Reconstituted Nuclei Depleted of a Vertebrate GLFG Nuclear Pore Protein, p97, Import But Are Defective in Nuclear Growth and Replication

Maureen A. Powers, Colin Macaulay, Frank R. Masiarz,*‡ and Douglass J. Forbes

Department of Biology 0347, University of California at San Diego, La Jolla, California 92093-0347; *Protein Structure Analysis Group, Chiron Corporation, Emeryville, California 94608-2916; and ‡Department of Pharmaceutical Chemistry, University of California at San Francisco, San Francisco, California 94143

Abstract. Xenopus egg extracts provide a powerful system for in vitro reconstitution of nuclei and analysis of nuclear transport. Such cell-free extracts contain three major N-acetylglucosaminylated proteins: p200, p97, and p60. Both p200 and p60 have been found to be components of the nuclear pore. Here, the role of p97 has been investigated. Xenopus p97 was isolated and antisera were raised and affinity purified. Immunolocalization experiments indicate that p97 is present in a punctate pattern on the nuclear envelope and also in the nuclear interior. Peptide sequence analysis reveals that p97 contains a GLFG motif which defines a family of yeast nuclear pore proteins, as well as a peptide that is identical at 11/15 amino acids to a specific member of the GLFG family, NUP116. An additional peptide is highly homologous to a second sequence found in NUP116 and other members of the yeast GLFG family. A monoclonal antibody to the GLFG domain cross-reacts with a major Xenopus protein of 97 kD and polyclonal antiserum to p97 recognizes the yeast GLFG nucleoporin family. The p97 antiserum was used to immunodeplete Xenopus egg cytosol and p97-deficient nuclei were reconstituted. The p97-depleted nuclei remained largely competent for nuclear protein import. However, in contrast to control nuclei, nuclei deficient in p97 fail to grow in size over time and do not replicate their chromosomal DNA. ssDNA replication in such extracts remains unaffected. Addition of the N-acetylglucosaminylated nuclear proteins of Xenopus or rat reverses these replication and growth defects. The possible role(s) of p97 in these nuclear functions is discussed.

Nuclear pores are highly complex structures of ~120 million daltons which control the bi-directional traffic of ribosomes and RNA out of the nucleus, and proteins and snRNAs into the nucleus. Nuclear proteins possess specific nuclear localization signals which bind to one or more cytosolic receptors; a complex of nuclear protein and receptor(s) is then thought to bind to the exterior of the nuclear pore, followed by translocation through the pore (Feldherr et al., 1984; Newmeyer and Forbes, 1988; Richardson et al., 1988; Akey and Goldfarb, 1989; Adam and Gerace, 1991; Moore and Blobel, 1992). The nuclear pore contains an estimated 1,000 proteins of ~60–100 different types. At the electron microscopic level the pore consists of three parallel rings: (*a*) a central ring of spokes which holds a potential transporter at its hub; (*b*) an adjacent cytoplasmic ring from which short fibers emanate into the cytoplasm; and (*c*) a nucleoplasmic ring from which an elaborate basket-like structure extends (Akey, 1989; Hinshaw et al., 1993; for reviews see Forbes, 1992; Gerace, 1992; Osborne and Silver, 1993; Pante and Aebi, 1993; Rout and Wente, 1994). Long fibers stretch from this basket further into the nucleus (Cordes et al., 1993).

