Nuclear Protein Import Is Inhibited by an Antibody to a Lumenal Epitope of a Nuclear Pore Complex Glycoprotein

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Abstract. Gp210 is a major transmembrane glycoprotein associated with the nuclear pore complex that is suggested to be important for organizing pore complex architecture and assembly. A mouse monoclonal IgG directed against an epitope in the lumenal domain of rat gp210 was expressed in cultured rat cells by microinjection of mRNA prepared from a hybridoma cell line. The expressed IgG, which becomes assembled into a functional antibody in the lumen of the endoplasmic reticulum, bound to the nuclear envelope in vivo. Expression of anti-gp210 antibody in interphase cells specifically reduced approximately fourfold the mediated nuclear import of a microinjected nuclear protein (nucleoplasmin) coupled to gold particles. The antibody also significantly decreased nuclear influx of a 10-kD dextran by passive diffusion. This transport inhibition did not result from removal of pore complexes from nuclear membranes or from gross alterations in pore complex structure, as shown by EM and immunocytochemistry. A physiological consequence of this transport inhibition was inhibition of cell progression from G2 into M phase. Hence, binding of this antibody to the lumenal side of gp210 must have a transmembrane effect on the structure and functions of the pore complex. These data argue that gp210 is directly or indirectly connected to pore complex constitutents involved in mediated import and passive diffusion.

THE nuclear envelope, which forms the boundary of the nuclear compartment in eukaryotes, consists of inner and outer membranes joined at nuclear pore complexes. The pore complex is a large supramolecular structure that spans the nuclear envelope and provides a channel for the transport of molecules between the cytoplasm and nucleus (for reviews see Franke et al., 1981; Dingwall and Laskey, 1986; Gerace and Burke, 1988; Goldfarb, 1989; Burke, 1990; Silver, 1991). Regulated transport of macromolecules across the pore complex could be of major importance for the control of gene expression, and has been implicated in both growth and developmental control mechanisms (Gerace and Burke, 1988; Burke, 1990).

EM of ultrathin sections of cells and of negatively stained nuclear envelopes reveals that the pore complex has prominent eightfold rotational symmetry when viewed along an axis perpendicular to the plane of the membrane (e.g., Unwin and Milligan, 1982; Akey, 1989; Reichelt et al., 1990). It appears as a tripartite structure, composed of two coaxial "rings" (with a diameter of ~120 nm) on the cytoplasmic and nucleoplasmic sides of the pore complex that are connected to a central "plug-spoke" complex (Reichelt et al., 1990). The "plug-spoke" complex provides the major spacial constriction for molecular movement across the pore complex and contains an expandable "central channel" (Akey, 1989). The total mass of the pore complex determined by scanning transmission EM is ~124 × 10^6 daltons (Reichelt et al., 1990).

The general features of pore complex permeability have been determined by a variety of physiological studies. The pore complex contains an aqueous channel of a functional diameter of ~10 nm that permits rapid nonselective diffusion of molecules smaller than ~20–40 kD between nucleus and cytoplasm (Bonner, 1975; Paine et al., 1975; Peters, 1986). Mediated mechanisms are thought to be responsible for transport of most macromolecules across the pore complex, since diffusion cannot account for their rapid translocation seen in vivo. Mediated import of proteins is specified by short amino acid regions called nuclear location sequences (NLS)[1] (for review, see Gerace and Burke, 1988), which appear to interact with specific cellular receptors (Goldfarb et al., 1986; Adam and Gerace, 1991). NLS-mediated nuclear import requires ATP (Newmeyer and Forbes, 1988; Richardson et al., 1988) and appears to involve expansion of the central channel of the pore complex to allow transport of particles up to at least 26 nm in diameter (Dworetzky and Feldherr, 1988; Feldherr and Akin, 1990). However, ligands can bind to the pore complex in the absence of transport at reduced temperature or under conditions of ATP depletion

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1. *Abbreviations used in this paper*: N/C, nuclear/cytoplasmic; NLS, nuclear location sequence.

(Newmeyer and Forbes, 1988; Richardson et al., 1988), possibly reflecting transport intermediates.

Understanding the process of protein transport across the nuclear envelope will require a substantial amount of information on pore complex biochemistry. Since it has not been possible to isolate highly purified pore complexes by biochemical procedures, positive identification of pore complex components has relied on the use of electron microscope immunocytochemical localization. By this approach, a relatively small number of pore complex proteins have been identified in vertebrate cells. These include a group of peripheral membrane proteins containing O-linked *N*-acetylglucosamine (Snow et al., 1987; Davis and Blobel, 1987), some homologues of which have been identified recently in budding yeast (Davis and Fink, 1990; Nehrbass et al., 1990). A second pore complex component present in a wide range of higher eukaryotes is a major integral membrane glycoprotein with N-linked carbohydrate called gp210 (Gerace et al., 1982; Berrios et al., 1983; Wozniak et al., 1989).

Interest has focused on gp210 as a protein that may anchor the pore complex to nuclear membranes and participate in pore complex assembly. Topological studies involving site-specific antibodies and protease digestions have demonstrated that gp210 has a cytoplasmic tail of ~58 residues and a single transmembrane region, with the remaining mass located in the lumen of the perinuclear space as a single continuous segment containing the N-linked carbohydrate (Greber et al., 1990). The cytoplasmic tail, at least in part, may interact with other components of the pore complex in a manner important for pore complex structure and assembly.

In this report, we have used a microinjection approach to analyze a possible interaction of the pore complex glycoprotein gp210 with the transport apparatus of the pore complex. A mouse monoclonal IgG directed to a lumenal epitope of gp210 was expressed in normal rat kidney (NRK) cells by microinjecting mRNA from IgG-secreting hybridoma cells. Heavy and light chains of immunoglobulins are secretory proteins that are translocated into and assembled inside the lumen of the endoplasmic reticulum following injection of hybridoma mRNA (Burke and Warren, 1984; Hurtley and Helenius, 1989). The assembled antibody has access to the perinuclear space between inner and outer nuclear membranes, since the ER is continuous with the nuclear envelope, and in addition the outer nuclear membrane functions as rough ER (Franke, 1974).

We observed that the expressed anti-gp210 antibody bound to the nuclear envelope in vivo. As a consequence, both mediated transport and passive diffusion of proteins from the cytoplasm to the nucleus was severely reduced, and progression of cells from G2 into M phase was effectively inhibited. This indicates that binding of the antibody to the lumenal domain of gp210 causes malfunctioning of the nuclear pore complex by a mechanism that involves transmission of a structural change from the lumen to the cytoplasmic side of nuclear membranes where the major components of the transport apparatus are located. These results suggest that gp210 is directly or indirectly in contact with the apparatus important for mediated protein import and passive diffusion. In addition, these results suggest a novel potential mechanism by which the cell may regulate pore complex permeability from the lumenal side of the nuclear envelope.

Materials and Methods

Cell Cultures and Microinjections

NRK cells were maintained in a humidified incubator at 37°C under a 5% CO_2 atmosphere in DME containing high glucose (Gibco Laboratories, Grand Island, NY) that was supplemented with 10% FBS (Hyclone Laboratories, Logan, UT) and penicillin/streptomycin. Cells were removed from nearly confluent plastic dishes 24 to 48 h before microinjection by trypsinization and were replated on glass coverslips in growth medium supplemented with 15 mM Hepes-NaOH, pH 7.4, at a density of $\sim 1 \times 10^5$ cells per 9.5-cm^2 petri dish. For experiments involving sequential microinjections, cells were plated on scored glass coverslips obtained from Bellco Glass, Inc. (Vineland, NJ) (for fluorescence analysis) or on scratched plastic dishes (for electron microscopic analysis), which facilitated subsequent identification of the injected cells. Coverslips and plastic dishes were pretreated with Cell-Tak (Collaborative Research Inc., Lexington MA) to enhance cell attachment. Populations enriched in G2 cells were obtained by a double thymidine block, involving two sequential single thymidine block (Greber et al., 1990) separated by a growth interval of 12 h in medium lacking added thymidine. Mouse hybridoma cells were grown in DME containing high glucose and supplemented with 20% FBS, nonessential amino acids, and penicillin/streptomycin.

The following fluorescent samples and concentrations were used in microinjection experiments: rhodamine-conjugated nucleoplasmin (1 mg/ml; prepared as in Dingwall et al., 1982), rhodamine-conjugated goat anti-rabbit IgG (5 mg/ml; Pierce Chemical Co., Rockford, IL), FITC-conjugated bovine serum albumin (5 mg/ml; Cappel Laboratories, Cochranville, PA), 10 kDa FITC-dextran (2 mg/ml; Sigma Chemical Co., St. Louis, MO), and 150 kD FITC-dextran (5 mg/ml; Sigma Chemical Co.). All the above microinjection samples were briefly dialysed against a solution containing 10 mM Hepes-KOH, pH 7.4, 120 mM KCl before microinjection. Samples of mRNA were prepared for injection by adding 1/5 vol of stock solutions of fluorescently labeled dextran or IgG to solutions containing 1.2 mg/ml mRNA. To prepare stock solutions of fluorescent materials for dilution into mRNA solutions, samples were concentrated in a collodium bag system using a 25-kD cut off membrane (Schleicher & Schuell, Keene, NH). All samples, except the nucleoplasmin-gold conjugates, were centrifuged for 10 min at 13,000 *g* before microinjection.

Samples were injected into the cytoplasm of NRK cells at room temperature using an Eppendorf microinjector (5242; Brinkman Instruments Inc., Westbury, NY) equipped with an Eppendorf micromanipulator (5170; Brinkman Instruments Inc.) and Eppendorf Femptotips under an inverted microscope (Carl Zeiss, Oberkochen, Germany) equipped with an Achrostigmat 32× objective. Injections were performed at a rate of about 10 cells per minute with a needle pressure of about 100 hPa for the nucleoplasmin and dextran preparations and at a pressure of about 150 hPa for mRNA samples.

