Epidermal Growth Factor and Insulin Stimulate Nuclear Pore-mediated Macromolecular Transport in Isolated Rat Liver Nuclei

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Abstract. Fluorescence photobleaching was used to measure the effect of epidermal growth factor (EGF), insulin, and glucagon on the nuclear transport of fluorescent-labeled dextrans across the nuclear pore complex. EGF and insulin were found to stimulate transport ~200%, while boiling these polypeptide growth factors greatly diminished this enhancement activity. Glucagon demonstrated no enhancement effect. The nuclear transport enhancement effects were observed at EGF and insulin concentrations that elicit the various physiological responses, e.g., nanomolar range.

BINDING of hormones and growth factors to plasma membrane receptors constitutes the first element in a communication pathway between the cell interior and the external environment (15, 17). For polypeptide growth factors, the ligand binds its receptor, resulting in the internalization of the complex (15–17, 32). These recognition and activation steps lead to cellular responses, particularly enhanced nuclear activity, e.g., mRNA synthesis, mitosis, and macromolecular transport (5, 26, 35). The step(s) in the response pathway that directly initiate nuclear activity are unknown. The focus of past work has been: (*a*) to examine the possibility that the plasma membrane–binding event is sufficient to activate a cytoplasmic biochemical cascade resulting in activated molecules or second messengers that can initiate a nuclear response (3, 17, 23); and (*b*) to search for nuclear membrane or nucleoplasmic receptors capable of binding internalized effectors or effector–receptor complexes (14, 19, 28, 35) producing a direct effect. In the case of insulin and epidermal growth factor (EGF)[1], a number of investigations have demonstrated specific polypeptide growth factor binding to the nucleus (12, 14, 19, 35, 38). This binding leads to stimulation of RNA metabolism and mRNA transport (5, 26, 33). To examine the potentially direct role of hormones and growth factors on nuclear activity, we report the use of the fluorescence photobleaching technique (fluorescence redistribution after photobleaching) to measure insulin and epidermal growth factor–stimulated nuclear transport of fluorescein-labeled 64,000-mol wt dextrans across the nuclear pore complexes in isolated rat liver nuclei (18, 24). The data obtained constitute the first reported evidence for a direct effect of these growth factors on bi-directional macromolecular transport across the nuclear envelope.

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1. *Abbreviations used in this paper*: EGF, epidermal growth factor; WGA, wheat germ agglutinin.

Materials and Methods

Nuclear Isolation

Rat liver nuclei were isolated with minor modifications and characterized as described (20, 29). All experiments were performed within 2 d of preparation with nuclei that had been stored at 4°C in 0.25 M sucrose, 10 mM Hepes, 1 mM Mg^{++}, 1 mM phenylmethylsulfonylfluoride, pH 7.4 buffer. Little variation in results occurred over the 2-d period.

Reagents

Fluorescein-labeled dextrans and bovine insulin chain A were products of Sigma Chemical Co. (St. Louis, MO). Porcine insulin, EGF, and glucagon were products of Gibco (Grand Island, NY), Collaborative Research, Inc. (Lexington, MA), and Sigma Chemical Co., respectively. Wheat germ agglutinin was obtained from Vector Laboratories, Inc. (Burlingame, CA).

Fluorescent Dextran Influx Assay

A typical nuclear transport experiment was performed as described (18, 30). Briefly, isolated nuclei were incubated for 30 min at 37°C in storage buffer containing 1 μM fluorescein-labeled 64,000-mol wt dextrans (Mw:Mn < 1.25). Insulin, EGF, wheat germ agglutinin, and glucagon were individually added to the nuclei before the addition of dextrans, and remained present in the measuring solution throughout the experiment. An aliquot (5 μl) of nuclear suspension was pipetted onto a slide and a cover slip was sealed on top of the sample with melted paraffin. Photobleaching experiments and subsequent analyses were done as described by Peters (24, 25), and Jiang and Schindler (18). Nuclei bathed in fluorescent dextran solution were photobleached (fluorescence was photochemically destroyed in the nucleoplasm by a burst of high intensity excitation laser light) resulting in a transient spatial gradient of fluorescent-labeled dextrans between the surrounding solution and the nucleoplasm. The recovery of fluorescence in the nucleoplasm was followed as a function of time resulting in a calculated dextran flux rate. This recovery has been correlated to nuclear pore–mediated transport of the dextrans across the nuclear envelope (18, 24, 25). A typical experiment is presented in Fig. 1. Note the steady state level of fluorescence indicating