Recent developments have allowed assignment of specific proteins to different substructures of the nuclear pore complex. Progress in this area has come from the combined study of four different organisms. In humans, a protein of the cytoplasmic ring/filament structures has been identified and is encoded by a potential oncogene (p214/CAN). In rats, proteins from the cytoplasmic filaments (p250, p75), the central region of the pore (p62, p58, and p54), the pore membrane (gp210, POM121, and POM152), and the nuclear basket (NUP153, p62) have been identified by protein purification and production of specific antibodies (reviewed in Pante and Aebi, 1993; Rout and Wente, 1994). The high resolution of electron microscopy on Xenopus oocyte nuclear pores, combined with the crossreactivity of many of the anti–rat antisera, have allowed many of these structural assignments to be made (Cordes et al., 1991; Cordes et al., 1993; Pante et al., 1994). In Xenopus, an additional protein of the cytoplasmic filaments, p180, has been identified using an autoimmune antiserum (Wilken et al., 1993). Further useful information has been obtained following the cloning of quite a few

Address all correspondence to D. J. Forbes, Department of Biology 0347, University of California at San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0347. Ph.: (619) 534-3398. Fax: (619) 534-0555.

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of the rat genes encoding pore proteins, although in no case has there been found a sequence indicating a possible enzymatic mechanism of action for a pore protein, such as a kinase domain, motor domain, etc.

Rapid progress has been made in cloning the genes for yeast nuclear pore proteins. Using monoclonal antibodies directed against different conserved motifs found in the rat pore proteins, cross-reactive families of yeast pore proteins have been identified. Antibodies against the conserved motif XFXFG demark one such family of pore proteins (Davis and Blobel 1986; Davis and Fink, 1990). Anti-XFXFG antibodies, which recognize p62, NUP153, and POM121 in rats and p214/CAN in humans, cross-react with NSP1, NUP1, and NUP2 in yeast. Only one direct homology has as yet been reported between the yeast and rat XFXFG proteins: yeast NSP1 is thought to be the homolog of rat p62 and Xenopus p60 (Carmo-Fonseca et al., 1991; Cordes et al., 1991). Mutation of NSP1 in yeast blocks nuclear import; similarly, removal of p62 in reconstituted vertebrate nuclei blocks import (Nehrbass et al., 1990; Dabauvalle 1990; Finlay et al., 1991).

A second family of yeast pore proteins is characterized by possession of GLFG repeats. The yeast GLFG family includes NUP49, NUP54, NUP100, NUP116 and NUP145 (Wente et al., 1992; Wimmer et al., 1992; Grandi et al., 1993; Fabre et al., 1994; Wente and Blobel, 1994). The GLFG pore proteins have many sequence redundancies among themselves. To date, one of the most interesting of these proteins is NUP145 which binds to homopolymeric RNA in vitro and, when mutated, results in the loss of mRNA export (Fabre et al., 1994; Wente and Blobel, 1994).

In Xenopus, proteins have been identified that are specifically important for pore function. The Xenopus egg contains three highly abundant *N*-acetylglucosaminylated (GlcNAc) proteins of 200, 97, and 60 kD, as well as additional minor proteins (Finlay and Forbes, 1990; Miller and Hanover, 1994). A large family of N-acetylglucosaminylated pore proteins have also been found in rat (Hanover et al., 1987; Holt et al., 1987; Snow et al., 1987). A nuclear reconstitution extract derived from Xenopus eggs allows one to identify nuclear pore proteins, assemble nuclei lacking specific proteins, and assess their defects. Membrane and cytosolic fractions of egg extracts, when combined with a source of chromatin or DNA, readily form nuclei (Lohka and Masui, 1983; Newmeyer et al., 1986; Newport, 1987; Sheehan et al., 1988; for reviews see Leno and Laskey, 1990; Almouzni and Wolffe, 1993). The N-acetylglucosaminylated proteins, p200, p97 and p60, bind to the lectin wheat germ agglutinin (WGA)[1] and can be removed from an extract by WGA-Sepharose depletion. Nuclear reconstitution extracts depleted of all the WGA-binding proteins were found to form nuclei which are defective in nuclear transport (Finlay and Forbes, 1990, 1991; Dabauvalle, 1990, 1991; Miller and Hanover, 1994). The next step was clearly to raise antisera to each of the individual WGA-binding proteins, p60, p97, and p200, in order to assess which are pore proteins, which are required for transport, or which may have other roles in the nucleus. Both p60 and p200 have been found to be proteins of the nuclear pore (Dabauvalle et al., 1988*a*; Finlay and Forbes, 1990; Cordes et al., 1991; Finlay et al., 1991; Powers, M. A., C. Macaulay, and D. J. Forbes, manuscript in preparation). Immunogold labeling of oocyte nuclear envelopes with antisera to p60 reveals labeling at the very central region of the nuclear pore, in or near the transporter, as well as on the ring of the nuclear basket (Wilken et al., 1993; Pante et al., 1994). Both WGA and anti-p60/62 antisera block nuclear import (Finlay et al., 1987; Dabauvalle et al., 1988*a*,*b*; Yoneda et al., 1987; Wolff et al., 1988). Indeed, immunodepletion of a nuclear reconstitution system with anti-rat-p62 antiserum results in nuclei containing nuclear pores which are defective for protein import (Dabauvalle et al., 1988*a*; Finlay and Forbes, 1990). The GlcNAc-modified pore protein, p200, is localized on or near the cytoplasmic ring and filaments of the nuclear pore and the effects of immunodepletion of p200 are now being examined (Powers, M. A., C. Macaulay, and D. J. Forbes, manuscript in preparation). An interest in identifying nuclear glycoproteins of functional importance, as well as in identifying possible novel pore proteins, prompted us to investigate the third highly abundant soluble glycoprotein of Xenopus eggs, p97.

