EGF (50 ng/ml) in the nuclear transport assay	2.3 ± 0.6 (8)	– 4
3T3-NR6 EGF receptor construct	3.7 ± 1.1 (7)	+ 54
3T3-NR6 EGF receptor construct with EGF (50 ng/ml) in the transport assay	6.5 ± 0.8 (9)	+171
3T3-NR6 EGF receptor construct pretreated with chloroquine (15 μM) and containing EGF (50 ng/ml) in the nuclear transport assay	8.3 ± 1.3 (9)	+246
3T3-NR6 nuclei coincubated with EGF receptor fraction containing no EGF in the nuclear transport assay	1.9 ± 1.0 (3)	– 20
3T3-NR6 nuclei coincubated with EGF receptor fraction and then stimulated with EGF (50 ng/ml) in the transport assay	7.5 ± 1.1 (12)	+213

* Transport measurements for 70K dextrans (isolated nuclear measurements) were performed after adding 50 ng/ml EGF to the isolated nuclei and incubation at 37°C for 2.5 h.
‡ Mean ± SD.
§ Number of experiments (in parentheses).

whole cell (Table I) and isolated nuclei (Table II) data obtained for 3T3-NR6 cells support the notion that the absence of an EGF effect on nuclear transport may most simply be explained by the absence of nuclear-localized EGF binding capacity. Under these conditions, no EGF-mediated enhancement of nuclear transport is observed when monitored in whole cells or nuclei isolated from them (Table I and II). If, however, nuclei are isolated from 3T3-NR6 cells after EGF receptor fraction reconstitution into the plasma membrane and stimulated with EGF, nuclear transport is increased (Table II) to approximately the same extent as reported for nuclei isolated from the primed parent (3T3-1) cells (Table II). This is directly related to an EGF-mediated process in the reconstituted cells since the nuclei from 3T3-NR6 receptor constructs not incubated with EGF did not demonstrate enhanced transport. The 3T3-NR6 receptor construct also responded to chloroquine treatment in the same manner as the parent 3T3-1 cell line, in that nuclear transport in nuclei isolated from the chloroquine-treated cells was somewhat enhanced over nonchloroquine treatment. To examine whether the rat liver EGF receptor fraction by itself was sufficient to enhance transport, isolated 3T3-NR6 nuclei were directly coincubated with EGF receptor fraction. Incubation was performed as described for whole cell reconstitution, using isolated NR-6 nuclei instead of cells (Materials and Methods). Under these conditions, the nuclei again demonstrated enhanced transport in the presence, but not absence, of EGF (50 ng/ml in the transport assay) to the extent observed for nuclei isolated from primed 3T3-1 cells and 3T3-NR6 EGF receptor constructs.

Correlation between Nuclear Accumulation of EGF and the Transnuclear Dextran Transport Rate in Whole Cells

In an attempt to establish a minimal level for transnuclear transport activity, we measured EGF-mediated enhancement of nucleocytoplasmic transport in whole 3T3-1 fibroblasts not exposed to EGF in the growth medium (2% calf serum) and maintained at G_0 in the cell cycle (see Materials and Methods). The result for transnuclear dextran transport measurements in whole 3T3-1 cells at G_0 is $3.2 \pm 0.7 \times 10^{-3}$ s^{-1} (five measurements on different cells $\pm$ SD). To examine the correlation between whole cell nuclear transport and EGF receptor nuclear occupancy, we have plotted this value (Fig. 6, point *A*) with the other experimentally derived values (Fig. 6, points *B–D*, are from Table I). As pointed out in the legend to Table I, ^{125}I binding measurements were performed in the presence of 20 ng/ml ^{125}I-EGF, while dextran transport was performed at 50 ng/ml EGF. Considering that Fig. 1 *A* demonstrates saturation binding for 3T3-1 nuclei at 10 ng/ml, we believe this comparison is valid. A linear relationship is observed between the extent of nuclear ^{125}I-EGF accumulation and enhanced transport.

