Inhibition of In Vitro Nuclear Transport by a Lectin that Binds to Nuclear Pores

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Abstract. Selective transport of proteins is a major mechanism by which biochemical differences are maintained between the cytoplasm and nucleus. To begin to investigate the molecular mechanism of nuclear transport, we used an in vitro transport system composed of a *Xenopus* egg extract, rat liver nuclei, and a fluorescently labeled nuclear protein, nucleoplasmin. With this system, we screened for inhibitors of transport. We found that the lectin, wheat germ agglutinin (WGA), completely inhibits the nuclear transport of fluorescently labeled nucleoplasmin. No other lectin tested affected nuclear transport. The inhibition by WGA was not seen when *N*-acetylglucosamine was present and was reversible by subsequent addition of sugar. When rat liver nuclei that had been incubated with ferritin-labeled WGA were examined by electron microscopy, multiple molecules of WGA were found bound to the cytoplasmic face of each nuclear pore. Gel electrophoresis and nitrocellulose transfer identified one major and several minor nuclear protein bands as binding ^{125}I-labeled WGA. The most abundant protein of these, a 63–65-kD glycoprotein, is a candidate for the inhibitory site of action of WGA on nuclear protein transport. WGA is the first identified inhibitor of nuclear protein transport and interacts directly with the nuclear pore.

THE transport of macromolecules between the nucleus and cytoplasm appears to occur through the nuclear pores (Stevens and Swift, 1966; Feldherr et al., 1984). Structural studies based on electron microscopic observations indicate that the pore is composed of two prominent rings, one of which is located on the cytoplasmic surface of the nuclear envelope and the other on the nucleoplasmic surface. These rings define the periphery of the pore, each ring being composed of eight globular subunits (Maul, 1977; Franke et al., 1981; Unwin and Milligan, 1982). Located in the center of the pore is a central granule that appears to be connected to the ring subunits by spokes (Unwin and Milligan, 1982). At the molecular level, Gerace et al. (1982) have identified a concanavalin A (Con A)–binding glycoprotein of 180 kD molecular mass located at or near the nuclear pore. Fisher and colleagues have proposed that a 190-kD Mg^{++}-ATPase may also be a functional pore component (Berrios et al., 1983; Berrios and Fisher, 1986; Fisher, 1987). Most recently, Davis and Blobel (1986) identified a 62-kD protein that is a constituent of the nuclear pore. Although much is known about the structural morphology of the nuclear pore, relatively little is known about the pore proteins that play an active role in the transport of molecules through the pore.

The effective diameter of the nuclear pore has been measured as ~90 Å (Paine et al., 1975). Macromolecules of small diameter are able to enter the nucleus by passive diffusion, whereas macromolecules of large diameter cannot (Bonner, 1975; Feldherr and Ogburn, 1980; Einck and Bustin, 1984; for reviews see Paine and Horowitz, 1980; De Robertis, 1983; Dingwall, 1985). Nuclear proteins of large size contain one or more signal sequences that direct them to the nucleus, possibly by an active transport mechanism (Dingwall et al., 1982; Hall et al., 1984; Kalderon et al., 1984*a*, *b*). During transport, the signal sequence of the nuclear protein is thought to interact either with the nuclear pore itself or possibly with a carrier protein, which then ferries the protein to or through the nuclear pore.

The *Xenopus laevis* nuclear protein, nucleoplasmin, has been the transport substrate of choice in a number of studies. Nucleoplasmin is an abundant oocyte nuclear protein of ~150 kD molecular mass and is composed of five identical subunits. Because it is easy to isolate, is stable, and is transported efficiently into the nucleus (Mills et al., 1980; Krohne and Franke, 1980; Dingwall et al., 1982), it is an ideal substrate for nuclear transport studies. Radiolabeled nucleoplasmin has been shown to accumulate to high levels within the oocyte nucleus when injected into oocyte cytoplasm and such accumulation requires a signal domain (Dingwall et al., 1982). Feldherr et al. (1984) have shown that nucleoplasmin–gold complexes enter the nucleus through the nuclear pore. Recently, Newmeyer et al. (1986*b*) found, using an autoradiographic assay, that radiolabeled nucleoplasmin was transported into synthetic nuclei and that such transport required ATP. We have subsequently developed an in vitro fluorescence assay that allows us to follow the transport of rhodamine isothiocyanate (RITC)-labeled nucleoplasmin into a nucleus as it occurs (Newmeyer et al., 1986*a*).

We have used this rapid in vitro assay of nuclear protein

transport to screen for inhibitors of transport. The assay, which uses an extract of *Xenopus* eggs, added rat liver nuclei, and RITC-labeled nucleoplasmin, allows the direct microscopic observation of transport within 30 min of the addition of nucleoplasmin. The in vitro assay faithfully mimics in vivo nuclear transport. Using this assay, we have identified an inhibitor of nuclear protein transport, the lectin wheat germ agglutinin (WGA).[1] Electron microscopy using ferritin-labeled WGA supports a direct interaction of this inhibitor with the nuclear pore. Our results indicate that the pore contains a novel glycoprotein that plays an essential role in the mechanism of nuclear protein transport. Further experiments point to a nuclear glycoprotein of 63–65 kD as a possible target of action for WGA in inhibition of nuclear transport.

Materials and Methods

Materials

Tetramethyl rhodamine-labeled nucleoplasmin was isolated essentially by the method of Dingwall et al. (1982), and was RITC-labeled as described by Newmeyer et al. (1986*a*). Fluorescein isothiocyanate (FITC)-labeled lectins were purchased from Polysciences, Inc., Warrington, PA. *N,N',N''*-triacetyl chitotriose was obtained from Sigma Chemical Co., St. Louis, MO. Unlabeled WGA and *N*-acetyl-D-glucosamine were obtained from Calbiochem Behring Corp., La Jolla, CA. An autoimmune antilamin antiserum, which reacts with the rat nuclear lamins A and C, was a gift from Frank McKeon (Harvard University Medical School, Boston, MA). Iodobeads were purchased from Pierce Chemical Co., Rockford, Illinois.

Preparation of Nuclei, Nuclear Fractions, and Egg Extracts

Rat liver nuclei were prepared essentially by the method of Blobel and Potter (1966) with slight modifications and the addition of 0.5 mM spermidine. Rat liver nuclei (1–5 × 10^5/μl) were stored frozen at −70°C in the same buffer plus 250 mM sucrose.

Rat liver nuclear envelopes (ghosts) were isolated as described by Dwyer and Blobel (1976). Demembranated sperm nuclei were prepared by the method of Lohka and Masui (1983) and stored frozen at −70°C at a concentration of 1–4 × 10^5/μl. *Xenopus* embryonic nuclei, isolated from 9-h-old embryos, were prepared by gently Dounce-homogenizing the embryos and centrifuging the embryo extract in a clinical centrifuge to remove yolk granules. The majority of embryonic nuclei were not removed by this centrifugation and could be assayed for transport by the addition of tetramethylrhodamine isothiocyanate (TRITC)-labeled nucleoplasmin and an ATP-regenerating system. Nuclei reconstituted from bacteriophage DNA were prepared as described in Newmeyer et al. (1986*a*) and in Newport, 1987.

Xenopus egg extracts were prepared essentially by the method summarized in Newport and Forbes (1985). *Xenopus* eggs were dejellied with a 5-min incubation in 2% cysteine, pH 8, activated with the calcium ionophore A23187, and packed and lysed by centrifugation in buffer containing 250 mM sucrose, 50 mM KCl, 1 mM DTT, 2.5 mM MgCl, 0.02 mg/ml cycloheximide, and 0.005 mg/ml cytochalasin B. The cleared extract was recentrifuged and used for the transport assay either immediately or within several hours if stored on ice.