Fluorescence Microscopy

To examine nuclear protein import, microinjected NRK cells growing on glass coverslips were fixed in 3% paraformaldehyde in PBS for 7 min at room temperature and mounted in PBS containing 0.1% para-phenylenediamine (Baker Co., Inc., Sanford, ME) as an anti-quenching agent. For immunofluorescence microscopy, formaldehyde fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 4 min, quenched with 0.2% gelatin (Sigma Chemical Co.; 60 bloom) and stained for expressed mouse IgG with rhodamine conjugated goat anti-mouse IgG (2 μg/m; Cappel Laboratories). Indirect staining for pore complex antigens was performed using purified mouse monoclonal RL1 antibody (5 μg/ml; Snow et al., 1987) followed by rhodamine conjugated goat anti-mouse IgG (Pierce Chemical Co.). Cells were viewed through a Planapochromat 63× immersion objective on an Axiophot microscope (Carl Zeiss) equipped with rhodamine and fluorescein fluorescence filters and photographs were taken on T-Max 400 film (Eastman Kodak Co., Rochester, NY).

Electron Microscopy

After nuclear import reactions, cells attached to the plastic dish were fixed by adding 1 vol of 4% glutaraldehyde (Ted Pella, Inc., Irvine, CA) in 0.2 M cacodylate-NaOH, pH 7.3, 0.2% tannic acid (Mallincrodt) directly to the culture. After 1 h at room temperature, the cells were rinsed in 0.1 M cacodylate-NaOH, pH 7.3, and postfixed for 1 h at 0°C in 1% osmium

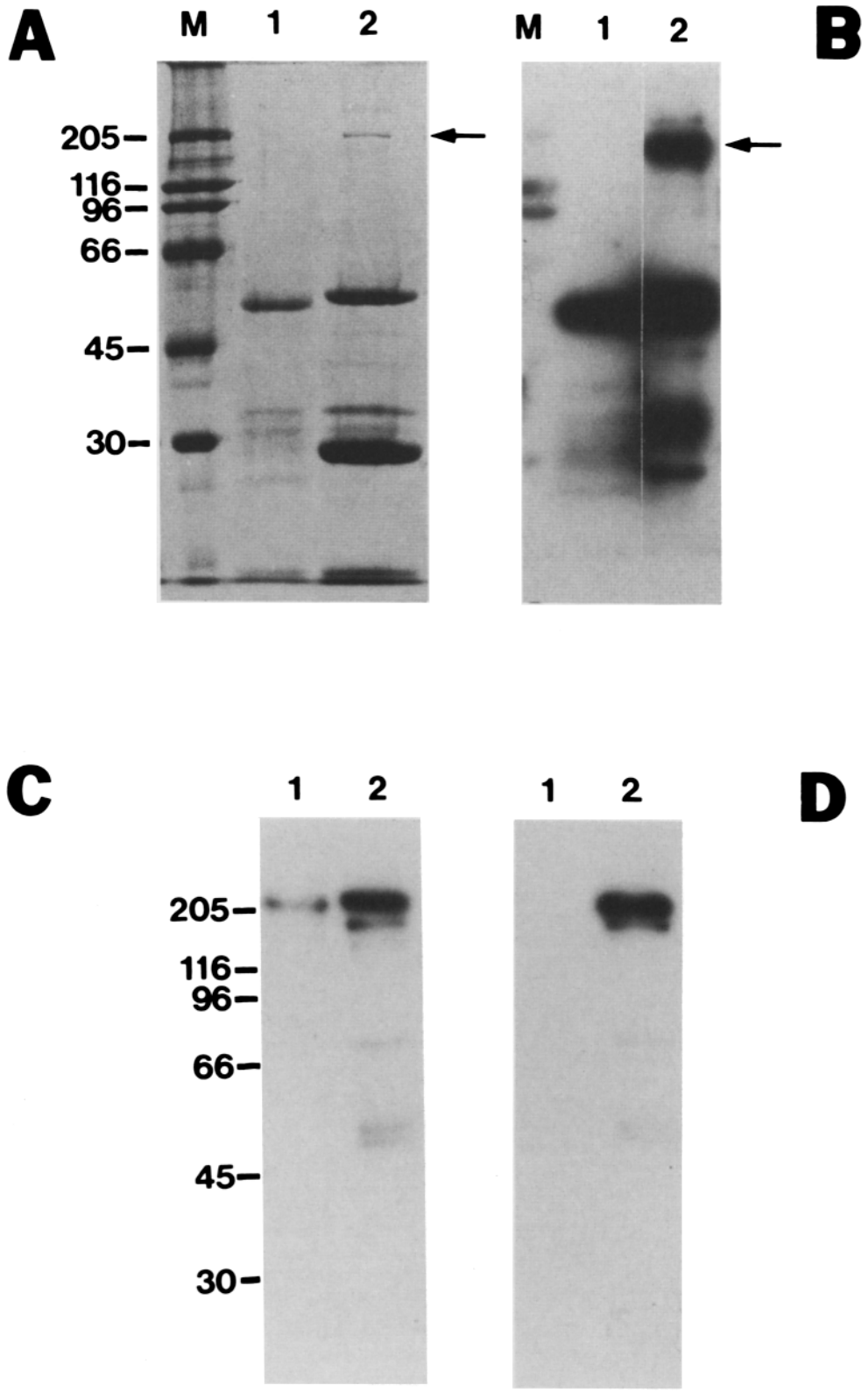


Figure 1. The mAb RL27 specifically recognizes a lumenal epitope of gp210. (*A* and *B*) Exponentially growing NRK cells were solubilized and the antigen recognized by RL27 was immunoadsorbed to immobilized antibody and subsequently analyzed by SDS-PAGE (lanes *2*). As a specificity control, cell lysates were immunoadsorbed with RL23, a mouse mAb that reacts with an uncharacterized protein of the endoplasmic reticulum (lanes *1*). *A* shows a Coomassie blue–stained gel and *B* shows an immunoblot probed with RL16, which is specific for gp210 (Greber et al., 1990). The arrows point to gp210. Note that the RL27-adsorbed samples (lanes *2*) contain a minor band migrating more slowly than gp210 and reactive with RL16. This band is also seen in gel analysis of lectin-isolated gp210 (Greber et al., 1990), and is likely to represent a higher molecular weight aggregate of gp210. Molecular weight markers are electrophoresed in lanes *M*. The heavily labeled bands migrating at ~50 and 30 kD in lane *2* represent antibody heavy and light chains that react strongly with protein A and are eluted from the Sepharose beads with SDS. The heavy chain of IgG is the heavily labeled band seen in lane *1*. (*C* and *D*) Isolated rat liver nuclear envelopes were digested with 10 μg/ml papain, separated into a soluble supernatant (lanes *1*) and a membrane pellet (lanes *2*) by centrifugation and fractionated by SDS-PAGE (Greber et al., 1990). *C* shows an immunoblot with purified RL27 and *D* is an immunoblot with the polyclonal antibody Rb-68, which is directed against a COOH-terminal peptide of gp210 (Greber et al., 1990).

tetroxide (Ted Pella, Inc.) in veronal acetate buffer (Farquhar and Palade, 1955). Cells were rinsed in deionized water, dehydrated in steps of increasing ethanol concentrations, and embedded in Epon resin (Polyscience Inc., Warrington, PA). After curing, thin sections (90-nm gold sections) were cut from the cells of interest, mounted on grids, stained with uranyl acetate, and lead citrate and examined in an electron microscope (CM12; Philips Electronic Instruments, Inc., Mahwah, NJ) at 100 kV.

For determining the frequency of pore complexes in microinjected NRK cells, nuclei were randomly selected and images were photographed at a magnification of 6,300×. Micrographs were printed and analyzed blindly

to determine the number of pores per μm of nuclear envelope contour length. Only regions where the inner and outer membranes were clearly connected were scored as nuclear pores.

For quantitation of the import of nucleoplasmin-gold conjugates into nuclei of mRNA-injected cells (Table I), sections through double-injected cells having a comparable ratio of cytoplasmic to nuclear area (2.33 +/− 0.21) were selected for analysis. One section per cell was analyzed. The gold grains in the entire cytoplasm and the nucleoplasm were counted in photographic prints from electron microscope negatives taken at a magnification of 9,600×.

Preparation of mRNA

Hybridoma cells (one liter) were grown in roller bottles (1,750 cm^2; Fisher Laboratories, Pittsburgh, PA) under CO_2 atmosphere to a density of ~8 × 10^5/ml. Cells were collected by centrifugation at 500 *g*, washed briefly in PBS, and lysed in a guanidinium hydrochloride based buffer (Sambrook et al., 1989). Total RNA was obtained after centrifugation through 4 M cesium chloride and poly A^+ RNA was isolated by two subsequent chromatography steps over oligo dT cellulose (Collaborative Research Inc., Lexington, MA) according to established protocols (Sambrook et al., 1989). mRNA was stored in 70% ethanol at −70°C at a concentration of about 0.1 mg/ml. Before a microinjection experiment, 3 M sodium acetate, pH 5.2, was added to 10 μg mRNA to give a final concentration of 0.3 M. mRNA was precipitated at −70°C for several hours, collected by centrifugation, washed twice in 70% ethanol, briefly dried, and resuspended in microinjection buffer.

The isolated mRNAs were shown to be functional for directing synthesis of IgG by in vitro translation in the presence of dog pancreas microsomes (Promega Biotec, Madison, WI) and [^{35}S]methionine/cysteine (Amersham Corp.), since labeled polypeptides with an M_r of 150,000 (nonreduced) and an M_r of ~53,000 and 25,000 (reduced) were isolated from translation mixes by binding to Protein G-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ).