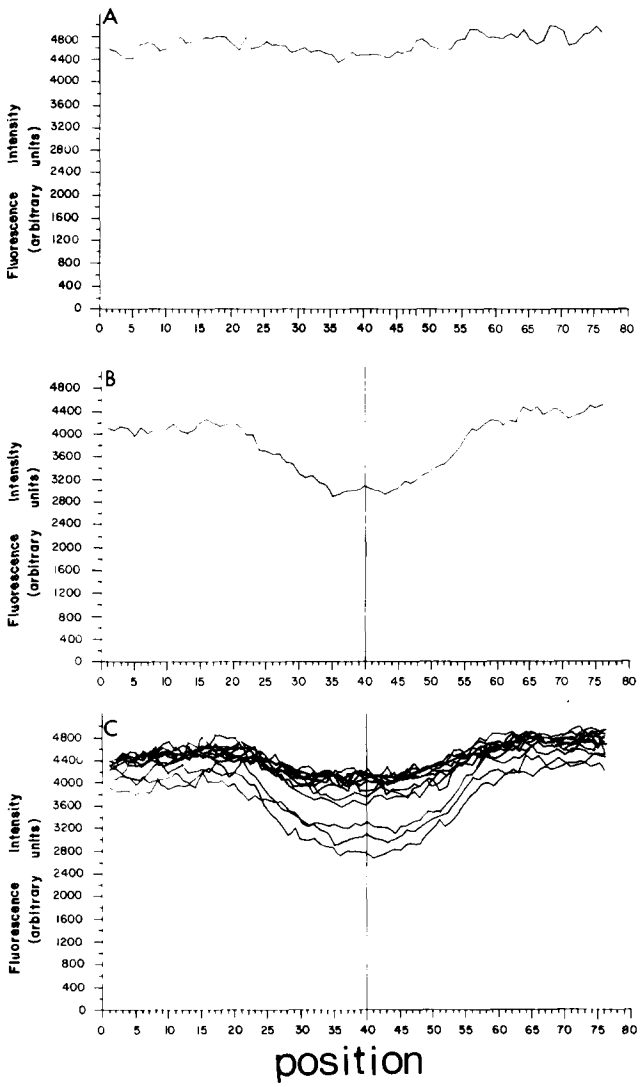

Figure 1. Fluorescence photobleach of an isolated rat liver nucleus suspended in a solution of fluorescein-derivitized dextrans (64,000 mol wt). Laser beam–excited emission pattern of a nucleus in equilibrium with labeled dextran (*A*). The ordinate is an arbitrary scale of fluorescence intensity, while the abscissa represents the position of the beam along a programmed scan. Each scan is a pass of the excitation laser beam across the nucleus. The interval between two scans, i.e., scan time with delay, is 5–10 s. A bleaching pulse is initiated (laser intensity is increased ~5,000 times) resulting in the depletion of nuclear fluorescence. This is represented by the dip observed in *B*. The line represents the center of the bleaching pulse. The recovery of fluorescence is observed by subsequent scans in a monitoring period of 150–300 s (*C*) and is proportional to the dextran flux rate (18, 24). Beam diameter is ~6 μm.

equilibration of fluorescein-labeled 64,000-mol wt dextran between the nucleoplasm and external solution (Fig. 1 *A*). After photobleaching the entire nucleus, a trough appeared representing the fluorescence-depleted intranuclear volume (Fig. 1 *B*); the vertical line represents the center of the bleach.

The recovery of this trough to prebleach intensity was observed in subsequent scans (a scan is the laser beam moving across the nucleus) (Fig. 1 *C*). Considering that the fluorescence intensities measured are representative of the dextran concentration (18, 24, 25), then a plot of the equation:

$$\frac{F(-) - F(t)}{F(-) - F(0)} = e^{-kt} \qquad (1)$$

with F(−), F(0), and F(*t*), fluorescence signals before (prebleach), after, and at time *t* after a photobleach, respectively, results in a calculated dextran flux rate (*k*).