Nucleoplasmin Transport Assay

Transport was assayed as described (Newmeyer et al., 1986*a*). Briefly, nuclei (0.5–3 × 10^5 in 1 μl) were added to 20 μl of egg extract supplemented with 1–2.75 mM ATP, 9 mM creatine phosphate, and 100 U/μl of creatine kinase. For assays using rat liver nuclei, the nuclei were incubated in the extract for 30 min to allow equilibration with the extract and/or healing of any small perforations. At this time 1 μl of TRITC-labeled nucleoplasmin (final concentration, ~15 ng/μl) was added. Transport of nucleoplasmin was assayed microscopically by taking aliquots at various times after nucleoplasmin addition. For this, 4–5 μl of the transport reaction mixture was placed on a slide and mixed with 0.5 μl 37% formaldehyde and 0.5 μl of 10 μg/ml bisbenzimide DNA dye (Hoechst 33258) before the coverslip was added. For experiments with demembranated sperm nuclei, the sperm nuclei were added to the extract and allowed to reacquire a nuclear envelope, decondense their DNA, and swell to nuclei several times the size of rat liver nuclei (~30–60 min) before the addition of TRITC-labeled nucleoplasmin.

Effects of Lectins on Nuclear Transport

To assay for binding of FITC-labeled lectins to nuclei in egg extract, one of two procedures that gave equivalent results was followed. In one, the labeled lectin was added to the extract to a concentration of 0.1 mg/ml and the nuclei observed microscopically 30 min later. In the second, the FITC-labeled lectin was added (0.5 μl of a 1 mg/ml stock solution) to a slide with a 5-μl sample of extract containing nuclei and the mixture immediately examined.

To assay the effect of lectin addition on transport, rat liver nuclei were added to 20 μl of egg extract (final concentration of 1–5 × 10^5 nuclei/20 μl) and allowed to incubate for 20 min. At this time, FITC lectin (1 mg/ml in PBS) was added to the extract (final concentration of 0.1 mg/ml) and incubated for 5 min at ambient temperature. An equivalent volume of PBS was added to control incubations instead of lectin. After the 5-min preincubation with lectin, 1/20th volume of nucleoplasmin was added to a final concentration of 15 μg/ml. Aliquots were assayed microscopically for accumulation or lack thereof 20 min later. In some incubations, *N*-acetyl-D-glucosamine was added to a final concentration of 0.5 M before the addition of WGA, or *N,N',N''*-triacetyl chitotriose was added to a concentration of 1 mM.

To determine whether the inhibition observed with WGA resulted from an interaction of the lectin with the nuclei or, instead, with the extract, 10 μl of rat liver nuclei were added to 100 μl of egg extract. WGA (50 μl) was added to one-half of this mixture (final concentration 0.1 mg/ml), and 50 μl of PBS was added to the other half. Each was allowed to incubate for 5 min at ambient temperature before dilution fivefold with buffer (80 mM KCl, 5 mM EDTA, 15 mM Pipes, pH 7.0, 200 mM sucrose, 7 mM $MgCl_2$). The diluted nuclei were layered on a 15/40% Percoll step gradient, which was then centrifuged at 1000 *g* for 10 min. The nuclei, which band at the interface between the two Percoll phases, were removed, diluted fivefold (final volume 300–500 μl) with the above buffer, and pelleted for 30 s in a microfuge. The nuclear pellet (1–2 μl) was resuspended in 20 μl of egg extract and incubated for 20 min before addition of 1/20th volume of TRITC-labeled nucleoplasmin. The percent of nuclei accumulating nucleoplasmin in the nuclei preincubated with WGA and the control nuclei was assayed microscopically in the usual manner.

Fluorescence Microscopy

Samples were observed using a Zeiss Photomicroscope II fitted for fluorescence visualization of FITC, RITC, and the DNA dye, bisbenzimide. The level of TRITC-nucleoplasmin accumulation in a single accumulating nucleus relative to external concentration could be quantitated by densitometric scanning of photographic negatives.

Gel Electrophoresis, Immunoblotting, and Radiolabeled Lectin Blotting

The proteins present in nuclear or extract samples were prepared for gel electrophoresis by solubilization in 10% glycerol, 4% SDS, 0.125 M Tris-HCl, pH 6.8, 0.5% bromophenol blue, and 0.05% 2-mercaptoethanol, and by boiling for 3 min. The solubilized samples were loaded onto 10% polyacrylamide SDS gels prepared by the method of Laemmli (1970) and electrophoresed at 200 V for 4 h. The proteins were electrophoretically transferred onto nitrocellulose (Schleicher & Schuell, Inc., Keene, NH) using a Bio-Rad transblot apparatus (100 V for 4 h to overnight) in 25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% SDS, and 20% methanol.

Protein blots were probed for the presence of WGA-binding glycoproteins by incubation with ^{125}I-labeled WGA using the method of Bartles and Hubbard (1984), which involves incubation at 4°C in 15 ml of PBS containing 2% polyvinylpyrrolidone and 0.5–4 × 10^6 cpm (per 15 ml) of iodinated WGA. The method of incubation was slightly modified in that the polyvinyl-

1. *Abbreviations used in this paper*: GlcNAc, *N*-acetylglucosamine; WGA, wheat germ agglutinin.

pyrrolidone used was either 360 or 40 kD in size and periodate-aniline-cyanoborohydride treatment was omitted. The blots were exposed for autoradiography with Kodak X-OMAT AR5 film.

To prepare iodinated WGA, Iodobeads were preincubated with 0.5 mCi of ^{125}I-iodine (Amersham Corp., Arlington Heights, IL) for 5 min at ambient temperature in 0.1 M potassium phosphate, pH 7.5, 0.2 M *N*-acetyl-D-glucosamine. WGA in 0.1 M potassium phosphate, pH 7.5, 0.2 M *N*-acetyl-D-glucosamine was added to a concentration of 2 mg/ml and incubated for 15 min at ambient temperature. The supernatant was removed from the Iodobead to stop the reaction, and passed twice over a Sephadex G25 Fine column (Pharmacia Fine Chemicals, Piscataway, NJ), which had been equilibrated in 0.1 M potassium phosphate, pH 7.5, to remove unbound ^{125}I-iodine.

The position of the nuclear lamin proteins A and C on the protein blots was determined by incubating a blot identical to that probed with radioactive WGA with antilamin antiserum. For this, the blot was first incubated with 5% bovine serum albumin, 0.1% Tween 20 in PBS for 1 h at ambient temperature. The blot was then incubated for 2 h with 15 ml of a 1:15,000 dilution of antilamin antiserum (LS1; McKeon et al., 1983) in 5% bovine serum albumin, 0.1% Tween 20 in PBS. The blot was washed three times in the same buffer but with no added antiserum and twice with PBS alone. The washed blot was incubated for 2 h at ambient temperature with ^{125}I-labeled protein A (0.033 mg/ml) at a concentration of 2–4 $\times$ 10^6 cpm/15 ml. After this incubation, the blot was washed as after the incubation with antiserum and exposed for autoradiography.

Electron Microscopy

Rat liver nuclei were diluted in PBS to a concentration of 0.2–1 $\times$ 10^5/μl. In some samples, 1 mM *N,N',N"*-triacetyl chitotriose was added. Ferritin-labeled WGA (0.1 mg/ml; Polysciences, Inc.) was added and allowed to incubate for 20 min. At this time, samples were fixed for 30 min on ice in 2% glutaraldehyde and 2.5% formaldehyde in 0.2 M cacodylate buffer (pH 7.4). The samples were centrifuged briefly to form a loose pellet. The fixative was discarded and the pellets resuspended in a small volume of 2% agarose at 30°C, followed by chilling. Samples were postfixed with 2% OsO_4 in 0.2% cacodylate, washed, dehydrated through a graded series of ethanol, and embedded in Spurr's low viscosity resin (Spurr, 1969). Sections were stained with uranyl acetate and lead citrate and examined in a Philips 300 electron microscope operated at 60 kV with a 50-μm objective aperture.

Results

WGA Binding to the Nuclear Periphery

One approach to identifying molecular components involved in nuclear transport would be to first identify specific inhibitors of transport and then to ask which nuclear proteins those inhibitors bind to. Several studies have pointed towards a possible involvement of one or more glycoproteins in nuclear transport. For example, Gerace et al. (1982) identified a 180-kD Con A-binding glycoprotein located at the nuclear pore. In a separate study, two lectins, Con A and wheat germ agglutinin, were reported to block the ATP-dependent release of ribonucleoprotein from isolated rat liver nuclei (Baglia and Maul, 1983), a biochemical assay thought to reflect the export of ribonucleoproteins from the nucleus (Agutter et al., 1979). In contrast, a study by Jiang and Schindler (1986), which measured the influx of a dextran molecule into rat liver nuclei, found no effect of WGA, but found that dextran influx was inhibited by Con A.