Antibodies and Nucleoplasmin-Gold

The mAbs RL24 (anti-lamin B_2) and RL27 (anti-gp210) were obtained after immunizing mice with rat liver nuclear envelopes (Snow et al., 1987). Production of ascites was achieved as described (Greber et al., 1990). Purified IgG was obtained from ascite fluids or culture supernatants of hybridomas grown in serum-free Ex-Cell-300 medium (J. R. Scientific, Woodland, CA). IgG was isolated by binding to Protein G-Sepharose (Pharmacia Fine Chemicals) and eluting with 0.1 M Tris-Glycine, pH 3.0. Control mRNA used for cell microinjection experiments was prepared from hybridomas secreting RL24, and from the CRL-1713 and TIB-131 cell lines. The mouse hybridoma cell line CRL-1713 (secreting IgG, anti-DNA polymerase III, *Escherichia coli*) and TIB-131 (secreting IgG, anti-intermediate filament proteins) were purchased from American Type Culture Collection (Rockville, MD). They had been established by fusion of mouse spleen cells with the same mouse P3 cell line that we have used to create the RL24 (IgG, anti-lamin B_2) and RL27 (IgG, anti-gp210) lines. The polyclonal rabbit anti-gp210 antibody Rb-68 was obtained as described (Greber et al., 1990). Nucleoplasmin-coated gold complexes were prepared from purified *Xenopus* egg-nucleoplasmin and 10-nm colloidal gold particles (Janssen Life Sciences Products, Piscataway, NJ) according to published procedures (Dworetzky and Feldherr, 1988; Leunissen and DeMey, 1989).

Immunoadsorption and Immunoblotting

For immunoadsorptions, 10^7 NRK cells were lysed in 2 ml of PBS (Gibco Laboratories) containing 1% Empigen-BB (Calbiochem-Behring Corp., Indianapolis, IN), 1 mM EDTA, 1 mM PMSF, 1 μg/ml leupeptin and pepstatin, and 2 μg/ml aprotinin. The sample was cleared by centrifugation for 15 min at 13,000 *g* and gp210 and immunoadsorbed to purified RL27-IgG immobilized on CNBr-activated Sepharose (Pharmacia Fine Chemical). A control adsorption involved immobilized RL23 IgG, an antibody directed against a membrane protein of the endoplasmic reticulum (L. Gerace, unpublished observation). Immunocomplexes were washed four times with lysis buffer, once with PBS, dissolved in SDS sample buffer (Gerace and Blobel, 1980), and separated by SDS-PAGE (8% acrylamide; Laemmli, 1970). Molecular weight markers (Sigma Chemical Co.) comprised myosin (205 kD), beta-galactosidase (116 kD), phosphorylase (96 kD), BSA (66 kD), ovalbumin (45 kD), and carbonic anhydrase (30 kD). Immunoblotting was performed as described (Greber et al., 1990) and antigen was detected using rabbit anti-mouse IgG (2 μg/ml; Cappel Laboratories) and ^{125}I-protein A (10^8 cpm/μg) at 10^6 cpm/ml (Featherstone et al., 1988).

Results

Expression of a Functional Antibody to a Lumenal Epitope of Gp210 by Microinjection of mRNA

We have obtained a number of mAbs that react with the lumenal domain of gp210 (Greber et al., 1990). To investigate a possible interaction of this protein with the transport apparatus of the pore complex, we decided to examine whether binding of antibodies to the lumenal domain of gp210 influences pore complex functions. Such an effect would necessarily require a transmembrane structural change in the pore complex, since protein transport is carried out by machinery located on the cytoplasmic side of nuclear membranes (Gerace and Burke, 1988).

Antibodies cannot be delivered to the lumen of the ER by injection of purified IgG into the cytoplasm. However, this can be accomplished indirectly by microinjection of mRNA isolated from hybridoma lines secreting the appropriate antibody, which leads to production of assembled IgG in the ER lumen (Burke and Warren, 1984). In pilot studies where polyA+ mRNA from three anti-gp210 hybridoma lines was injected into cultured rat cells, we found that the highest level of IgG expression was obtained using a hybridoma line secreting RL27, an antibody that was not previously characterized. Because of this efficient in vivo expression and the lumenally disposed epitope of RL27 (see below), we have performed the work described in this study with RL27 mRNA.

A biochemical characterization of RL27 IgG is shown in Fig. 1. RL27 specifically immunoprecipitated a band of about 205 kD from detergent-solubilized NRK cells (Fig. 1 *A*). On immunoblots, this immunoadsorbed protein strongly reacted with the RL16 mAb (Fig. 1 *B*), which we previously found to be specific for gp210 (Greber et al., 1990). To determine whether RL27 recognizes an epitope in the lumenal domain of gp210, isolated nuclear envelopes from rat liver were digested with papain, and supernatant and pellet fractions were analyzed by immunoblotting (Fig. 1, *C* and *D*). Our previous studies showed that papain digestion of isolated nuclear envelopes released an ~200-kD fragment of the lumenal domain of gp210 into a supernatant fraction (Greber et al., 1990). RL27 reacted with both the intact gp210 in the membrane fraction (Fig. 1 *C*, lane *2*) and also with the ~200-kD lumenal fragment of gp210 released into the supernatant (Fig. 1 *C*, lane *1*). This contrasts with the polyclonal anti-peptide antibody Rb-68 specific for the cytoplasmic tail of gp210, which recognizes only the intact gp210 present in the membrane pellet fraction (Fig. 1 *D*, lane *2*). These data demonstrate that gp210 reacts with the lumenal domain of gp210, like the RL16 and RL20 anti-gp210 antibodies previously characterized (Greber et al., 1990). In immunofluorescence staining of NRK cells, culture supernatants from the RL27 cell line gave a similar pattern and intensity of immunofluorescence staining as previously obtained with staining of cultured rat cells with RL20 IgG (Greber et al., 1990; data not shown). This further confirms that RL27 reacts strongly with native gp210.

Direct immunofluorescence microscopy was used to ana-

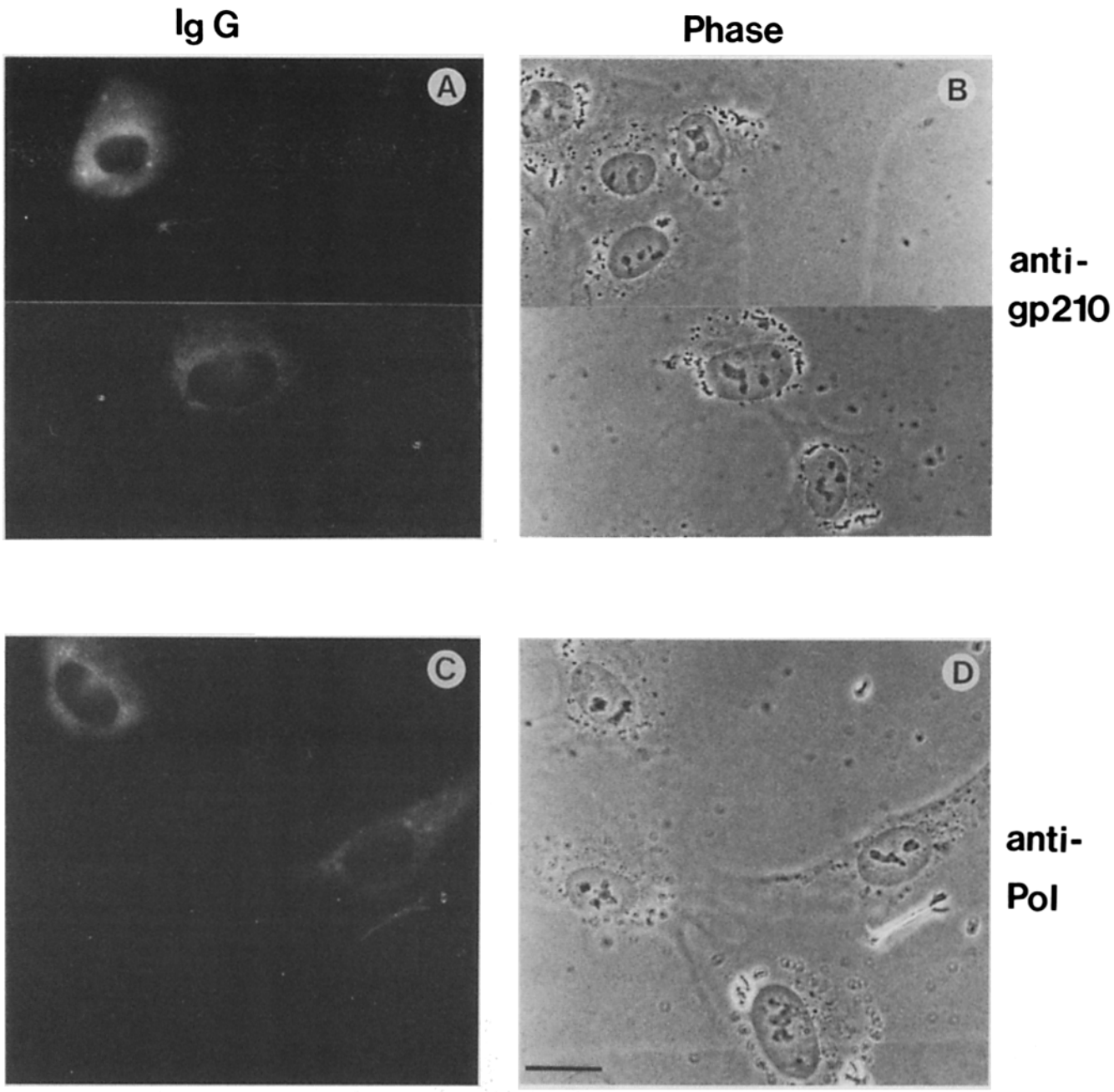


Figure 2. RL27 and anti-DNA polymerase IgGs are expressed in vivo following injection of mouse hybridoma mRNAs. NRK cells were microinjected with RL27 mRNA (anti-gp210, *A* and *B*) and control mRNA from a hybridoma line secreting antibodies to DNA polymerase III from *E. coli* (*anti-Pol*, *C* and *D*). After incubation for 2 1/2 h, cells were fixed in paraformaldehyde and stained with rhodamine-conjugated goat anti-mouse IgG (*A* and *C*). Corresponding phase contrast images are shown in *B* and *D*. Bar, 20 μm.

lyze the expression of mouse IgGs in NRK cells following microinjection of mRNAs isolated from hybridomas secreting RL27 and other control antibodies (Fig. 2). In cells that had been incubated for 2 1/2 h after mRNA injection (Fig. 2), anti-mouse IgG labeled a cytoplasmic reticular compartment, presumably corresponding to the ER/Golgi systems. Mouse IgG also was readily detectable in this reticular compartment 1 h post mRNA injection (data not shown). Microinjected mRNA from a cell line secreting an anti-DNA polymerase antibody led to the production of a qualitatively very similar level of mouse IgG compared to mRNA from the RL27 cell line (Fig. 2). Cells injected with anti-DNA polymerase mRNA are shown as controls in this study, although similar results were obtained with cells injected with mRNA isolated from hybridoma lines secreting antibodies directed against lamin B_2 (RL24) and cytoplasmic intermediate filaments (TIB-131; see Materials and Methods).