Here we report that peptide sequence analysis of isolated Xenopus p97 reveals the possession of: (*a*) a GLFG repeat; (*b*) a unique sequence with a high degree of homology to the yeast pore protein NUP116; and (*c*) a sequence with homology to a sequence found in NUP116, NUP100, and NUP145. Thus, Xenopus p97 is the first vertebrate example of a GLFG-family-pore protein. Immunofluorescence staining gives both a punctate nuclear rim pattern and an intranuclear stain. When nuclei which lack p97 are reconstituted in vitro, these nuclei carry out nuclear protein import, but remain small and fail to replicate their chromosomal DNA. Potential models for p97 action are discussed.

Materials and Methods

Preparation of Xenopus Laevis Nuclear Reconstitution Extracts

Xenopus egg cytosol was prepared as described in Smythe and Newport (1991). Briefly, eggs were dejellied with 2% cysteine (pH 7.8) and washed in egg lysis buffer (ELB; 10 mM Hepes, pH 7.4, 250 mM sucrose, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, 20 μg/ml cycloheximide, 5 μg/ml cytochalasin B, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Eggs were then lysed by a 15 min centrifugation at 12,000 *g* and the crude soluble fraction was collected. Following centrifugation at 200,000 *g* for 90 min, the soluble and membrane fractions were collected and the pellet containing mitochondria, glycogen, and ribosomes was discarded. The soluble fraction was recentrifuged for 30 min to remove any residual membrane and the resulting cytosol was used immediately for immunodepletion or aliquoted and frozen for subsequent use. The membrane fraction was collected, washed by dilution in ELB containing 250 mM KCl, and pelleted (34,000 *g*, 20 min) through a cushion of ELB containing 0.5 M sucrose. The membrane pellet was resuspended in a minimal volume of buffer and used as a 10× stock. Xenopus sperm were isolated and demembranated as described (Smythe and Newport 1991) and the resulting sperm chromatin was stored frozen in aliquots.