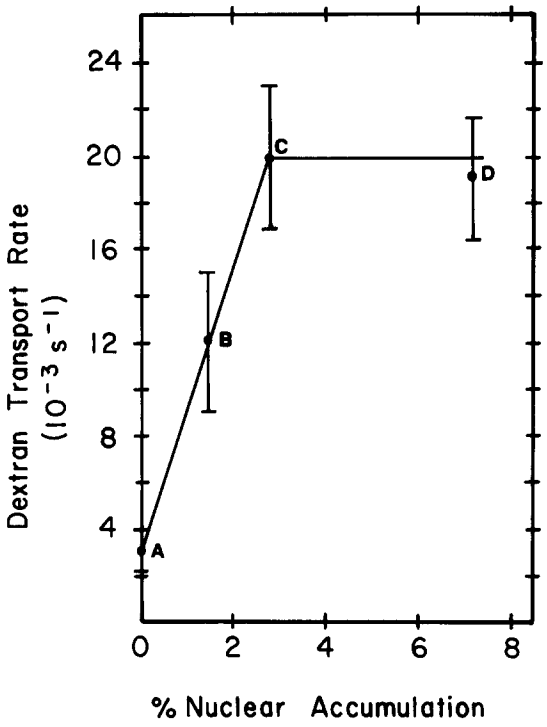

Figure 6. Transnuclear dextran transport rate as a function of EGF receptor nuclear occupancy. Using data from Table I and transport measurements performed on 3T3-1 cells during G_0, a relationship was plotted between nuclear transport and nuclear receptor occupancy. Point *A* is the transnuclear transport rate at G_0, while points *B–D* are obtained from data taken from Table I.

Discussion

Receptor Translocation Activation Mechanism for Direct Modulation of Nuclear Function

The data presented provide evidence that modulation of a significant nuclear function, nucleocytoplasmic communication in fibroblasts stimulated with EGF, is concomitant with the appearance of EGF binding activity at the cell nucleus. Reconstitution studies in cells not expressing EGF receptor demonstrated that functional EGF receptors, as functionally defined by ^{125}I-EGF binding, originally introduced at the plasma membrane, can migrate to the nucleus and affect transnuclear transport. That the incorporated receptor fraction, in fact, induces changes in nuclear transport is supported by our observation that direct addition of the receptor fraction to isolated NR-6 nuclei enhances nuclear transport. ^{125}I-EGF binding studies in whole cells in the presence of chloroquine and leupeptin further showed that the observed nuclear accumulation of EGF would appear to be a consequence of a transport process characteristic of an endosomal-mediated translocation from the plasma membrane to the nucleus. These results are consistent with our previous observations of an activational role for EGF receptor at the nucleus (16, 32) and are compatible with reports from other laboratories that polypeptide growth factors and polypeptide growth factor receptors can be found to accumulate at the nucleus in whole cells (17, 25, 28, 33, 37, 46, 47). Of additional relevance to our proposal for a receptor translocation activation mechanism are the observations presented in this communication and others demonstrating that (*a*) nuclei isolated from growth factor sensitive cells are capable of polypeptide growth factor–mediated changes in nuclear function (2, 27, 32, 36); (*b*) cells reconstituted with a rat liver EGF receptor fraction, in which photoreactive ^{125}I-EGF was shown to covalently bind EGF receptor, result in responsive isolated nuclei; and (*c*) isolated nonresponsive nuclei may be directly

reconstituted with rat liver EGF receptor fraction and then demonstrate EGF-mediated enhanced nuclear transport. An alternative to such a direct nuclear activation mechanism that has been proposed depends on nonnuclear localized receptor-dependent changes in a combination of cellular parameters: i.e., phosphatidylinositol turnover (44), free Ca^{++} fluxes (5), or alkalinization of cytoplasmic pH (29). A recent publication by Escobedo and Williams (9) provides evidence that such types of indirect nuclear signaling may not be sufficient for the mitogenic response. Through the use of a variety of mutant PDGF receptors, they showed that the stimulation of phosphatidylinositol turnover, cytoplasmic pH alkalinization, and transient intracellular calcium concentration changes are not sufficient to explain PDGF-induced mitogenesis. If we view transnuclear transport changes as an element of the mitogenic response, the results of Escobedo and Williams (9) would appear to suggest that indirect activation may be necessary but not sufficient for nuclear activation.