To identify possible inhibitors of nuclear protein transport, we used an assay developed to allow us to measure transport quickly in vitro (Newmeyer et al., 1986*a*). This assay uses, as a transport medium, an extract of activated *Xenopus laevis* eggs that contains all the components, with the exception of DNA, necessary to assemble nuclei in vitro (Forbes et al., 1983; Newport and Forbes, 1985; Newport et al., 1985; Newmeyer et al., 1986*b*; Newport, 1987). For the assay, nuclei are added to the egg extract and allowed to equilibrate for 30 min, at which time RITC-labeled nucleoplasmin is added. Aliquots, removed and examined under the fluorescence microscope, show nuclear accumulation of TRITC nucleoplasmin within minutes, with accumulation reaching a maximum at 30–45 min. When isolated rat liver nuclei are added to this extract, the majority maintain a functional nuclear envelope by three criteria: (*a*) large fluorescently labeled non-nuclear proteins (fluorescein-labeled immunoglobulin; phycoerythrin) are excluded from the nuclei, indicating that the nuclear envelopes are intact; (*b*) the nuclear envelope grows in size, gaining an adjacent extension of nuclear membrane that contains components derived from the egg extract; and (*c*) the nuclei transport and accumulate the fluorescently labeled nuclear protein, TRITC nucleoplasmin, up to 17-fold. (The number of accumulating nuclei ranges from 50 to 90% of the nuclei added, with freshly isolated nuclei showing a higher percentage of accumulating nuclei.) Nucleoplasmin accumulation in this assay is specific in that the signal domain of nucleoplasmin is required. Moreover, transport is temperature- and ATP-dependent. Finally, transport is observed using nuclei that possess their own nuclear envelopes when added to the extract (rat liver nuclei, *Xenopus* embryonic nuclei) or nuclei that acquire a nuclear envelope from the egg extract (demembranated *Xenopus* sperm nuclei) (Newmeyer et al., 1986*a*). This assay thus mimics transport as it occurs in vivo, and, because of its convenience, is appropriate for the quick direct identification of nuclear transport inhibitors.

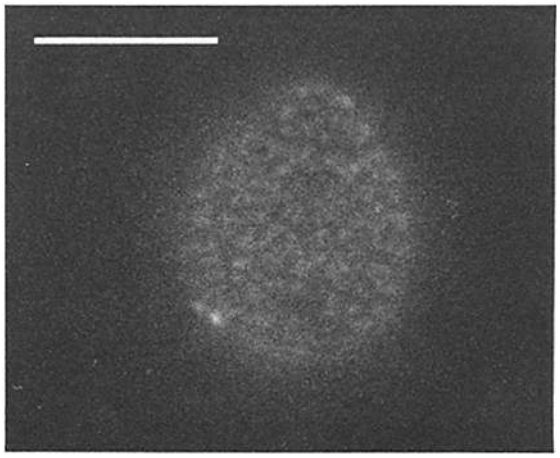

Figure 1. Punctate staining of a rat liver nuclear envelope by FITC-WGA. FITC-WGA was added to rat liver nuclei that had been incubated in egg extract. The view shown was photographed by focusing on the top surface of the nucleus. Bar, 10 μm.

Before testing a variety of lectins for possible inhibition of nucleoplasmin transport, we first asked whether specific lectins bound to rat liver nuclei under our conditions. Nuclei were added to an egg extract for 20 min. An aliquot of the mixture was placed on a slide, 0.5 μl of FITC-labeled lectin (0.1 mg/ml final concentration) added, and the aliquot examined by fluorescence microscopy. We found that WGA bound strongly to the nuclear envelopes of all rat liver nuclei. Interestingly, FITC-WGA stained the periphery of nuclei in a finely punctate manner (Fig. 1), even after fixation for 30 min with 2.5% glutaraldehyde (not shown). FITC-Con A stained the nuclear envelopes of damaged nuclei but not intact

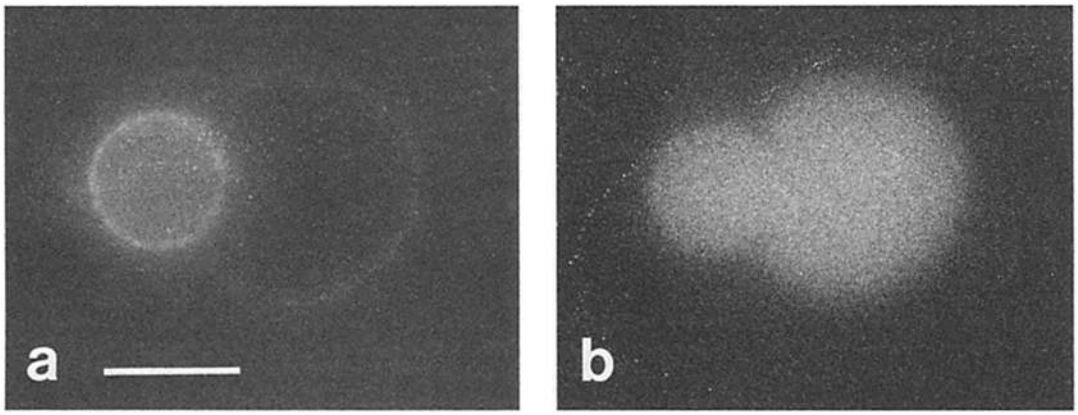

Figure 2. Differential staining of the original rat liver nuclear envelope and newly added nuclear envelope. Rat liver nuclei were added to an egg extract and allowed to equilibrate with the extract for 30 min. TRITC-labeled nucleoplasmin was then added. After 30 min, an aliquot was examined microscopically for accumulation and ability to bind FITC-labeled WGA by adding 0.5 μl FITC-WGA on the slide. The rat liver nucleus shown has undergone membrane growth; the small hemisphere derives from the original nucleus and contains the DNA, as determined by bisbenzimide staining (not shown). (*a*) FITC-WGA stains the original envelope brightly (the smaller hemisphere) and the newly added membrane weakly (larger hemisphere). (*b*) TRITC nucleoplasmin accumulation by the same rat liver nucleus as in *a*, shown to indicate the intactness of the nuclear envelope. Note: In this experiment, TRITC nucleoplasmin accumulation was allowed to occur for 30 min before the addition of WGA. Bar, 10 μm.

nuclei, as described previously (Newmeyer et al., 1986*a*). Unlike WGA, FITC-Con A stained the nuclear periphery in a continuous manner (see below). Other FITC-labeled lectins, *Bauhinia purpurea* agglutinin, *Dolichos biflorus* lectin, *Griffonia simplicifolia* lectins, *Maclura pomifera* lectin, *Arachis hypogaea* lectin, soy bean lectin, and *Ulex europaeus* lectin, gave no or only faint nuclear staining. Several of these lectins strongly stained non-nuclear membranous vesicles in the egg extract. Thus, of 10 lectins tested, only WGA and Con A were found to stain nuclei and did so at the nuclear periphery.

As stated, wheat germ agglutinin stained all rat liver nuclei. When rat liver nuclei are added to *Xenopus* egg extracts, they gain membranous extensions of their nuclear envelope. This envelope growth most often appears as an adjacent membrane bleb attached to the original nuclear envelope (Newmeyer et al., 1986*a*). We found that the newly added nuclear envelope that was acquired from the egg ex-