Production of Polyclonal Antisera and Affinity Purification

To prepare p97 antigen, N-acetylglucosaminylated, WGA-binding proteins were first isolated from Xenopus egg cytosol as follows. 10 ml of Xenopus

1. *Abbreviations used in this paper*: ECL, enhanced chemiluminescence; EGS, ethylenglycol*bis*(succinimidylsuccinate); ELB, egg lysis buffer; MAP, multiple antigen peptide; pre-RC, pre-replication centers; RE, rat eluate; RPA, replication factor A; RT, room temperature; TB, transport buffer; WGA, wheat germ agglutinin.

egg cytosol was diluted with 4 vol of 5× TEN (1× TEN is 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, 100 mM NaCl), centrifuged at 12,000 *g* for 1 h to remove insoluble material, and subsequently incubated (overnight at 4°C) with 1 ml of WGA-Sepharose (E. Y. Laboratories; San Mateo, CA). After washing three times with 10 bead volumes of 5× TEN, followed by 5 vol of TE (100 mM Tris-Cl, pH 6.8 1 mM EDTA) the WGA-binding proteins were eluted from the lectin-Sepharose by boiling in gel sample buffer (Laemmli, 1970) and resolved by electrophoresis through a preparative 8% polyacrylamide gel. Proteins were detected by staining with Coomassie blue and the band corresponding to p97 was excised from the gel, washed in water, emulsified with Freunds complete adjuvant, and injected intramuscularly into a 2.5 kg female rabbit. For later boosts, p97 was prepared in the same manner from 5 ml of Xenopus egg cytosol and the gel slice was emulsified with Freunds incomplete adjuvant before injection.

Antibodies were affinity purified from rabbit serum as described by Robinson et al., (1988) using p97 bound to nitrocellulose. For this, p97 derived as above from 10 ml of Xenopus cytosol was used to purify antibodies from 1 ml of rabbit serum. The eluted antibodies were concentrated by ammonium sulfate precipitation, resuspended in half the original serum volume of dialysis buffer (25 mM Tris, pH 8.0, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT) and dialyzed with several changes. Antibodies to Xenopus p200 (Powers M. A., C. Macaulay, and D. J. Forbes, manuscript in preparation) were prepared and affinity purified in the same manner. Antiserum to rat p62 (Finlay et al., 1991) was affinity purified on nitrocellulose strips containing the Xenopus homolog, p60. The yield of IgG was quantitated by immunoblotting several dilutions of affinity purified antibody and comparison to standard amounts of rabbit IgG.

For production of anti-peptide sera, p97 peptides 1 and 3 were synthesized as multiple antigen peptide (MAP) conjugates and injected into rabbits by Research Genetics (Huntsville, AL). To affinity purify anti-peptide sera, 3 mg of MAP conjugate was coupled to 6 ml of Affigel 10. The appropriate antiserum in 5× PBS was passed three times over the affinity resin to allow binding. After washing the column with 5× PBS, specific antibodies were eluted in 100 mM citrate, pH 2.5, and immediately neutralized with a half volume of 1 M Tris, pH 8.5. Ovalbumin was added to act as carrier and the antibodies were precipitated with ammonium sulfate, resuspended, and dialyzed against PBS.

Preparation of p97 Peptides and Sequence Analysis

The WGA-binding proteins were isolated from 20 ml of Xenopus egg cytosol and resolved on two preparative 7.5% polyacrylamide gels. Proteins were localized by staining the gels with 0.3 M $CuCl_2$ (Lee et al., 1987) and p97 was recovered from the excised gel slice by electroelution. Following vinylpyridinylation of cysteine resides and digestion with the *Achromobacter* endoproteinase Lys C, peptides were resolved by reverse phase HPLC in acetonitrile. Individual peak fractions were collected and rechromatographed by reverse phase HPLC in isopropanol to yield single peptides. The peptides were sequenced by Edman degradation using an Applied Biosystems (Foster City, CA) sequencer. Sequence comparisons were performed using programs described by Doolittle (1987). Weighted scores (where, in addition to identities, similar amino acids contribute) were derived using a conventional Dayhoff matrix (Schwartz and Dayhoff, 1978).