In viewing our quantitation of nuclear EGF receptor accumulation and those for other polypeptide growth factors from other laboratories (12, 25, 33, 37), it is clear that nuclear occupancy reflects a small population of total cell accumulation ($\leqslant 5\%$). Although all such recent investigations have gone to great extents to rule out nonfunctional and artifactual whole cell contamination, a point of view has persisted from earlier work that the nuclear occupancy values for polypeptide growth factors are sufficiently low to justify an interpretation in terms of contamination. Yet, the number of receptors found to localize to the nucleus are within amounts required to provide between 0.5 and 1 receptor per nuclear pore complex ($\sim$1,000 and 2,000 nuclear pore complexes are observed in fibroblast and hepatocyte nuclei, respectively [21]). The growth factor–stimulated receptor (previously termed receptorzyme [34]), in conjunction with growth factor and/or other associated protein(s) (21, 23, 42), may localize to the nucleus through a receptor-encoded sequence. The recent reports of a translocation of activated protein kinase C from the plasma membrane to the nucleus may be representative of such a translocation complex possibly containing a growth factor receptor (10). At the nuclear level, the activated receptorzyme complex could directly initiate a series of enzymatic modifications, leading to changes in nuclear activity as a consequence of modifications in the conformation of nuclear pore complex proteins, nuclear envelope/matrix structure, and chromatin organization.

This work was supported by National Institutes of Health grant GM 30158.

Received for publication 17 January 1989 and in revised form 10 November 1989.