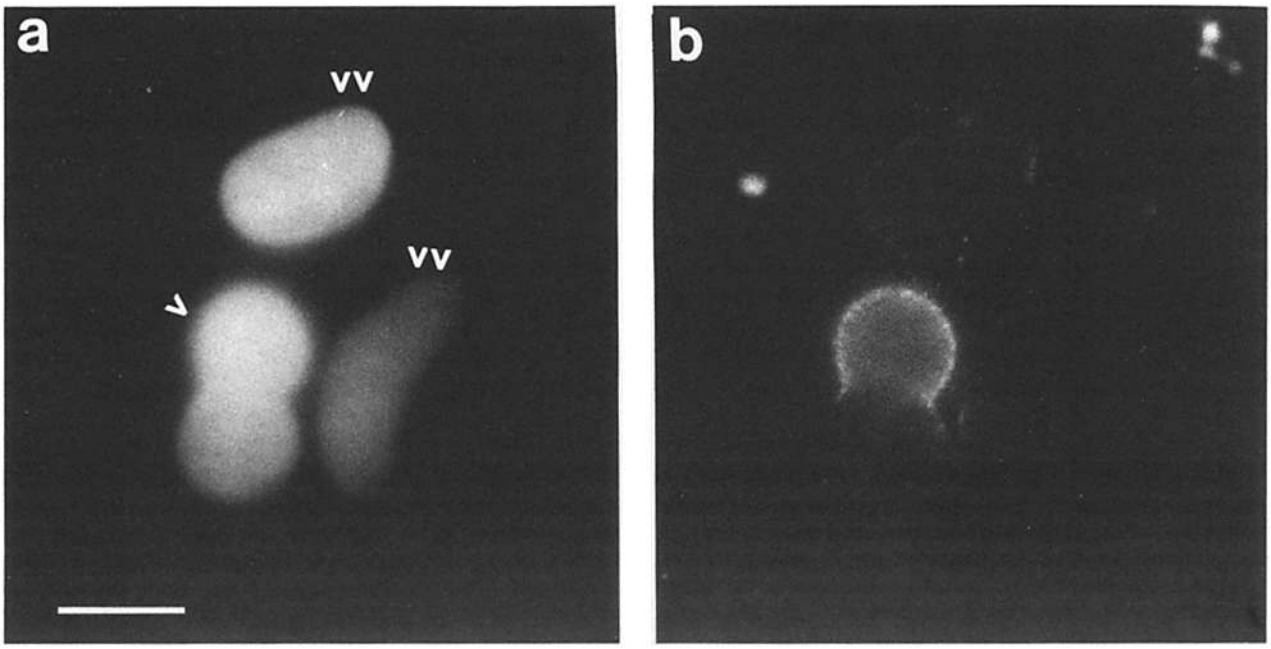

Figure 3. Differential staining of the nuclear envelopes of regrown sperm nuclei and rat liver nuclei. Demembranated *Xenopus* sperm nuclei were added to an egg extract. Within 30 min, the DNA decondensed and the nuclei acquired nuclear envelopes from components present in the extract. Rat liver nuclei were then added, followed by addition of TRITC-labeled nucleoplasmin. 30 min later, an aliquot was applied to a slide and FITC-WGA added. (*a*) TRITC nucleoplasmin accumulation is obvious in all three nuclei, indicating that intact nuclear envelopes are present. (*b*) FITC-WGA staining. Only the original rat nuclear envelope is stained brightly. The rat liver nucleus is designated by the single arrowhead and sperm nuclei by double arrowheads. Note: WGA was added 30 min after nucleoplasmin addition. Bar, 10 μm.

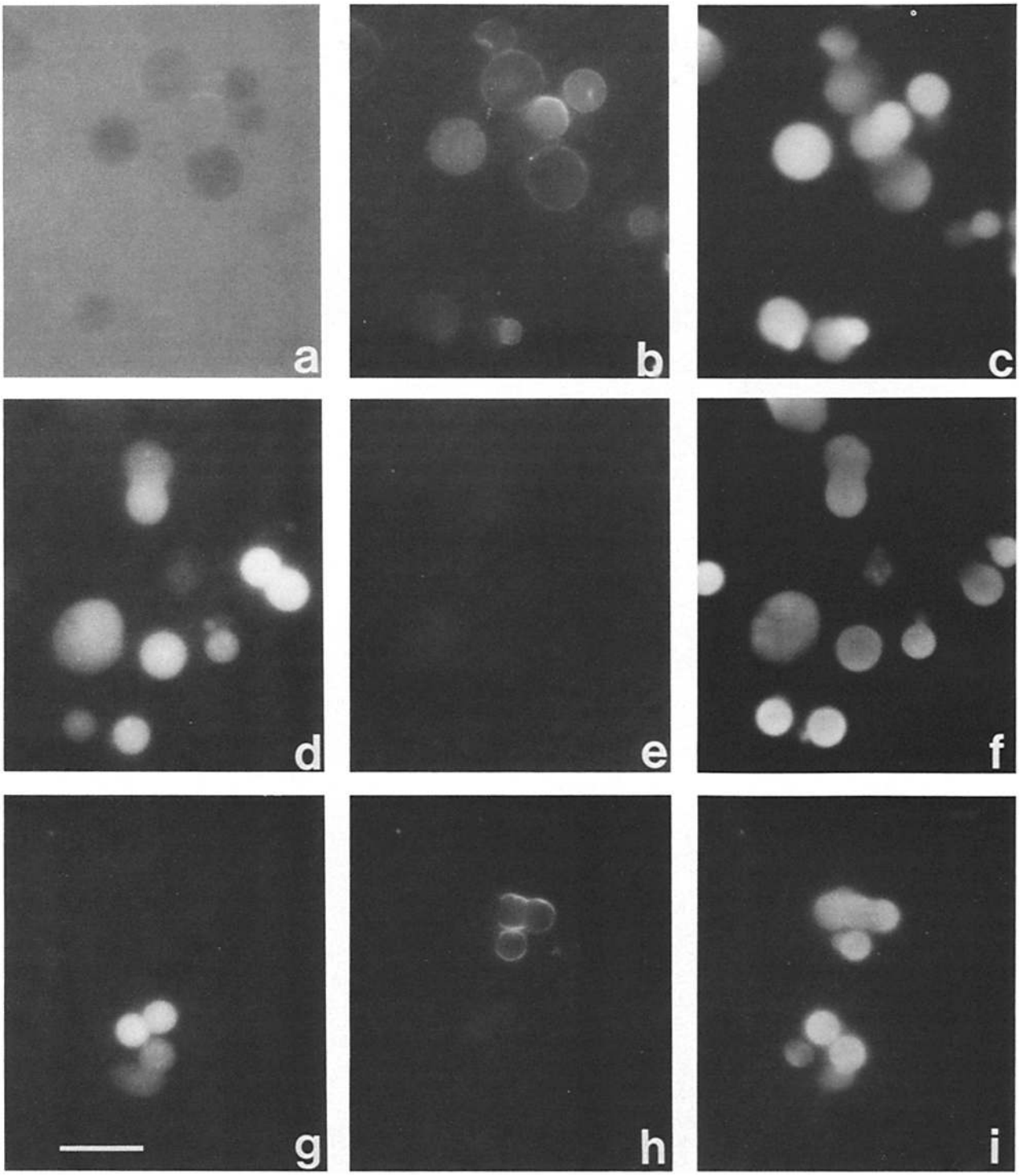

Figure 4. Nucleoplasmin transport is inhibited by the lectin WGA, but not by lectins Con A or *Arachis hypogaea*. Rat liver nuclei were added to egg extract and allowed to incubate for 30 min before the addition of WGA (*a–c*), *Arachis hypogaea* (*d–f*), or Con A (*g–i*) to a final concentration of 0.1 mg/ml. After 5 min, TRITC nucleoplasmin was added and aliquots observed by fluorescence microscopy 30 min later. (*a*, *d*, and *g*) TRITC nucleoplasmin fluorescence; (*b*, *e*, and *h*) FITC lectin fluorescence; (*c*, *f*, and *i*) DNA fluorescence as visualized with bisbenzimide. (The nuclei in *g–i* were chosen to include a number of broken nuclei to demonstrate FITC-Con A staining of broken nuclei. WGA addition results in the exclusion of TRITC nucleoplasmin (*a*) by intact nuclei, while PNA (*d*) and Con A (*g*) have no effect on TRITC nucleoplasmin accumulation. Bar, 20 μm.

tract stained more weakly than the original nuclear envelope, indicating that the added envelope is of a different protein composition (Fig. 2 *a*). To aid in visualizing the boundaries of the nuclear envelope in Fig. 2, TRITC nucleoplasmin (Fig. 2 *b*) was added to the extract containing nuclei 30 min before the addition of FITC-WGA. Efficient accumulation of nucleoplasmin can be seen to have taken place and fills both lobes of the enlarged rat liver nucleus. Thus, this nucleus is