To determine whether the anti-gp210 IgG could bind to its antigen in vivo after it was synthesized, RL27 mRNA and control anti-DNA polymerase mRNA were injected into the cytoplasm of NRK cells and cells were incubated for 2 1/2 h at 37°C. Subsequently, cells were extracted with buffer con-

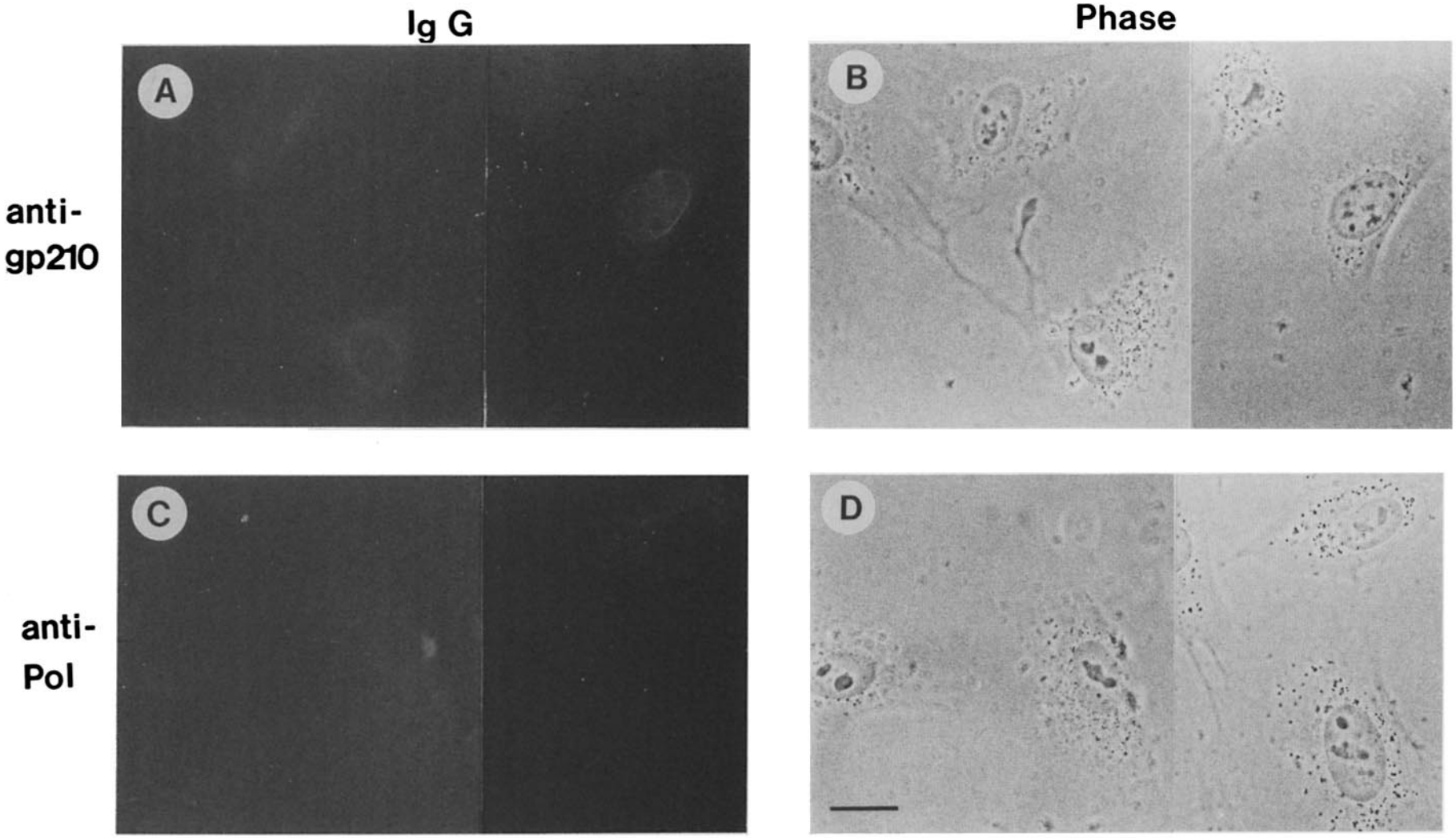


Figure 3. RL27 IgG specifically binds to the nuclear envelope after expression from microinjected mRNA. NRK cells were microinjected with hybridoma mRNAs encoding RL27 IgG (*anti-gp210, A* and *B*) and control anti-DNA polymerase IgG (*anti-Pol, C* and *D*). After incubation for 2 1/2 h, cells were extracted in ice cold buffer containing 0.1% Triton X-100, 0.02 M Hepes-NaOH, pH 7.4, 0.02 M NaCl, 0.001 M $MgCl_2$ for 45 s, immediately fixed in paraformaldehyde and processed for fluorescence microscopy using rhodamine conjugated goat anti-mouse IgG (*A* and *C*). Corresponding phase contrast images are shown in *B* and *D*. Cells injected with anti-Pol mRNA were identified based on their location on a gridded coverslip. Bar, 20 μm.

taining Triton X-100 and low salt to solubilize membranes and then were fixed for immunofluorescence microscopy to localize the extraction-resistant antibody. Under these conditions, most soluble proteins are removed from the ER lumen and from the cytoplasmic space, while proteins bound to insoluble structures are not extracted. Previous work demonstrated that gp210 remains associated with the detergent-stable pore complex under these conditions (Gerace et al., 1982; Greber et al., 1990). Following detergent extraction of cells injected with RL27 mRNA, mouse IgG was localized to the nuclear envelope in a perinuclear "rim" pattern by direct immunofluorescence, while almost no mouse antibody remained in more peripheral cytoplasmic regions (Fig. 3 *A*; compare to Fig. 2 *A*). In contrast, cells injected with control anti-DNA polymerase mRNA had no detectable IgG at the nuclear periphery and only very low levels of diffuse cytoplasmic staining (Fig. 3 *C*). These data indicate that anti-gp210 IgG synthesized by microinjected mRNA was functional in the lumen of the nuclear envelope and bound to gp210.

Lumenal Anti-gp210 Antibody Inhibits Mediated Nuclear Protein Import

We next examined whether binding of anti-gp210 antibody to the pore complex had an effect on mediated nuclear protein import in vivo. NRK cells were microinjected with RL27 mRNA or with anti-DNA polymerase mRNA together with an FITC-conjugated large dextran to identify the RNA-injected cells (Fig. 4, *A* and *D*). After incubation for 2 1/2 h at 37°C, cells were chilled to 0°C (to arrest ongoing nuclear import during the microinjection session), and injected a second time with rhodamine-conjugated nucleoplasmin, a large nuclear protein of *Xenopus* oocytes that is useful as a probe for studying mediated nuclear import in mammalian cells (e.g., Robbins et al., 1991). Cells then were incubated for 30 min at 37°C before being fixed and visualized by fluorescence microscopy (Fig. 4, *B* and *E*, *arrows*). As a further control, rhodamine-conjugated nucleoplasmin also was injected into nearby non-mRNA-injected cells.

The nuclei of cells injected with RL27 mRNA contained little nucleoplasmin, although some fluorescence could be detected in the nucleoli where the intranuclear nucleoplasmin tended to concentrate (Fig. 4 *B*). In contrast, nucleoplasmin-injected cells which had not been preinjected with mRNA (Fig. 4 *A*) showed strong nuclear rhodamine fluorescence and only a faint cytoplasmic signal (Fig. 4 *B*). Cells which were injected with control anti-DNA polymerase mRNA accumulated nucleoplasmin in the nucleus to a similar extent as did non-RNA-injected controls (Fig. 4 *E*). Therefore, expression of RL27 antibody had a specific inhibitory effect on import of nucleoplasmin, although it did not totally block nucleoplasmin uptake with our injection conditions (see Materials and Methods). It was not possible to determine whether expression of higher levels of RL27 antibody would completely inhibit nucleoplasmin import, since higher concentrations of mRNA could not be injected into cells due to the viscosity of these RNA solutions.

We used EM to quantify the effects of microinjected RL27 mRNA on mediated nuclear import. NRK cells were injected with RL27 mRNA or anti-DNA polymerase mRNA and after a 2 1/2-h incubation at 37°C, they were chilled to 0°C and injected with 10-nm colloidal gold particles coated with nucleoplasmin. Cells were then incubated for 10 or 30 min at 37°C to permit mediated transport, or were kept on ice for 10 min to inhibit mediated import. After fixation and processing for thin section EM, the number of gold particles in the nucleus vs. cytoplasm was quantified in cell sections having a similar ratio of cytoplasmic to nuclear area. This data was expressed as the ratio of nuclear to cytoplasmic particles (N/C ratio).