References

1. Agutter, P. S. 1984. Nucleocytoplasmic RNA transport. *Subcell. Biochem.* 10:231–257.
2. Baglia, F. A., and G. G. Maul. 1983. Nuclear ribonucleoprotein release and nucleoside triphosphatase activity are inhibited by antibodies directed against one nuclear matrix glycoprotein. *Proc. Natl. Acad. Sci. USA.* 80:2285–2289.
3. Bishayee, S., J. Feinman, M. Pittenger, H. Michael, and M. Das. 1982. Cell surface insertion of exogenous epidermal growth factor receptors into receptor-mutant cells: demonstration of insertion in the absence of added fusogenic agents. *Proc. Natl. Acad. Sci. USA.* 79:1893–1897.
4. Carpentier, J.-L., M. F. White, L. Orci, and R. C. Kahn. 1987. Direct visualization of the phosphorylated epidermal growth factor receptor during its internalization in A-431 cells. *J. Cell Biol.* 105:2751–2762.
5. Chen, W. S., C. S. Lazar, M. Poenie, R. Y. Tsien, G. N. Gill, and M. G. Rosenfeld. 1987. Requirement for intrinsic protein tyrosine kinase in the immediate and late actions of the EGF receptor. *Nature (Lond.).* 328: 820–823.
6. Dean, R. I., W. Jessup, and C. R. Roberts. 1984. Effects of exogenous amines on mammalian cells, with particular reference to membrane flow. *Biochem. J.* 217:27–40.
7. Dunn, W. A., T. P. Connolly, and A. L. Hubbard. 1986. Receptor-mediated endocytosis of epidermal growth factor by rat hepatocytes: receptor pathway. *J. Cell Biol.* 102:24–36.
8. Emmelot, P., C. J. Box, R. P. van Hoeven, and W. J. van Blitterswijk. 1974. Isolation of plasma membranes from rat and mouse livers and hepatomas. *Methods Enzymol.* 31:75–85.
9. Escobedo, J. A., and L. T. Williams. 1988. A PDGF receptor domain essential for mitogenesis but not for many other responses to PDGF. *Nature (Lond.)* 335:85–87.
10. Fields, A. P., G. R. Pettit, and W. S. May. 1988. Phosphorylation of lamin B at the nuclear membrane by activated protein kinase C. *J. Biol. Chem.* 263:8253–8260.
11. Glenney, J. P., Jr., W. S. Chen, C. S. Lazar, G. M. Walton, L. M. Zokas, M. G. Rosenfeld, and G. N. Gill. 1988. Ligand-induced endocytosis of the EGF receptor is blocked by mutational inactivation and by microinjection of anti-phosphotyrosine antibodies. *Cell.* 52:675–684.
12. Goldfine, I. D., and G. J. Smith. 1976. Binding of insulin to isolated nuclei. *Proc. Natl. Acad. Sci. USA.* 73:1422–1431.
13. Harford, J., K. Bridges, G. Ashwell, and R. D. Klausner. 1983. Intracellular dissociation of receptor-bound asialoglycoproteins in cultured hepatocytes: a pH-mediated nonlysosomal event. *J Biol. Chem.* 258:3191–3197.
14. Hokin, L. E. 1985. Receptors and phosphoinositide-generated second messengers. *Annu. Rev. Biochem.* 54:205–235.
15. James, R., and R. A. Bradshaw. 1984. Polypeptide growth factors. *Annu. Rev. Biochem.* 53:259–292.
16. Jiang, L.-W., and M. Schindler. 1988. Nuclear transport in 3T3 fibroblasts: effects of growth factors, transformation, and cell shape. *J. Cell Biol.* 106:13–19.
17. Johnson, L. K., I. Vlodavsky, J. D. Baxter, and D. Gospodarowicz. 1980. Nuclear accumulation of epidermal growth factor in cultured rat pituitary cells. *Nature (Lond.).* 287:340–343.
18. Lin, P. H., R. Selinfreund, E. Wakshull, and W. Wharton. 1987. Rapid and efficient purification of plasma membrane from cultured cells: characterization of epidermal growth factor binding. *Biochemistry.* 26:731–736.
19. Livneh, E., R. Prywes, O. Kashles, N. Reiss, I. Sasson, Y. Mory, A. Ullrich, and J. Schlessinger. 1986. Reconstitution of human epidermal growth factor receptors and its deletion mutants in cultured hamster cells. *J. Biol. Chem.* 261:12490–12497.
20. Margolis, B., S. G. Rhee, S. Felder, M. Mervic, R. Lyall, A. Levitzki, A. Ullrich, A. Zilberstein, and J. Schlessinger. 1989. EGF induces tyrosine phosphorylation of phospholipase C-II: a potential mechanism for EGF receptor signaling. *Cell.* 57:1101–1107.
21. Maul, G. G. 1977. The nuclear and the cytoplasmic pore complex: structure, dynamics, distribution, and evolution. *Int. Rev. Cytol.* 6:75–186.
22. Meisenhelder, J., P.-G. Suh, S. G. Rhee, and T. Hunter. 1989. Phospholipase C-γ is a substrate for the PDGF and EGF receptor protein tyrosine kinases in vivo and in vitro. *Cell.* 57:1109–1122.
23. Merion, M., and W. S. Sly. 1983. The role of intermediate vesicles in the adsorptive endocytosis and transport of ligand to lysosomes by human fibroblasts. *J. Cell Biol.* 96:644–650.
24. Murthy, U., M. Basu, A. Sen-Majumdar, and M. Das. 1986. Perinuclear location and recycling of epidermal growth factor receptor kinase: immunofluorescent visualization using antibodies directed to kinase and extracellular domains. *J. Cell Biol.* 103:333–342.
25. Podlecki, D. A., R. M. Smith, M. Kao, P. Tsai, T. Hueckstead, D. Brandenburg, R. S. Lasher, L. Jarett, and J. M. Olefsky. 1987. Nuclear translocation of the insulin receptor—a possible mediator of insulin's long-term effects. *J. Biol. Chem.* 262:3362–3368.
26. Pruss, R. M., and H. R. Herschman. 1977. Variants of 3T3 cells lacking mitogenic response to epidermal growth factor. *Proc. Natl. Acad. Sci. USA.* 74:3918–3921.
27. Purrello, F., D. B. Burnham, and I. D. Goldfine. 1983. Insulin regulation of protein phosphorylation in isolated rat liver nuclear envelopes: potential relationship to mRNA metabolism. *Proc. Natl. Acad. Sci. USA.* 80:1189–1193.
28. Rakowicz-Szulczynska, E. M., U. Rodeck, M. Herlyn, and H. Koprowski. 1986. Chromatin binding of epidermal growth factor, nerve growth factor, and platelet-derived growth factor in cells bearing the appropriate surface receptors. *Proc. Natl. Acad. Sci. USA.* 83:3728–3732.
29. Reuss, L., D. Cassel, P. Rothenberg, B. Whiteley, D. Mancuso, and L. Glaser. 1986. Mitogens and ion fluxes. *Curr. Top. Membr. Transp.* 27:3–54.
30. Rozengurt, E. 1986. Early signals in the mitogenic response. *Science (Wash. DC).* 234:161–166.
31. Savion, N., I. Vlodavsky, and D. Gospoderowicz. 1981. Nuclear accumulation of epidermal growth factor in cultured bovine corneal endothelial and granulosa cells. *J. Biol. Chem.* 256:1149–1154.