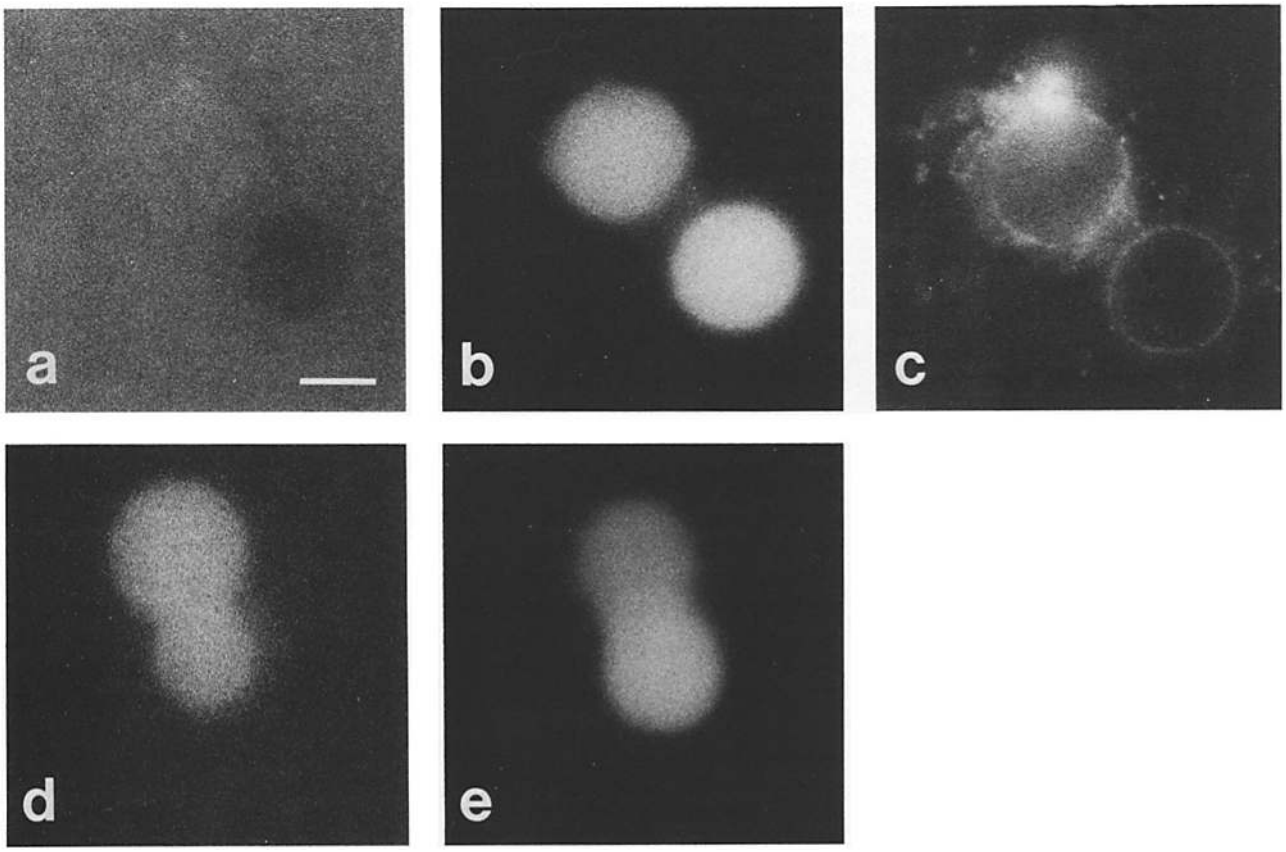

Figure 5. WGA inhibition of nucleoplasmin transport is reversed by incubation with a competing sugar. Rat liver nuclei were added to egg extract and allowed to incubate for 20 min before the addition of FITC-WGA to a final concentration of 0.1 mg/ml without (*a–c*) or with (*d* and *e*) co-addition of the competing sugar, *N*-acetyl-glucosamine (0.5 M). 5 min after lectin addition, TRITC nucleoplasmin was added and aliquots observed by fluorescence microscopy 30 min later. (*a* and *d*) TRITC nucleoplasmin fluorescence; (*b* and *e*) bisbenzimide DNA fluorescence; (*c*) FITC-WGA fluorescence. Bar, 10 μm.

intact and contains a hybrid nuclear envelope as revealed by subsequent FITC-WGA staining.

Consistent with the finding that FITC-WGA stains the newly added nuclear membrane more weakly, we found that regrown *Xenopus* sperm nuclei, which acquire a nuclear envelope derived exclusively from components in the egg extract (Lohka and Masui, 1983), also stained weakly with FITC–wheat germ agglutinin. To visualize directly the difference in staining intensity, demembranated *Xenopus* sperm nuclei were added to an egg extract and allowed to reacquire a nuclear envelope, decondense their DNA, and swell to sizes larger than rat liver nuclei. We previously showed that such nuclei are capable of TRITC–nucleoplasmin transport (Newmeyer et al., 1986*a*). Rat liver nuclei were then added to the extract containing regrown sperm nuclei. TRITC nucleoplasmin was added and accumulation allowed to take place. An aliquot containing both types of nuclei was then removed and tested for FITC-WGA binding. Fig. 3 shows a representative rat liver nucleus and two regrown sperm nuclei. All three have accumulated nucleoplasmin and thus contain intact nuclear envelopes (Fig. 3 *a*). Only the original nuclear envelope of the rat liver nucleus stains brightly with FITC-WGA (Fig. 3 *b*). The nuclear envelope contributed by the extract thus appears to contain less WGA-binding glycoproteins or to contain proteins altered in their glycosylation or accessibility to WGA. (Weak staining with FITC-WGA is not a characteristic of *Xenopus* nuclear envelopes in general, since the envelopes of *Xenopus* embryonic nuclei stained as brightly as those of rat liver nuclei; data not shown). We conclude that the amount, accessibility, or level of glycosylation of the glycoprotein(s) recognized by WGA in the nuclear envelope varies between nuclei of different types.

Inhibition of Nuclear Protein Transport by WGA

To test whether the nuclear binding of WGA affected transport of nucleoplasmin, lectin was added to the transport assay itself prior to the addition of TRITC-labeled nucleoplasmin. We found that the addition of wheat germ agglutinin, either FITC-labeled or unlabeled, completely blocked nucleoplasmin transport (Figs. 4, *a–c* and 5, *a–c*). Nuclei were often seen that clearly excluded TRITC nucleoplasmin (Figs. 4 *a* and 5 *a*), indicating that they were intact but incapable of transport. The inhibition of nucleoplasmin accumulation was complete at a concentration of 0.1 mg/ml WGA. Concentrations between 0.02 and 0.1 mg/ml gave partial inhibition, i.e., the amount of accumulation per nucleus was lower than in controls lacking WGA. When the concentration of WGA was reduced to 0.01 mg/ml WGA, no inhibition was seen (Table I).

When those lectins that failed to stain nuclei were tested, it was found that none blocked nucleoplasmin transport. The

high levels of nucleoplasmin accumulation observed with one such lectin, *Arachis hypogaea* lectin are shown in Fig. 4, *d–f*. FITC-Con A, as described previously (Newmeyer et al., 1986*a*) stained damaged rat liver nuclei at the nuclear periphery, but neither stained nor affected the transport of nucleoplasmin into intact nuclei (Fig. 4, *g–i*). Thus, only WGA was found to inhibit nuclear transport of fluorescently labeled nucleoplasmin.

Because FITC-WGA stained regrown *Xenopus* sperm nuclear envelopes more weakly than those of rat liver nuclei, it was possible that addition of WGA would not block nucleoplasmin accumulation in sperm nuclei. We found, however, that accumulation was as efficiently blocked in sperm nuclei as in rat liver nuclei (Table I). WGA also blocked nuclear accumulation of nucleoplasmin in *Xenopus* embryonic nuclei and nuclei reconstituted from bacteriophage DNA (Table I).

The inhibition of nucleoplasmin transport by WGA appears to be due to a specific interaction between WGA and a carbohydrate residue, since the inclusion of competing sugar (500 mM *N*-acetylglucosamine or 1 mM *N,N',N"*-triacetyl chitotriose) at the time of WGA addition resulted in completely normal nucleoplasmin transport (Fig. 5, *d–e*). Furthermore, it was possible to reverse WGA inhibition of nucleoplasmin transport by the addition of competing sugar 30 min later (Table I).