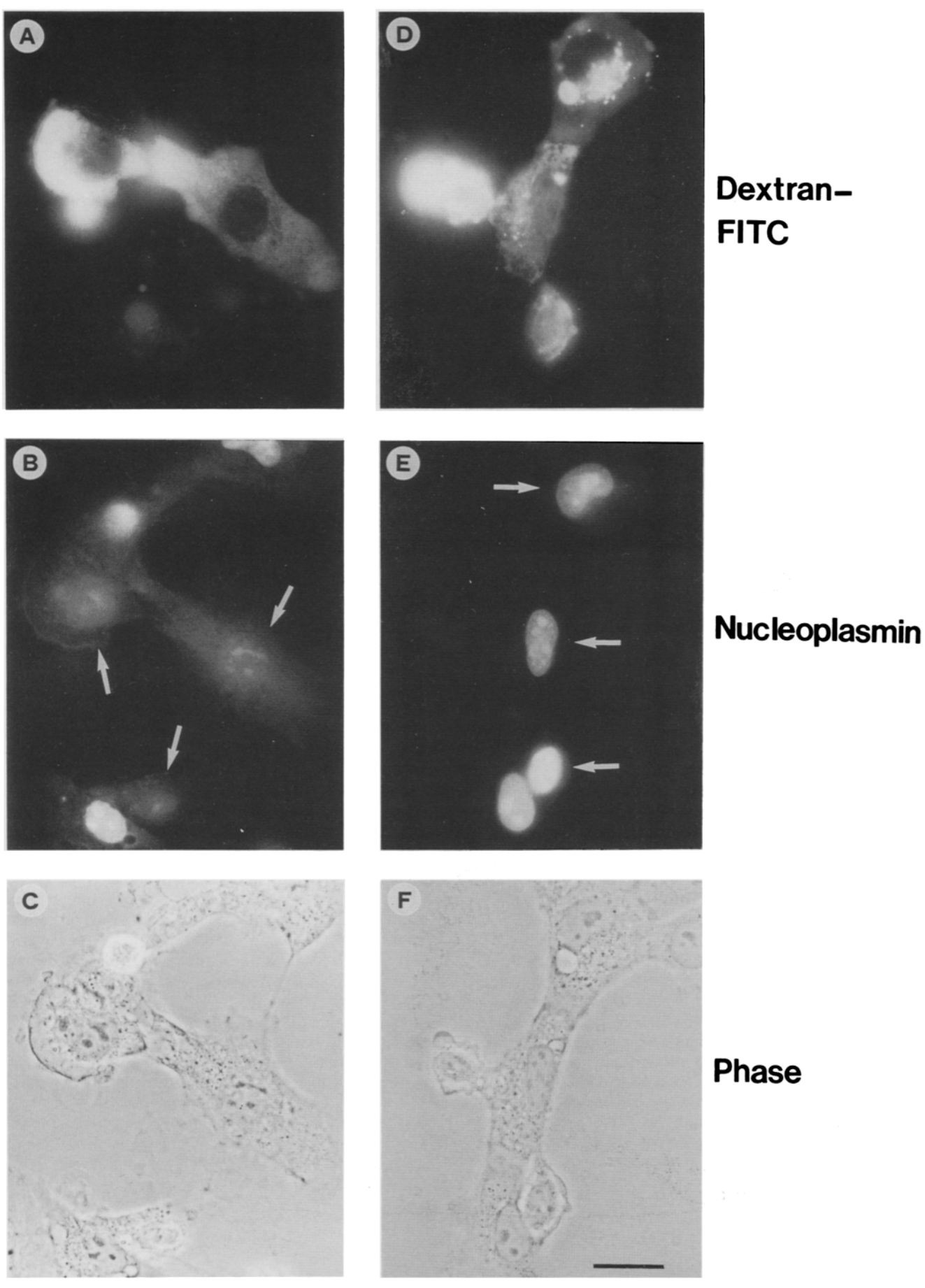
After 30 min at 37°C, control cells injected with anti-DNA polymerase mRNA had a N/C ratio of approximately 2, whereas cells injected with RL27 mRNA had an average N/C ratio of about 0.5 (Table I). After only 10 min, the N/C ratio in anti-DNA polymerase–injected cells had reached a similar level as seen after 30 min with RL27-injected cells. As expected from previous studies (Richardson et al., 1988), cells injected with anti-DNA polymerase mRNA and incubated at 0°C showed almost no import of nucleoplasmin-gold, with a N/C ratio of 0.04. Considered together, these data show that expression of RL27 IgG inhibits nuclear import of nucleoplasmin-gold particles approximately fourfold at 30 min, consistent with the qualitative results of fluorescence microscopy (Fig. 4).

Electron micrographs of cells injected with nucleoplasmin–gold conjugates are shown in Fig. 5. There was no detectable difference in morphology of pore complexes between cells injected with RL27 mRNA and cells injected with anti-DNA polymerase mRNA, although only low resolution information on the architecture of the pore complex can be deduced from thin section EM. In cells injected with RL27 mRNA as in cells injected with anti-DNA polymerase mRNA, pore complex–associated gold was frequently found near the cytoplasmic surface of pore complexes (Fig. 5 *A*), similar to previously published views of cells injected with nucleoplasmin–gold conjugates (Feldherr et al., 1984; Richardson et al., 1988).

Passive Diffusion from the Cytoplasm to the Nucleus Is Reduced by Anti-gp210

To examine whether the RL27 antibody affects the functional diffusion channel of the pore complex, NRK cells were injected with RL27 mRNA or control anti-DNA polymerase mRNA, together with rhodamine-labeled IgG as a marker for the injected cells. After incubation for 2 1/2 h to allow expression of IgG, cells were chilled on ice. A 10-kD fluorescent dextran was then injected into the cytoplasm of mRNA-injected cells and cells were further incubated for 30 min in the cold before fixation and examination by fluorescence microscopy (Fig. 6). Virtually all of the cells that were injected with RL27 mRNA had significantly lower levels of nuclear dextran fluorescence than cells injected with anti-DNA polymerase mRNA or cells that had not been injected with mRNA (Fig. 6, *B* and *E*). In three independent experiments comprising ~50 cells injected with RL27 mRNA and 10-kD dextran, ~40% of the cells excluded most dextran from the nucleus (similar to the two cells indicated by arrows in Fig. 6 *B*). The other 60% of the cells had some nuclear dextran fluorescence (similar to the cell indicated by an arrowhead in Fig. 6 *B*), although less than most control cells.

In all control cells, the 10-kD dextran was more concentrated in the nucleus than in the cytoplasm after the 30-min incubation in the cold (Fig. 6 *B*, internal controls and *E*, *arrows*). A similar phenomenon was also observed in previous


anti-
gp210
anti-
Pol
A
D
Dextran–
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B
E
Nucleoplasmin
C
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Phase

Table I. Nuclear Protein Import Is Specifically Inhibited by Anti-gp210 Antibody

	Nucleus	Cytoplasm	N/C
αGp210, 30′, 37°C	2,549	4,867	0.52 ± 0.05
αPol, 10′, 37°C	1,050	1,784	0.59 ± 0.09
αPol, 30′, 37°C	3,675	1,827	2.01 ± 0.55
αPol, 10′, chilled	90	2,174	0.04 ± 0.01

Exponentially growing NRK cells were microinjected with hybridoma mRNA coding for anti-gp210 IgG or anti-DNA polymerase IgG in defined areas of petri dishes. Cells were incubated for 2 1/2 h in growth medium, chilled on ice, and injected a second time with nucleoplasmin-coated 10-nm gold particles. After nuclear import reactions as indicated, cells were fixed, and processed for thin section EM. Gold grains in the nucleus (*N*) and cytoplasm (*C*) were quantified as described in Materials and Methods. In the first experiment with anti-gp210 IgG and a 30 min at 37°C uptake incubation, sections across four cells from two independent experiments were analyzed. In the second and fourth experiments with anti-DNA polymerase IgG import reactions for 10 min at 37°C and 10 min at 0°C, respectively, two cells from one experiment were analyzed in each case. In the third experiment with anti-DNA polymerase IgG and an import reaction for 30 min at 37°C, three cells from two independent experiments were analyzed for gold particles. Values of the N/C particle ratio are shown with the standard deviation of this ratio within each group of different cells analyzed.

studies involving microinjected 10-kD dextran (Paine et al., 1975; Peters, 1986), possibly because of a higher level of solute exclusion from the cytoplasm compared to the nucleus (Horowitz and Moore, 1974), or to higher nonspecific interaction with intranuclear components compared to cytoplasmic structures.

In cells injected with RL27 mRNA, we also have observed a clearly decreased rate of nuclear entry at 0°C of microinjected HRP, another small macromolecule that can freely diffuse across the nuclear envelope (data not shown). Considered together, our results indicate that the RL27 antibody significantly reduces the rate of diffusion of small macromolecules into the nucleus in addition to inhibiting mediated nuclear import.

Expression of Anti-Gp210 IgG Does Not Remove RL1 Antigens from the Nuclear Pore Complex

Recently, it has been demonstrated by an in vitro reconstitution assay that nuclear pore complexes lacking O-linked glycoproteins that bind wheat germ agglutinin are not functional for mediated import of nuclear proteins (Finlay and Forbes, 1990). We therefore tested whether inhibition of nuclear protein import in our system was a result of the removal of O-linked glycoproteins of the pore complex, which are recognized by the mAb RL1 (Snow et al., 1987).

NRK cells were microinjected with RL27 mRNA and with control anti-DNA polymerase hybridoma mRNA and incubated for 2 1/2 h for expression of antibodies. After fixation, cells were examined for RL1 antigens by indirect immunofluorescence (Fig. 7). mRNA-injected cells were identified by coinjection of fluorescently labeled BSA (Fig. 7, *A* and *D*). Compared to a population of control cells, the population of microinjected cells displayed a qualitatively indistinguishable pattern of punctate staining in the nuclear envelope with a comparable signal intensity (Fig. 7, *B* and *E*). Each fluorescent point probably represents either a single pore complex or a small cluster of pore complexes (Maul, 1977). This implies that expression of RL27 antibody did not lead to detectable removal or rearrangement of RL1 antigens of the nuclear envelope.