Results

Representative plots of the data are presented in Fig. 2 for nuclear transport in the presence of insulin (Fig. 2 *A*) and boiled insulin (Fig. 2 *B*). In all cases, rebleaching on the same sample resulted in no observed differences in calculated flux rates. To examine the effect of hormone–growth factor concentration on nuclear transport, dose–response curves were prepared for insulin and EGF (Fig. 3). Half-maximal response from control (2.2×10^{-3} s^{-1}) for insulin and EGF were observed at 1.6 and 4.2 nM, respectively. When EGF (50 ng/ml) and insulin (30 ng/ml) were added together, the enhancement of transport [$K = (8.6 \pm 1.0) \times 10^{-3}$ s^{-1}, 10 experiments] was not significantly different than that of each polypeptide growth factor when added individually at saturating concentrations (Fig. 3). Glucagon, on the other hand, at a concentration of 60 nM (maximal stimulation by insulin or EGF) had no effect on nuclear transport (data not shown). Boiling both insulin and EGF for 30 min resulted in preparations that had minimal effects on transport (Table I). When insulin A chain was used at a concentration of 50 μg/ml, the concentration required to inhibit ~20% of the binding of labeled insulin to human skin fibroblasts (10, 11), the transport rate enhancement was ~26% of the native insulin enhancement of transport. At 0.1 μg/ml, insulin A chain had neither an effect on transport nor a reported inhibitory effect on the binding of labeled insulin to human skin fibroblasts (10, 11). Because lectins, particularly wheat germ agglutinin (WGA), have been demonstrated to have insulin-like activity when bound by the cell plasma membrane (8), transport measurements were performed in the presence of WGA (Table I). WGA bound to nuclei (30) and was observed to maximally enhance transport at ~3–14 μM. This enhancement of dextran transport was directly related to WGA binding to nuclei since 0.2 M GlcNAc (an inhibitor of binding) blocked the effect and WGA does not bind to the fluorescent dextrans.

Discussion

The biochemical events between the initial steps of effector–receptor recognition at the plasma membrane and subsequent nuclear activation of RNA metabolism and/or DNA replication compose a "black box." The limited number of biochemical investigations of direct nuclear interactions with hormones and growth factors have concluded that: (*a*) insulin, EGF, and nerve growth factor have nuclear binding sites (14, 19, 28, 39); (*b*) the K_d for such sites may vary between a high and low affinity (e.g., NGF, 0.08 and 9.0 nM; insulin, 6.0 and 66.0 nM) representative of two types of receptor or receptor states (14, 19); (*c*) a high affinity receptor or a receptor with covalent-bound ligand may be important for nuclear activation (6, 34); and (*d*) the nuclear receptor may differ structurally from the plasma membrane receptor (13, 38). Previously, in an attempt to provide dynamic evidence of growth factor effects on the nucleus, Schumm and Webb (33) observed that insulin increases the efflux of mRNA from isolated nuclei, while Purrello et al. (27) confirmed these obser-