Wheat Germ Agglutinin Recognizes a Nuclear Pore Protein

To determine whether WGA was binding to a nuclear component or to an extract component, rat liver nuclei were briefly exposed to WGA, then washed twice with buffer before the transport assay. For this procedure, WGA was added to nuclei in extract and incubated for 5 min (0.1 mg/ml final concentration). Washes were then performed by dilution of the mixture and centrifugation on a 15/40% Percoll step gradient. The interface between the two Percoll phases was withdrawn, diluted, and centrifuged for 30 s in a microcentrifuge to wash the nuclei further. The pelleted nuclei were resuspended in fresh egg extract and incubated for 10 min. TRITC nucleoplasmin was then added and transport assayed 30 min later. Nuclei preincubated with WGA and washed in this manner exhibited no nucleoplasmin transport when added to fresh extract (Table II). Control nuclei, which were not preincubated with WGA, but which were subjected to the same washing procedure, were capable of efficient transport (Table II). This result indicates that WGA is binding to the nucleus itself and that this binding is sufficient to block the transport of subsequently added nucleoplasmin.

Table I. Conditions Affecting Nucleoplasmin Accumulation

Rat liver nuclei	Accumulation
Control	+++
+ Con A (0.1 mg/ml)	+++
+ WGA (0.1 mg/ml)	–
+ WGA (0.05 mg/ml)	+
+ WGA (0.01 mg/ml)	+++
+ WGA + *N,N',N"*-triacetyl chitotriose	+++
+ WGA for 30 min, then *N,N',N"*-triacetyl chitotriose	+++
+ lectins BPA, DBA, MPA, GS I and II, PNA, SBA, or UEA I (0.1 mg/ml)	+++
Xenopus sperm nuclei	
RITC nucleoplasmin	+++
RITC nucleoplasmin + WGA (0.1 mg/ml)	–
Xenopus embryonic nuclei	
RITC nucleoplasmin	+++
RITC nucleoplasmin + WGA (0.1 mg/ml)	–
Nuclei reconstituted from bacteriophage DNA	
RITC nucleoplasmin	+++
RITC nucleoplasmin + WGA (0.1 mg/ml)	–

Transport assays were performed as described in Materials and Methods. Lectins were added, where noted, 5 min before nucleoplasmin addition. Nucleoplasmin accumulation was measured 30 min after addition of nucleoplasmin. Several hundred nuclei in multiple experiments were monitored for each of the conditions tested. The concentration of WGA was 0.1 mg/ml, except where noted. BPA, *Bauhinia purpurea* agglutinin; DBA, *Dolichos biflorus* lectin; MPA, *Maclura pomifera* lectin; GS I and II, *Griffonia simplicifolia* lectins; PNA, *Arachis hypogaea* lectin; SBA, soybean lectin; UEA I, *Ulex europaeus* lectin; +++, high levels of accumulation (up to 17-fold); +, accumulation that was visible but faint (approximately twofold); –, no accumulation seen in any of the nuclei examined.

Table II. Inhibition of Nuclear Transport by WGA

	WGA	Sugar	Accumulated nuclei	Total nuclei	Accumulated nuclei
			No.		%
Rat liver nuclei					
	–	–	41.0	87.0	47.0
	+	–	0.0	82.0	0.0
	+	+	37.0	84.0	44.0
Washed rat liver nuclei					
	–	–	23.0	62.0	37.0
	+	–	1.0	60.0	1.6

Transport assays were performed as described in Materials and Methods. Nuclei accumulating RITC-labeled nucleoplasmin were visualized by their rhodamine fluorescence. The total number of nuclei was determined by staining for DNA with the fluorescent DNA dye bisbenzimide. Observations were made 30 min after the addition of nucleoplasmin. When present, WGA and *N,N',N"*-triacetyl chitotriose were added 5 min before the addition of nucleoplasmin.

To determine the nuclear site of WGA binding, electron microscopy was performed. Rat liver nuclei in PBS were mixed with ferritin-labeled WGA for 20 min, then centrifuged to concentrate the nuclei. The pelleted nuclei were fixed, embedded, sectioned, and examined with the electron microscope. We found that ferritin WGA bound to the nuclei and did so almost exclusively on the cytoplasmic faces of the nuclear pores. Nearly all the nuclear pores (>95%) were decorated as in Fig. 6 *a*, *b*, *d*, and *e*, where numerous ferritin grains can be seen in each pore. Rare pores (<2%) also showed ferritin on the nucleoplasmic face of the pore, but ferritin binding to the nuclear membrane was not observed. The binding of ferritin WGA to the pores appears specific, since incubation of the nuclei with ferritin-labeled WGA and 1 mM *N,N',N"*-triacetyl chitotriose (a competing sugar) resulted in no ferritin WGA binding to the pores (<2%; Fig. 6, *c* and *f*). We conclude that WGA recognizes multiple copies of one or more glycoproteins present on the cytoplasmic face of the nuclear pore.

WGA-binding Nuclear Proteins

To identify the nuclear glycoprotein(s) to which WGA binds, proteins from rat liver nuclei, rat liver nuclear pore–lamina