Direct quantification of the frequency of pore complexes in different populations of cells was performed by thin section EM (see Materials and Methods). At least 100 μm of nuclear envelope were examined in NRK cells for each experimental condition involving injection with RL27 mRNA, control mRNA, or noninjected cells. In all three cases, an average of about one pore complex per 2 μm of nuclear envelope was obtained in two to four independent experiments. Considered together with the immunofluorescence staining discussed above, these data indicate that the observed nuclear import inhibition of nucleoplasmin caused by the RL27 antibody was not simply a result of removal of pore complexes, but rather was because of constraints on pore complex functions.

Cells Are Arrested in G2 Phase by Anti-gp210 IgG

Since the expression of the RL27 antibody in interphase cells drastically restricts the protein transport functions of the pore complex, we examined whether expression of this antibody influences the progression of G2 phase NRK cells into mitosis. Such an effect would be expected if nuclear import of proteins (or nuclear export of RNAs) were required for activation of MPF, a primary initiator of M phase progression (Pines and Hunter, 1990). Cells grown on coverslips were synchronized in S phase by a double thymidine block. Five hours after thymidine release when most cells were in G2 phase, cells were injected with RL27 mRNA or with control anti-DNA polymerase mRNA together with a large FITC-conjugated dextran as a tracer. Cells were subsequently incubated at 37°C and monitored for progression through mitosis into G1 phase, as indicated by the appearance of cell doublets with roughly mirror image symmetry arising from cell division (e.g., Fig. 8 *C*). Normally, 60 to 80% of the mRNA-injected cells were recovered on the dish for this analysis. 4 h postinjection, only 10% of the cells injected with RL27 mRNA had divided, whereas 78% of the control cells had separated into two daughter cells (Table II). Typically, G2-arrested cells that had been injected with RL27 mRNA appeared larger than G2 control cells and often contained a nucleus almost twice the diameter of control nuclei (Fig. 8). We have not directly determined whether the nu-

Figure 4. Microinjection of hybridoma mRNA encoding RL27 IgG inhibits mediated nuclear import of nucleoplasmin. NRK cells in a defined area on a gridded coverslip were microinjected with mRNA encoding RL27 IgG (*anti-gp210, A–C*) or control anti-DNA polymerase IgG (*anti-Pol, D–F*) and incubated for 2 1/2 h for IgG expression. Petri dishes containing the injected coverslips were chilled on ice for 30 min, and rhodamine-conjugated nucleoplasmin was then injected into the cytoplasm of cells in the previously defined areas of the coverslip (*B* and *E*). The cells were subsequently moved to prewarmed medium, incubated for 30 min at 37°C, fixed, and examined by fluorescence microscopy. mRNA-injected cells were identified with coinjected 150-kD FITC-dextran (*A* and *D*), and nucleoplasmin-injected cells were identified by rhodamine fluorescence (*B* and *E*). Arrows point to cells double injected with mRNA and nucleoplasmin (*B* and *E*). The corresponding phase contrast images are shown in *C* and *F*. Bar, 20 μm.

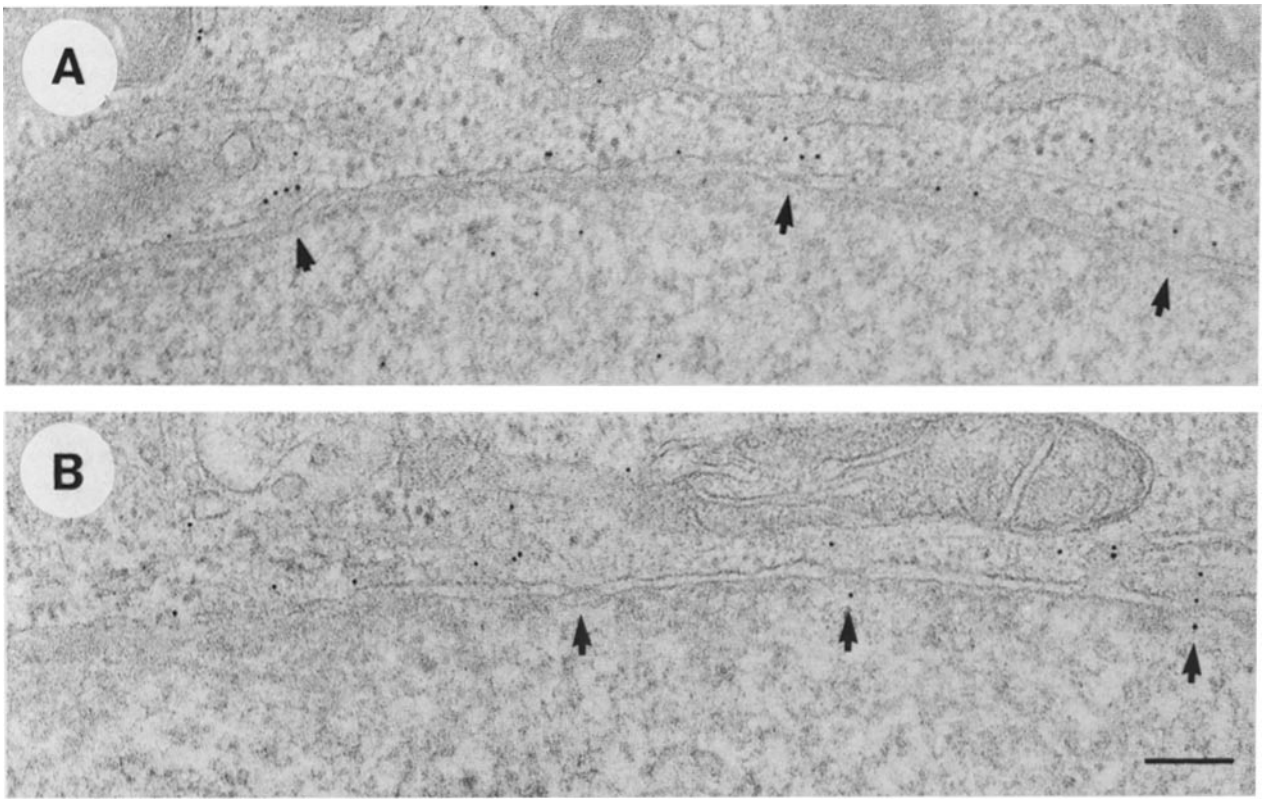


Figure 5. Visualization of nucleoplasmin-coated gold particles at the nuclear envelope of cells injected with RL27 mRNA. NRK cells grown in a defined area of a plastic dish were injected with mRNA encoding anti-gp210 IgG (*A*) and control anti-DNA polymerase IgG (*B*), incubated for 2 1/2 h at 37°C and chilled on ice for 30 min. Nucleoplasmin-coated gold (10 nm) was injected into the cytoplasm of cells in the defined areas of the dish, cold medium was removed and prewarmed medium was added, and the cells were incubated at 37°C for 30 min (*A*) or for 10 min (*B*). The cells were then processed for thin section EM. Arrowheads indicate examples of nuclear pore complexes, most of which have associated nucleoplasmin–gold particles. A 10-min timepoint is shown for cells preinjected with anti-DNA polymerase mRNA and incubated at 37°C, since by 30 min in these cells most gold had been imported in the nucleus (Table I) and little gold could be seen at the nuclear envelope (data not shown). Bar, 200 nm.

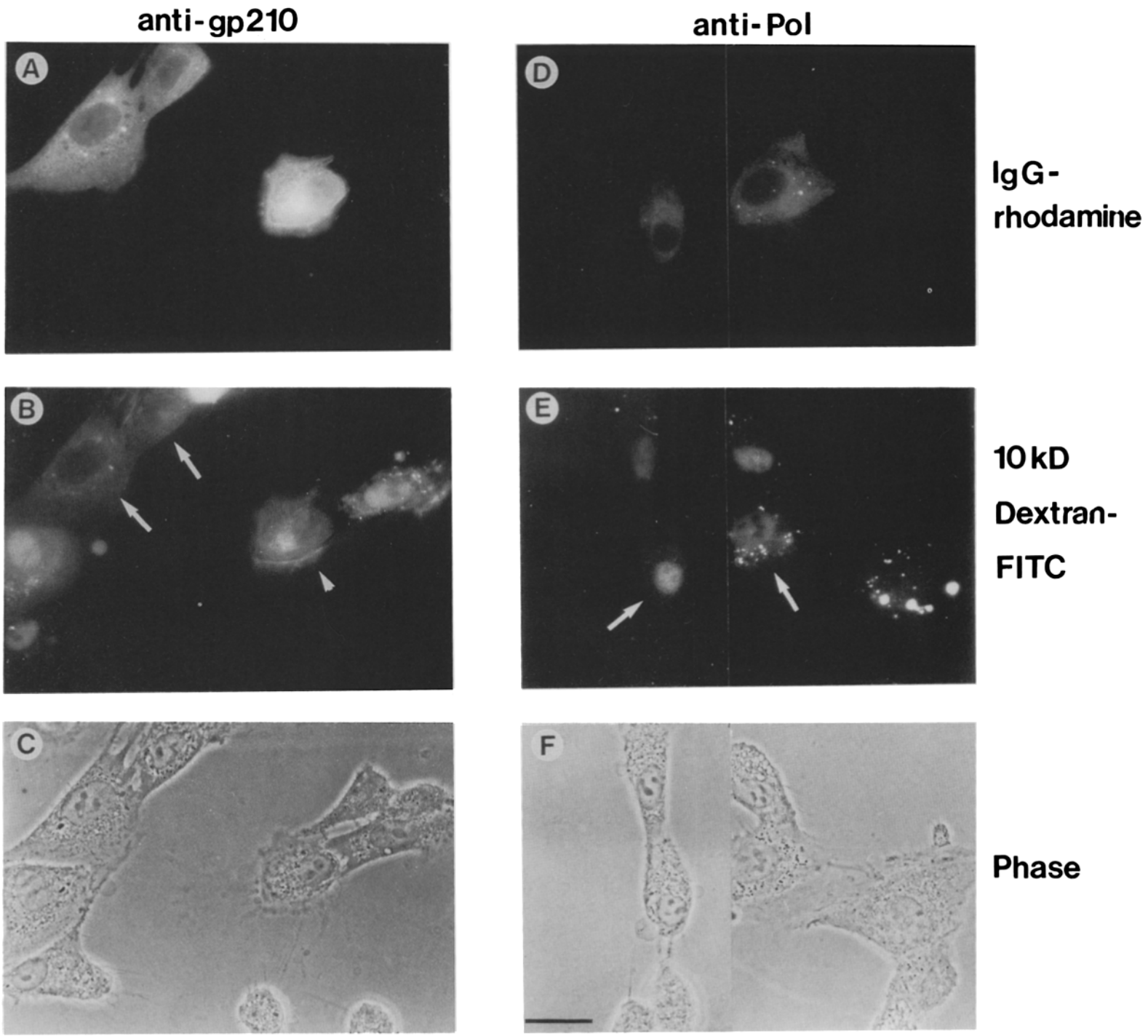
cleus of G2-arrested cells actually increased in volume as suggested by this morphological change, or whether they simply became more flattened. 24 h postinjection, only 44% of the cells injected with RL27 mRNA had divided, while 94% of the control cells had undergone division (Table II). This indicates that the arrest in G2 phase is reversible. This could be due either to turnover of the RL27 IgG and/or of the mRNA encoding RL27, or to the low levels of mediated import and passive diffusion encountered in RL27-injected cells.