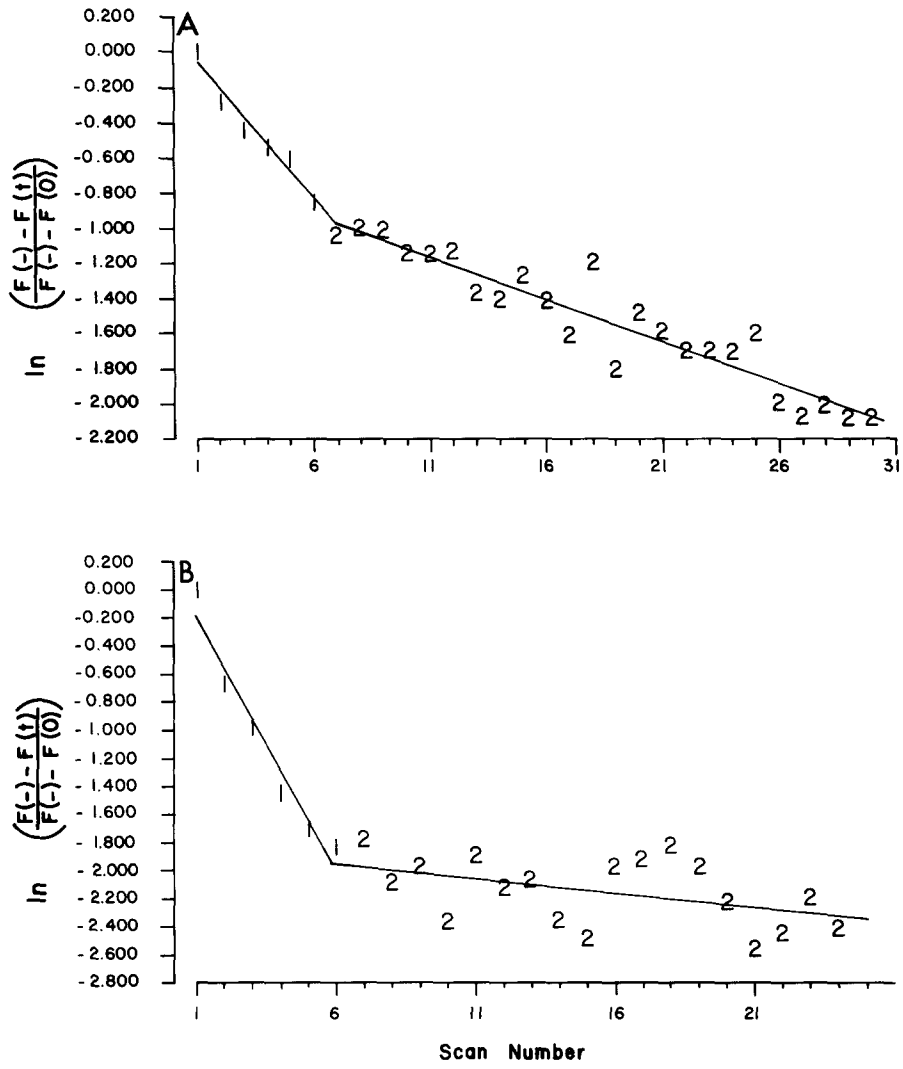

Figure 2. Analysis of fluorescence photobleaching recovery scans. Recovery is represented on a semi-logarithmic plot of ln $[F(-) - F(t)]/(F(-) - F(0)]$ as a function of scan number where $F(-)$, $F(0)$, and $F(t)$ are the fluorescence intensities before bleaching, after, and at time t after the bleach measured at the center of the bleach. Recovery is best represented by least squares fits to the sum of two first order terms as described (24). Component 1 represents dextran-surface adsorption, while component 2 corresponds to nuclear transport across the nuclear pore complex (18). *A* is the recovery curve for insulin (50 ng/ml), while *B* shows the recovery of fluorescence for boiled insulin (500 ng/ml).

vations and further demonstrated insulin-induced alterations in the phosphorylation of nuclear envelope proteins. Our present results extend these observations to show a direct effect of insulin and EGF on bi-directional macromolecular transport (unbleached dextrans diffuse into the nucleoplasm, while bleached dextrans diffuse out through nuclear pore complexes) in an in vitro nuclear transport system. The concentration range in which insulin and EGF affect dextran

Table I. Macromolecular Transport Rates Across the Nuclear Envelope

Treatment	64,000-mol wt Dextran transport rate coefficient	Change from control
	$\times 10^3\ s^{-1}$	%
Control	2.2 ± 0.8* (14)‡	–
+ Insulin (0.05 μg/ml)	8.6 ± 1.3 (8)	+ 291
+ A chain (0.1 μg/ml)	2.1 ± 0.7 (8)	–
+ A chain (50.0 μg/ml)	3.9 ± 0.6 (6)	+ 77
+ Boiled insulin (0.5 μg/ml)	3.2 ± 0.5 (5)	+ 45
+ EGF (0.5 μg/ml)	7.4 ± 1.5 (5)	+ 236
+ Boiled EGF (0.5 μg/ml)	2.6 ± 0.8 (5)	+ 18
+ WGA (5 μg/ml)	3.2 ± 1.4 (3)	+ 45
+ WGA (100 μg/ml)	4.6 ± 0.9 (15)§	+ 109
+ WGA (500 μg/ml)	5.5 ± 1.1 (4)	+ 150
+ WGA (100 μg/ml) + 0.2 M GlcNAc	2.6 ± 1.3 (5)	+ 18

* Mean ± SD.
‡ Number of experiments.
§ Previously reported by Jiang and Schindler (18).