32. Schindler, M., and L.-W. Jiang. 1987. Epidermal growth factor and insulin stimulate nuclear pore-mediated macromolecular transport in isolated rat liver nuclei. *J. Cell Biol.* 104:849–853.
33. Schindler, M., and L.-W. Jiang. 1987. Fluorescence redistribution after photobleaching as a tool for dissecting the control elements of nucleocytoplasmic transport. *Methods Enzymol.* 141:447–459.
34. Schindler, M., and L.-W. Jiang. 1987. Nuclear-plasma membrane-cytoplasmic communication—linkages, controllers, and pathways. *Comments Mol. Cell. Biophys.* 4:215–231.
35. Schneider, C. A., R. W. Lim, E. Terwilliger, and H. R. Herschman. 1986. Epidermal growth factor-nonresponsive 3T3 variants do not contain epidermal growth factor receptor-related antigens or mRNA. *Proc. Natl. Acad. Sci. USA.* 83:333–336.
36. Schumm, D. E., and T. E. Webb. 1981. Insulin-modulated transport of RNA from isolated liver nuclei. *Arch. Biochem. Biophys.* 210:275–279.
37. Smith, R. M., and L. Jarett. 1987. Ultrastructural evidence for the accumulation of insulin in nuclei of intact 3T3-L1 adipocytes by an insulin receptor mediated process. *Proc. Natl. Acad. Sci. USA.* 84:459–463.
38. Sorkin, A. P., L. V. Teslenko, and N. N. Nikolsky. 1988. The endocytosis of epidermal growth factor in A431 cells: a pH of microenvironment and the dynamics of receptor complex dissociation. *Exp. Cell Res.* 175: 192–205.
39. Deleted in proof.
40. Swanson, J. A., and P. L. McNeil. 1987. Nuclear reassembly excludes large macromolecules. *Science (Wash. DC).* 238:548–550.
41. Thompson, D. M., C. Cochet, E. M. Chambaz, and G. N. Gill. 1985. Separation and characterization of a phosphatidylinositol kinase activity that co-purifies with the epidermal growth factor receptor. *J. Biol. Chem.* 260:8824–8830.
42. Ullrich, A., L. Coussens, J. S. Hayflick, T. J. Dull, A. Gray, A. W. Tam, J. Lee, Y. Yarden, T. A. Libermann, J. Schlessinger, J. Downward, E. L. V. Mayes, N. Whittle, M. D. Waterfield, and P. H. Seeberg. 1984. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature (Lond.).* 309:418–425.
43. Ullrich, A., J. R. Bell, E. Y. Chen, R. Herrera, L. M., Petruzzelli, T. J. Dull, A. Gray, L. Coussens, Y.-C. Liao, M. Tsubokawa, A. Mason, P. H. Seeburg, C. Grunfeld, O. M. Rosen, and J. Ramachandran. 1985. Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. *Nature (Lond.).* 313:756–761.
44. Wahl, M., and G. Carpenter. 1988. Regulation of epidermal growth factor-stimulated formation of inositol phosphates in A-431 cells by calcium and protein kinase C. *J. Biol. Chem.* 263:7581–7590.
45. Wiley, H. S., W. VanNostrand, D. N. McKinley, and D. D. Cunningham. 1985. Intracellular processing of epidermal growth factor and its effect on ligand-receptor interactions. *J. Biol. Chem.* 260:5290–5295.
46. Wong, K. Y., D. Hawley, R. Vigneri, and I. D. Goldfine. 1988. Comparison of solubilized and purified plasma membrane and nuclear insulin receptors. *Biochemistry.* 27:375–379.
47. Yankner, B. A., and E. M. Shooter. 1979. Nerve growth factor in the nucleus: interaction with receptors on the nuclear membrane. *Proc. Natl. Acad. Sci. USA.* 76:1269–1273.
48. Yarden, Y., J. A. Escobedo, W.-J. Kuang, T. L. Yang-Feng, T. O. Daniel, P. M. Tremble, E. Y. Chen, M. E. Ando, R. N. Harkins, U. Francke, V. A. Fried, A. Ullrich, and L. T. Williams. 1986. Structure of the receptor for platelet-derived growth factor helps define a family of closely related growth factor receptors. *Nature (Lond.).* 323:226–232.