A similar G2 arrest phenotype was obtained by microinjecting a 1 mg/ml solution of wheat germ agglutinin into the cytoplasm of late G2 cells (data not shown). Wheat germ agglutinin is known to inhibit mediated nuclear protein import but not passive diffusion in vivo (Dabauvalle et al., 1988*b*; Yoneda et al., 1987) and in vitro (Finlay et al., 1987; Adam et al., 1990). This result supports the notion that the observed G2 arrest caused by expression of anti-gp210 antibody could be due to inhibition of mediated protein transport across the nuclear envelope.

Discussion

In this study we have obtained direct evidence that gp210, an integral membrane protein of the nuclear pore complex, plays an important role in organization of the pore complex associated with mediated transport and passive diffusion. We introduced a mAb (RL27) against gp210 into the lumen of the ER and nuclear envelope of cultured rat cells by microinjection of polyA+ mRNA from a hybridoma cell line secreting RL27. The RL27 antibody, which reacts with the lumenal domain of gp210, bound to the nuclear envelope

Figure 6. The rate of diffusion of a 10-kD dextran across the nuclear envelope is reduced in cells microinjected with RL27 mRNA. NRK cells in a defined area on a gridded coverslip were injected with hybridoma mRNA encoding RL27 IgG (*anti-gp210, A–C*) or anti-DNA polymerase IgG (*anti-Pol, D–F*). After an incubation of 2 1/2 h, the cells were chilled on ice for 30 min and subsequently injected with 10-kD FITC–dextran (*B* and *E*). After a further incubation for 30 min on ice, cells were fixed and observed in the fluorescence microscope. RNA-injected cells were visualized with coinjected rhodamine–labeled goat IgG (*A* and *D*). Arrows point to cells that were double injected with mRNA and dextran (*B* and *E*). Corresponding phase contrast images are shown in *C* and *F*. Bar, 20 μm.


anti-gp210
anti-Pol
A
D
IgG-
rhodamine
B
E
10kD
Dextran-
FITC
C
F
Phase

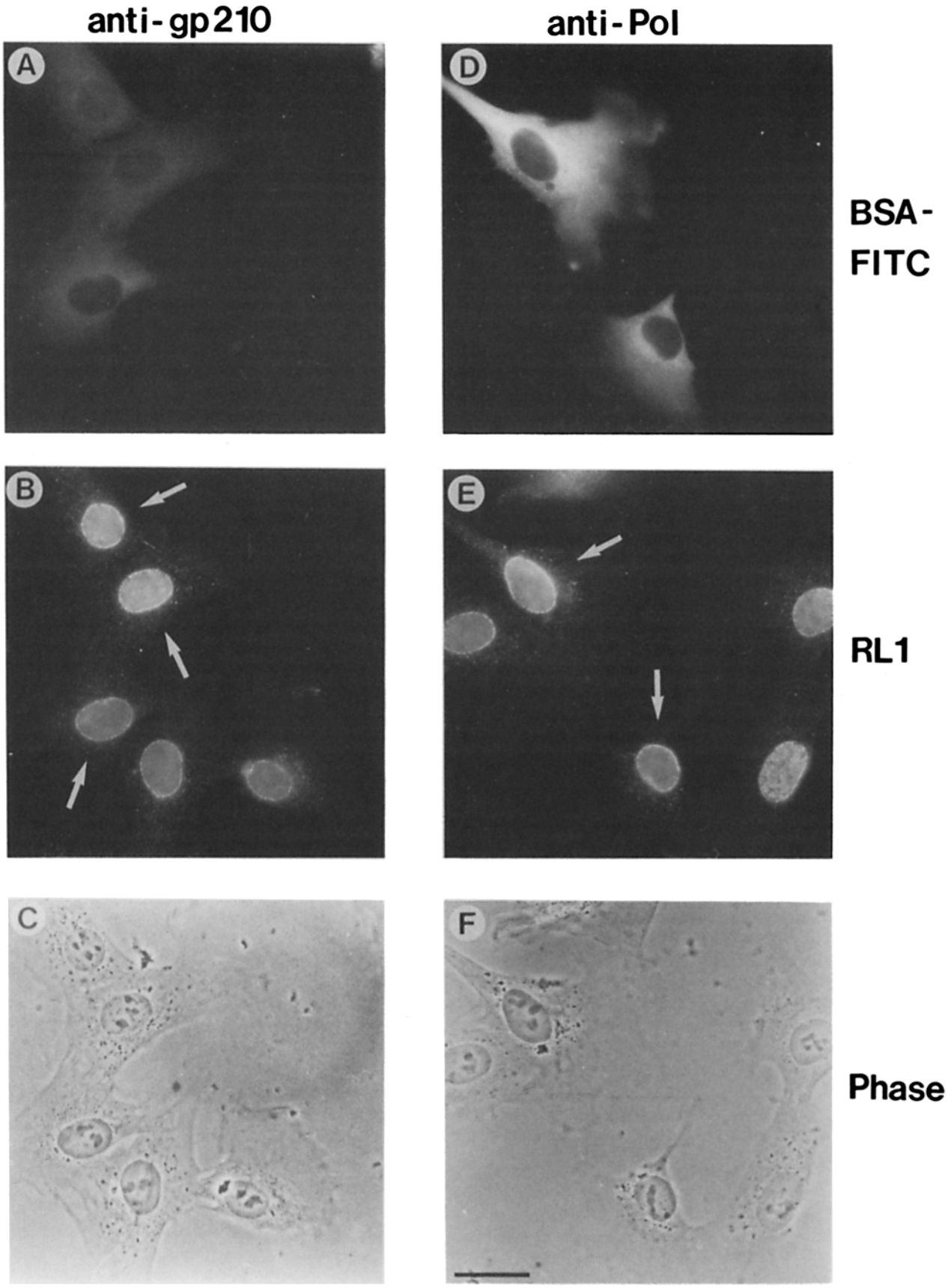


Figure 7. Pore complex antigens recognized by the RL1 antibody persist in the nuclear envelope of cells microinjected with RL27 mRNA. NRK cells were microinjected with hybridoma mRNAs encoding anti-gp210 IgG (*A–C*) and anti-DNA polymerase IgG (*D–F*) and incubated for 2 1/2 h at 37°C before fixation and further processing for immunofluorescence microscopy using the RL1 monoclonal antibody (*B* and *E*). mRNA injected cells were identified with coinjected FITC-BSA (*A* and *D*) and are indicated by arrows in *B* and *E*. The corresponding phase contrast images are shown in *C* and *F*. Bar, 20 μm.