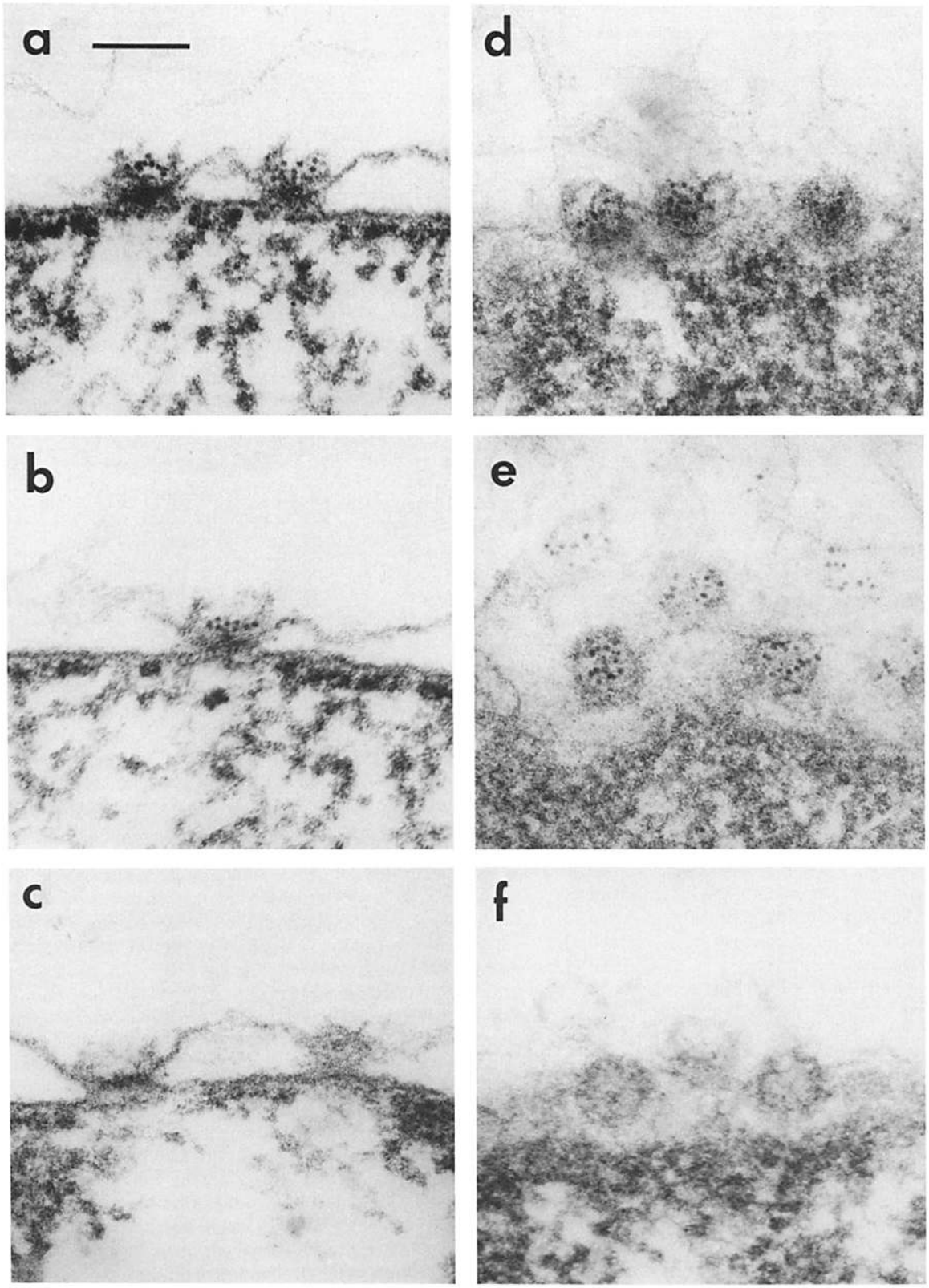

Figure 6. Ferritin-labeled WGA binds to the cytoplasmic side of the nuclear pore. Rat liver nuclei were incubated with ferritin-labeled WGA in PBS before preparation for electron microscopy. Control samples were incubated in PBS containing 1 mM *N,N',N''*-triacetyl chitotriose. *a*, *b*, *d*, and *e* show rat liver nuclear pores incubated with ferritin-labeled WGA, while panels *c* and *f* show nuclear pores of nuclei incubated with ferritin WGA and the competing sugar, chitotriose. (*a* and *b*) Cross sections of representative nuclear pores containing bound ferritin WGA. (*d* and *e*) Tangential views of representative nuclear pores containing bound ferritin WGA. (*c*) A cross section of a representative nuclear pore in nuclei incubated with WGA plus chitotriose. (*f*) A tangential view of a representative nuclear pore of a nucleus incubated with WGA plus chitotriose. Bar, 100 nm.

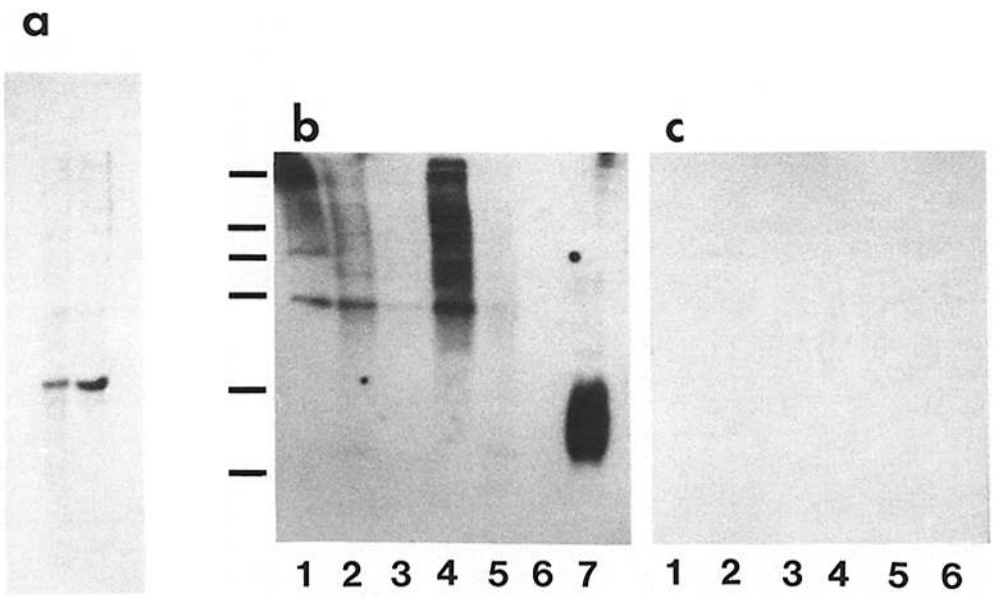

Figure 7. WGA-binding nuclear proteins. Proteins were separated on an SDS polyacrylamide gel, electrophoretically blotted onto nitrocellulose, and the blot probed with ^{125}I-labeled WGA. (*a*) Rat liver nuclear proteins of nuclei prepared with our usual buffers (*left lane*) and using the buffers of Blobel and Potter (1966) (*right lane*). (The protein aliquots resolved in the two lanes were not normalized for equal numbers of rat liver nuclei.) (*b*) The fractions probed were: egg extract, lane *1*; rat liver nuclei (1.5×10^6), lane *2*; rat liver nuclear pore complex–lamina fraction (1.25×10^6), lane *3*; supernatant of rat liver nuclei treated with 2% Triton X-100 (3×10^6), lane *4*; pellet of Triton-treated rat liver nuclei, lane *5*; and nucleoplasmin (5 μg), lane *6*. Lane *7* contained molecular mass markers, one of which is a WGA-binding glycoprotein (ovalbumin, 45 kD). The other markers were visualized by staining the blot with India ink. These are: myosin heavy chain (205 kD), beta-galactosidase (116 kD), phosphorylase B (97.4 kD), bovine plasma albumin (66 kD), ovalbumin (45 kD), and carbonic anhydrase (29 kD). (*c*) No bands were observed when a blot identical to that in *b* was incubated with ^{125}I-WGA and 0.5 M *N*-acetylglucosamine. Lanes *1–6* are as in *b*.

complexes, and egg extract were resolved on an SDS polyacrylamide gel, transferred to nitrocellulose, and incubated with ^{125}I-WGA. Autoradiography of the blot revealed that rat liver nuclei contain one major protein of 63–65-kD that binds ^{125}I-WGA (Fig. 7 *a*; Fig. 7 *b*, lane *2*), as well as several minor ones of higher molecular mass. Co-incubation of an identical blot with 500 mM *N*-acetylglucosamine blocked the binding of ^{125}I-WGA to all these proteins, indicating a specific interaction between WGA and the sugar residues present on the proteins (Fig. 7 *c*, lanes *1–6*). A *Xenopus* WGA-binding glycoprotein co-migrating with the 63–65-kD rat protein was also found in the egg extract, as were other WGA-binding glycoproteins (Fig. 7 *b*, lane *1*).

The 63–65-kD band was only weakly detectable in blots of rat liver nuclear pore–lamina complexes probed with ^{125}I-labeled WGA (Fig. 7 *b*, lane *3*). Although the 63–65-kD band is similar in size to the lamin proteins, its depletion in the nuclear pore complex–lamina fraction, which is enriched in lamins, argues against it being a lamin protein. (An identical blot, incubated with antilamin A and C antisera, indicated our pore complex–lamina preparation to be ~5–10-fold enriched in lamin protein with respect to total rat liver nuclei; data not shown). As an additional control, nucleoplasmin itself was tested for binding of ^{125}I-labeled wheat germ agglutinin (Fig. 7 *b*, lane *6*) and found to have no affinity for the lectin, indicating that WGA does not inhibit transport by direct interaction with nucleoplasmin.

To investigate the possibility that the 63–65-kD glycoprotein is a nuclear membrane protein and/or a nuclear pore constituent, rat liver nuclei were treated with 2% Triton X-100 to remove all or part of the nuclear membranes (Aaronson and Blobel, 1974). The membrane fraction (Fig. 7 *b*, lane *4*) and the depleted nuclear fraction (Fig. 7 *b*, lane *5*) were electrophoresed on an SDS polyacrylamide gel, transferred to nitrocellulose, and incubated with ^{125}I-labeled WGA. The 63–65-kD glycoprotein, as well as most of the other nuclear glycoproteins, were removed by Triton treatment of the nuclei and were present in the membrane fraction (Fig. 7, lane *4*). Thus, the 63–65-kD glycoprotein is extractable by Triton and is likely to be either a nuclear membrane protein or an extractable nuclear pore protein. Our electron microscopy indicates that an abundant WGA-binding protein is a nuclear pore protein.

Discussion

In this report, we used an in vitro nuclear transport assay that faithfully mimics in vivo nuclear protein transport to screen for inhibitors of nuclear transport. We thus hoped to identify proteins involved in transport. It had been previously shown that RNA efflux from isolated nuclei was inhibited by millimolar concentrations of either of two lectins, Con A or WGA (Baglia and Maul, 1983). When we tested these and other lectins for binding to nuclei or inhibition of nuclear transport, we found that WGA both bound to nuclei and completely blocked nucleoplasmin transport.