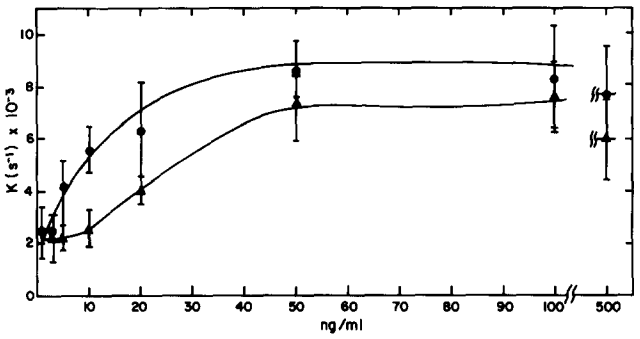

Figure 3. Effect of EGF and insulin concentration on transnuclear transport of 64,000-mol wt dextran in isolated rat liver nuclei. The ordinate represents the transport rate coefficient. At 0 nM polypeptide growth factor, the control value is 2.2×10^{-3} s^{-1}. The abscissa represents the concentration range of EGF or insulin. Indicated concentrations of EGF or insulin were added to the nuclear suspension before dextran addition. The suspension was then incubated for 30 min at 37°C. Solid circles represent the insulin dose-response, while solid triangles show the response to EGF.

transport (Fig. 3) correlates with the concentration range used for assessing the binding of insulin and EGF to nuclear and plasma membrane receptors (14, 19, 38). These observations are bolstered by the demonstration that the concentration of insulin A chain required for some transport activation is ~1,000 times higher than the concentration of native insulin required for maximal transport enhancement. This correlates well with the observed potencies and binding abilities of native insulin and A chain (10, 11). Our negative results with glucagon are consistent with observations that glucagon does not bind to nuclei (14). The enhanced transport rates in the presence of WGA may either be related to the demonstrations that lectins, and in particular WGA, bind to the insulin and EGF receptor in the plasma membrane (7, 8) causing, in the case of insulin, hormone-like activity (8), or that WGA binds to the nucleus (31), in particular, a nuclear pore protein that may be an NTPase (2, 9).

Models for nuclear pore-mediated transport have been presented that suggest that an NTPase in the nuclear pore complex may control transport (1, 27). Work by us (18, 30) and others (2, 22) have modified this model to suggest that ATP hydrolysis catalyzes transport through the activity of a nuclear pore complex ATPase, which has been proposed to be actomyosin (30). The modulation of transport may then be possible through modification of ATPase activity. Considering that polypeptide growth factors binding to the plasma membrane can initiate phosphorylation of proteins (7, 17, 27), as well as alter polyphosphoinositide metabolism (3, 23), it seems reasonable to propose that the activation of transport observed in the presence of insulin and EGF may be related to the role of hormone receptors as kinases. In this manner, elements of transport, particularly the ATPase, may be modulated by either direct phosphorylation mediated by the hormone-receptor kinase or indirectly by second messenger polyphosphoinositides that are produced by the activation of phosphatidylinositol kinase in the nuclear envelope (36). Support for both activation models exists. Phosphorylation processes are known to affect ATPase and actomyosin activity (4, 21), while ATP hydrolysis by an ATPase isolated from the nuclear envelope (37) is enhanced by polyphosphoinositides. That both these activities may in fact occur simultaneously is not ruled out.

The data presented were obtained by a dynamic assay measuring a significant nuclear activity, nuclear transport. The strong correlation observed for the hormone concentration requirements for maximal nuclear binding and maximal transnuclear transport provides suggestive functional evidence for a direct effect of hormone–receptor complex activation at the nuclear envelope.

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