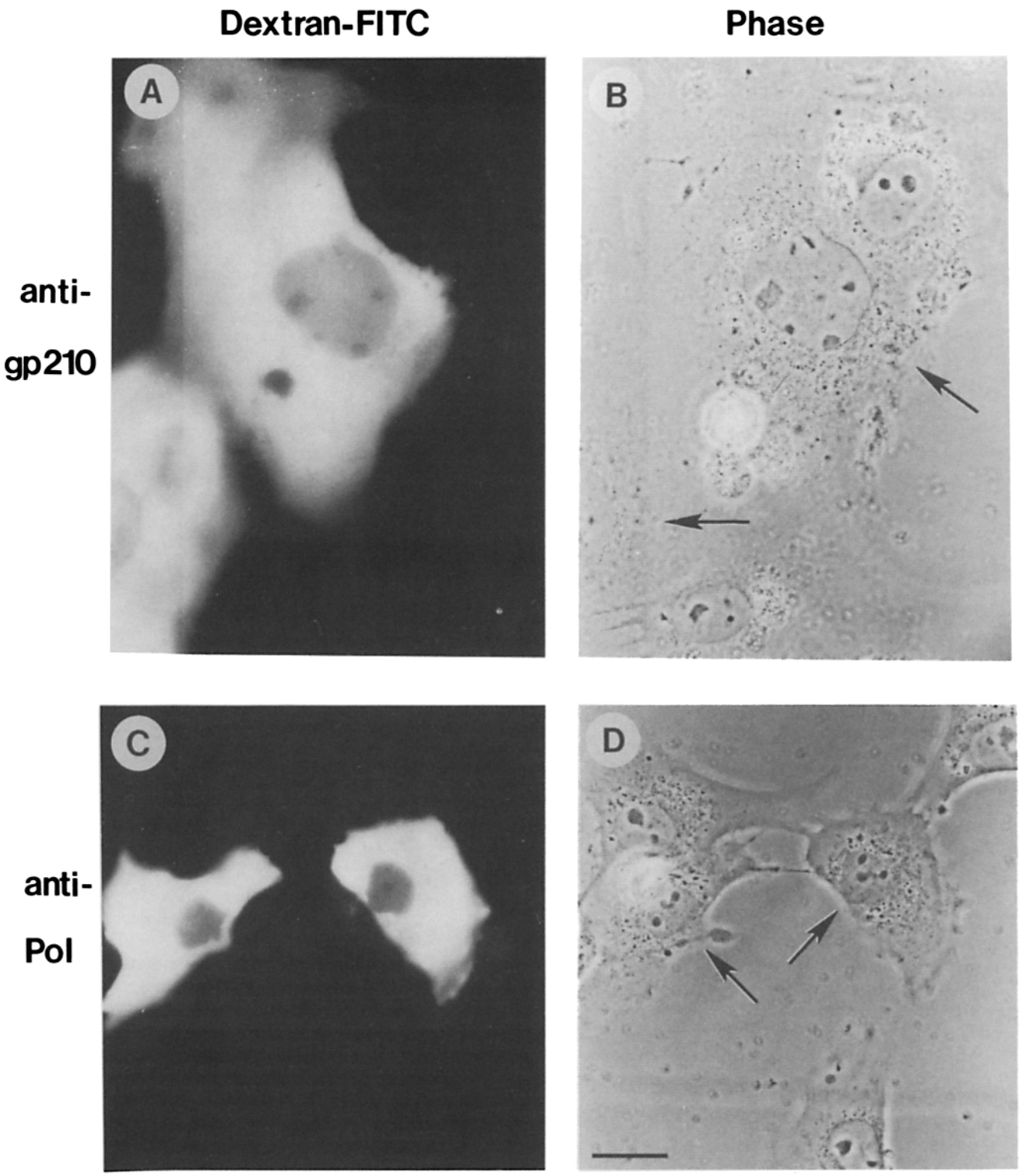


Figure 8. Microinjection of mRNA encoding RL27 IgG inhibits progression of G2 cells into M phase. NRK cells were synchronized in S phase by a double thymidine block and subsequently grown for 5 h to accumulate the populations in G2 phase. They then were microinjected with hybridoma mRNAs encoding RL27 IgG (*anti-gp210*, *A* and *B*) and anti-DNA polymerase IgG (*anti-Pol*, *C* and *D*). mRNA-injected cells were identified with coinjected 150-kD FITC–dextran (*A* and *C*). Corresponding phase contrast images are shown in *B* and *D*. Arrows point to cells injected with mRNA. Bar, 20 μm.

during the course of in vivo expression, as indicated by its specific retention in a detergent-insoluble structure of the nuclear envelope. It was possible to subsequently measure several functional properties of the pore complex, including mediated transport and passive diffusion.

While the epitope for RL27 was strongly detectable in gp210 of all cultured rat cells tested, it was absent from mouse gp210 based on immunofluorescence microscopy and immunoprecipitation studies performed on mouse 3T3 cells (data not shown). This lack of cross-reactivity with gp210

Table II. Expression of Anti-gp210 Antibody in G2 Cells Blocks Progression into Mitosis

	mRNA	Undivided cells	Divided cells	Divided cells
				%
4 h after injection	αGp210	71	8	10
	αPol	15	53	78
20 h after injection	αGp210	31	26	46
	αPol	3	47	94

The experiment was performed as described in Fig. 8. The data are from three independent experiments (4 h after injection) and two independent experiments (20 h after injection).

of mouse, the source of the hybridoma cells, is understandable considering the inhibitory properties of RL27 on cell cycle progression.

Inhibition of Mediated Protein Import and Passive Diffusion into the Nucleus by RL27

We found that in vivo expression of the anti-gp210 antibody dramatically inhibited the ability of the cell to import fluorescently labeled nucleoplasmin into the nucleus. Quantitative analysis showed that nuclear import of nucleoplasmin-gold conjugates was reduced approximately fourfold at 30 min after injection. Light microscope immunocytochemistry and EM demonstrated that this effect was not because of removal or large-scale structural alteration of pore complexes. Considered together, the light and electron microscopic analyses indicate that the anti-gp210 antibody acted by diminishing the rate of nuclear protein uptake. Whether all pore complexes are inhibited to a similar extent by this antibody is unknown.

In addition to influencing mediated protein import, the RL27 antibody also apparently reduced the size of the functional diffusional channel of the pore complex, since the rate of nuclear entry of a 10-kD dextran was significantly reduced by the RL27 antibody in essentially all cells. Considered together, the results of the nucleoplasmin and dextran transport studies indicate that gp210 is either directly or indirectly linked to structures important for mediated protein transport and passive diffusion across the pore complex.

Cells that were injected with RL27 IgG were found to be almost completely inhibited in their ability to progress from G2 phase to mitosis. This block in cell cycle progression persists for a long period of time, since even 20 h after mRNA injection, <50% of cells injected with RL27 mRNA had divided. This inhibition may be explained by the inhibitory effects of gp210 on nuclear protein import, and directly indicates that nucleocytoplasmic trafficking is required either directly or indirectly for activation of $p34^{cdc2}$ kinase (for review see Pines and Hunter, 1990) and as a consequence, for progression into M phase.

Transport Inhibition and Organization of the Pore Complex

How could binding of a mAb to the lumenal domain of gp210 influence the pathways for both mediated transport and passive diffusion, which are controlled by components located on the cytoplasmic side of nuclear membranes? This outcome clearly must be because of a transmembrane effect on pore complex structure. However, the RL27 antibody could have many conceivable effects on pore complex structure related either to static or dynamic interactions of gp210 with other components of the pore complex. Insight on the mechanism of transport inhibition should come from additional studies with the RL27 antibody and a detailed understanding of how gp210 interacts with other proteins of the pore complex.

The primary sequence (Wozniak et al., 1989) and transmembrane topology (Greber et al., 1990) of gp210 have been determined, but its precise localization within the context of the 3-D structure of the pore complex is unknown. Recently, thin section electron microscopy of isolated nuclear envelopes have clearly revealed "knobs" protruding into the perinuclear space immediately adjacent to the plug-spoke complex (Aebi et al., 1990). These knobs may correspond to "radial arms" that are seen to extend outward beyond the nuclear and cytoplasmic rings when pore complexes were viewed in projection (Akey, 1989). Considering that gp210 is a very abundant integral membrane protein of the pore complex, the "knobs" emanating from the plug-spoke region are good candidates for structures that contain, or consist of gp210. Hence, gp210 may be linked to the plug-spoke complex that defines the central channel of the pore complex, and which presumably is responsible for providing the major diffusional restriction across the pore as well as a gated channel for mediated transport of macromolecules (Feldherr et al., 1983; Akey, 1989; Reichelt et al., 1990). This is a very plausible notion in light of our results, which suggest that gp210 is either directly or indirectly connected to structures important for mediated transport and passive diffusion. While gp210 may be associated with the plug-spoke complex, the small size of the cytoplasmic tail of gp210 (58 amino acids; Greber et al., 1990) argues that gp210 itself is not present in the central channel of the plug-spoke region, which is ~30–50 nm from the membrane (Reichelt et al., 1990).

Is There Lumenal Regulation of the Pore Complex?

Nuclear import of proteins can be regulated by several different mechanisms, including controlled release of proteins containing NLSs from anchorage sites at cytoplasmic structures, activation of NLSs by posttranslational mechanisms, and changes in the components of the nuclear transport apparatus itself (Gerace and Burke, 1988; Silver, 1991). Several examples of the last mechanism have been described. In *Tetrahymena* where micronuclei and macronuclei are present in a common cytoplasm, both nuclei have morphologically similar pore complexes, yet micronuclei are incapable of importing many nuclear proteins that are readily taken up by macronuclei (White et al., 1989). A second example is provided by cultured mammalian cells, where the rate of mediated nuclear import of nucleoplasmin differs substantially in growth-arrested, compared to rapidly dividing cells (Feldherr and Akin, 1990).

These results suggest that the pore complex and/or soluble factors involved in nuclear import can be regulated in response to different growth states of the cell and in relation to the state of differentiation. A priori, it seems likely that much of this regulation will be mediated by signals transmitted through the cytoplasmic space that interact with these components. The data presented in this study on the effects

of the RL27 antibody raise the interesting possibility that pore complex permeability also may be regulated from the lumenal side of the ER/nuclear envelope system.

While it is possible that RL27 "nonphysiologically" perturbs pore complex functions via alteration of gp210 organization, it also is conceivable that inhibition mediated by binding of RL27 partially mimics some physiological pathway. According to this scenario, gp210 (or associated lumenal proteins) could serve as a conduit for transmitting signals from the lumen of the ER to the cytoplasmically localized transport apparatus. While binding of RL27 to gp210 diminishes the permeability of the pore complex, lumenal signals acting through a transmembrane pathway at the pore complex in principle could either inhibit or stimulate nuclear transport.

Lumenal regulation of nuclear envelope permeability could be important in a number of different physiological situations. For example, this could be a component of short-term responses to cells to growth factors (Berridge and Irvine, 1989). In numerous cases, growth factor signaling involves a transient decrease in the free pool of ER calcium, which in turn could directly or indirectly affect the structure of gp210. It could also be a part of an intermediate-term response of cells to environmental conditions that regulate secretion, membrane traffic, and the lumenal structure of the ER. Included in this latter category would be the heat shock response. Finally, different types of lumenal regulation of the pore complex could be associated with specific differentiation states of the cell.

In conclusion, the results of the present study underscore the importance of gp210 in the functional organization of the pore complex, and emphasize the need for high resolution biochemical and structural analysis of gp210 and associated proteins. Furthermore, this work suggests that a possible influence of the ER lumenal compartment on nucleoplasmic transport should be explored in more detail.

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