Lectins have previously been observed to bind to nuclei. The lectin Con A interacts with a 180-kD glycoprotein shown to be a nuclear pore protein, the carbohydrate portion of which lies within the cisternal space of the nuclear membranes (Gerace et al., 1982). Ferritin Con A binds to the cisternal faces of both the inner and outer nuclear membranes, but not to the nucleoplasmic or cytoplasmic faces of the membranes (Virtanen and Wartiovaara, 1976; Feldherr et al., 1977; Virtanen and Wartiovaara, 1978; Schindler et al., 1985). FITC-Con A in our assay bound exclusively to damaged nuclei, presumably by gaining access to the cisternal space, and had no effect on nucleoplasmin transport, which occurs only in intact nuclei (Newmeyer et al., 1986*a*).

Fluorescent WGA has also been seen by others to stain

nuclei. These studies observed FITC-WGA at the nuclear periphery but did not further localize the WGA-binding sites (Nicolson et al., 1971; Virtanen and Wartiovaara, 1976; Seve et al., 1984; Schindler et al., 1985). The FITC-WGA staining that we observe is of a punctate nature. Because of the capacity of WGA molecules to self-agglutinate, a punctate pore staining pattern would not be distinguishable in the light microscope from the pattern that might result from WGA agglutination of non-pore-associated nuclear membrane glycoproteins. However, electron microscopy with ferritin WGA indicates that the fluorescent punctate pattern is pore-related. We found no evidence from electron microscopy for WGA-mediated agglutination of nuclear pores, although pores were often seen in loose clusters, even when competing sugar was present. Since the rat liver nucleus has been estimated to contain ~3–4,000 nuclear pores (Maul, 1977) and we observe fluorescent punctate entities numbering in the hundreds, the punctate staining pattern presumably represents naturally occurring clusters of nuclear pores.

The addition of WGA to our system resulted in a complete inhibition of nucleoplasmin transport. It appeared from the experiment involving preincubation of nuclei with WGA and subsequent washing that WGA caused transport inhibition by binding to a component of the nucleus itself, rather than an extract component. This inhibition was not seen in the presence of 500 mM *N*-acetyl-D-glucosamine, indicating that inhibition was the result of specific recognition of one or more glycoproteins by WGA. Furthermore, inhibition of accumulation by WGA was reversible upon later addition of a competing sugar, indicating that continued binding of WGA was necessary for inhibition and that inhibition could be reversed by dissociation of WGA from its binding site.

WGA is known to bind to terminal *N*-acetylglucosamine (GlcNAc) and sialic acid residues. Typically, these residues are indicators that a protein has passed through the Golgi and contains complex oligosaccharide chains. In the cell, such proteins are found within membrane-enclosed vesicles or organelles (Kornfeld and Kornfeld, 1976). Recently, however, several groups have identified a new type of glycoprotein containing simple GlcNAc monomers (Schindler and Hogan, 1984; Torres and Hart, 1984; Holt and Hart, 1986). Each monomer is attached to a protein by an O-linkage (Holt and Hart, 1986). Proteins with these residues are highly concentrated in nuclear and cytosolic fractions. The most abundant protein of this type in rat liver nuclei is a protein of molecular mass approximating that of the 63–65-kD protein we observe (Holt and Hart, 1986). Our finding that all rat liver nuclei stain with FITC-WGA, while only damaged nuclei stain with FITC-Con A, suggests that the WGA-binding GlcNAc residues reside on the exterior of the nucleus. Although this would be an unexpected location for a glycoprotein containing a complex-type oligosaccharide chain, the unusual subcellular distribution of glycoproteins with single O-linked GlcNAc residues (Schindler and Hogan, 1984; Holt and Hart, 1986) makes it entirely possible that nuclear proteins with single GlcNAc residues face outward into the cytoplasm. There is additional precedent for glycoproteins in regions of the cell other than the cisternal spaces of organelles; several groups have found glycoproteins in the interior of the nucleus (Hozier et al., 1980; Seve et al., 1984; Kan and Pinto da Silva, 1986). Clearly, our electron microscopic results indicate that WGA binding sites are present on the cytoplasmic face of each nuclear pore.

If every pore contained an equal amount of the WGA-binding protein, the amount of fluorescent WGA binding could be used as an estimate of pore number for individual nuclei. If this were true, the newly added portion of hybrid rat liver nuclear envelopes or of regrown sperm nuclear envelopes must have fewer pores per unit area, since they stain much more faintly with FITC-WGA. An alternate explanation, however, for the fainter staining of some nuclear envelopes could be that individual nuclear pores vary in the amount or glycosylation level of WGA-binding protein they contain. Theoretically, variation in a protein involved in pore function might either regulate the state of the pore (i.e., open or closed) or affect the rate of transport through the pore. We find that the WGA-binding pore protein does affect pore function, at least when bound to WGA, and does vary in nuclei from different sources. We further find that, when intermediate concentrations of WGA are used in the transport assay, the same number of nuclei are active in transport, but the amount of TRITC nucleoplasmin observed per nucleus decreases as the concentration of WGA increases. Unfortunately, this latter result does not let us distinguish between (*a*) induction of an off state in an increasing number of pores and (*b*) a gradual slowing of the rate of transport through each pore as more WGA binds to the pore.

In a recent report, WGA was found to have no effect on the influx of a 64-kD fluorescent dextran into the nucleus (Jiang and Schindler, 1986). In support of this finding, we see no exclusion of 10- and 20-kD fluorescent dextrans by WGA in experiments where nucleoplasmin is excluded. Similarly, we see no difference in the largely non-nuclear distribution of 40-, 70-, and 150-kD FITC-labeled dextrans when WGA is present in our system (observations recorded at 30 min–3 h; Finlay, D., and D. Forbes, unpublished). Since WGA blocks nucleoplasmin transport, it appears that this nuclear protein enters the nucleus by a pore interaction different from that of the non-nuclear dextran molecule.

In investigating the target of action of WGA, we found that WGA bound strongly to one major (63–65-kD) and several minor rat liver nuclear proteins. The 63–65-kD protein is depleted from rat liver pore complex–lamina fractions (relative to the total nuclear membrane fraction) and is thus not a lamin protein. Although we have not presented direct evidence that the 63–65-kD protein is the target of inhibition of transport by WGA, it is strikingly the most abundant WGA-binding protein in rat liver nuclei. Our data clearly implicate at least one glycoprotein in the nucleus which, when bound by WGA, inhibits the transport of nucleoplasmin. In addition, our observation that ferritin-labeled WGA binds to pores demonstrates that there are WGA-binding glycoproteins in nuclear pores and that these glycoproteins are present in multiple copies. At the completion of this work, Davis and Blobel (1986) reported that a 62-kD protein, identified by a monoclonal antibody, is located in nuclear pores. Their work also indicates that there are several molecules of the 62-kD protein in the pore, and that, when isolated, this protein binds WGA. Thus it is likely that this protein and the 63–65-kD protein described here are the same, and, moreover, that the 63–65-kD protein is a target of WGA inhibition. We are presently testing our supposition that the 63–65-kD protein, when bound by WGA, inhibits nuclear protein transport by the production of antibodies to the 63–65-kD protein.

Possible explanations for the inhibition of nuclear transport by WGA would place the 63–65-kD (or other) glycopro-

tein in the nuclear pore. WGA when bound to the glycoprotein might either: (*a*) physically block the pore so that large nucleoplasmin molecules could no longer pass through, (*b*) bind to a recognition signal of the pore glycoprotein that is required for the binding and subsequent transport of nucleoplasmin, or (*c*) alter the glycoprotein so that pore function is destroyed. Our preliminary result that WGA does not interfere with the passage of fluorescent dextrans into the nucleus argues against a total blockage of the pore by WGA. In any event, the ease and specificity with which WGA inhibition of transport is reversed by subsequent sugar addition argue that the pore is not permanently altered by WGA.

In summary, the results reported here describe the first identified inhibitor of nuclear protein transport, the lectin WGA. We have shown that WGA completely blocks nuclear transport and binds directly to the nuclear pore. A pore glycoprotein thus appears to be either directly involved in nuclear transport or to be placed in such a position that WGA can, by binding to it, obstruct the passage of nuclear proteins through the pore. The most likely candidate for the target of WGA binding is the 62-kD pore glycoprotein recently observed by Davis and Blobel (1986) and independently by us as the major WGA-binding protein (63–65-kD) in rat liver nuclei. We hope by extending these studies to further probe the structure and function of the nuclear pore.

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