Immunoblotting and WGA Blotting

Total Xenopus egg cytosol and Xenopus WGA-binding proteins were prepared as described above. Proteins from Xenopus A6 kidney epithelial cells were obtained by trypsinization of the cells followed by lysis in boiling 2% SDS, 70 mM mercaptoethanol. The viscosity of the A6 cell lysate was reduced by shearing the DNA through a 26 g needle. Rat nuclear proteins were obtained from rat liver nuclei prepared by the method of Blobel and Potter (1966) with modifications as described by Newport and Spann (1987). Prior to gel electrophoresis, rat liver nuclei were treated with DNase I and RNase A, followed by extraction with 2 M NaCl. The remaining nuclear proteins were solubilized in Laemmli gel sample buffer. Yeast cell lysates were prepared by alkaline lysis and TCA precipitation according to the method of Yaffe and Schatz (1984). Proteins from the above samples were resolved on polyacrylamide gels and transferred to either polyvinyldifluoride or nitrocellulose membrane.

For detection with affinity purified rabbit antibodies, the membranes were blocked by incubation with 5% nonfat milk in PBS and 0.2% Tween-20 (5% milk/PBST). Affinity purified polyclonal antibodies to p97, p200, or p60 were used at 1:1,000 dilution in 5% milk/PBST, unless otherwise indicated. For detection of Xenopus proteins, the blots were incubated with antibodies for 1 h at room temperature (RT). For detection of proteins from other species, anti-p97 antibodies were incubated overnight at 4°C. Affinity purified anti-p97 peptide sera were used at 1:75 (peptide 1) or 1:300 (peptide 3) in 5% milk/PBST with 1 h incubations After washing with PBST, the blots were probed with a secondary goat anti-rabbit IgG conjugated to horseradish peroxidase (Jackson Immunoresearch, West Grove, PA; 1:2,000 in 5% milk/PBST). Following washes with PBST, the blots were developed with an enhanced chemiluminescent substrate (ECL; Amersham, Arlington Heights, IL; or Renaissance, Dupont-NEN, Boston, MA) and exposed to film.

For immunoblotting with monoclonal antibody 192 (Wente et al., 1992), blots were blocked with 2% milk in PBS with 0.05% Tween-20 (PBST-0.05%), and then incubated overnight at 4°C with monoclonal tissue culture supernatant diluted 1:100 in the blocking solution. With washing between each step (PBST-0.05%), the blots were further incubated with rabbit anti-mouse IgM (Zymed, So. San Francisco, CA; 1:1,000), and then with goat anti-rabbit IgG HRP conjugate (1:1,000). The blots were developed with chemiluminescent substrate.

Detection of WGA-binding proteins with ^{125}I-labeled WGA was performed as described by Finlay and Forbes (1990).

Immunodepletions and Nuclear Import Assays

To assemble nuclei lacking p97, Xenopus egg cytosol was first immunodepleted with affinity purified anti-p97 antibodies (0.3–0.5 μg IgG per μl of cytosol) or with an equivalent amount of nonspecific rabbit IgG. For a typical experiment, 60 μg of either anti-p97 or control IgG in dialysis buffer with 2 mg/ml ovalbumin was incubated overnight at 4°C with 50 μl of protein A-Sepharose beads. The Sepharose with bound antibody was washed with ELB and 25 μl was used in each of two sequential immunodepletions of cytosol (200 μl). For this, antibody-Sepharose was first pre-equilibrated with two volumes of WGA-depleted Xenopus cytosol (30 min, 4°C) to block non-specific sites and to avoid subsequent dilution of cytosolic proteins. Following this, freshly prepared undepleted cytosol (200 μl) was added to the pre-equilibrated antibody beads and incubated for 1 h at 4°C with gentle mixing. The partially immunodepleted cytosol was collected and incubated with a second batch of pre-equilibrated antibody-Sepharose (25 μl). This p97- or control-depleted cytosol was then used immediately for nuclear reconstitution or was aliquoted and frozen in liquid N_2 for future use. The extent of depletion was assessed by immunoblotting. (A total protein stain was not informative in determining whether p97 or possibly additional components of a p97 complex were removed. Due to the very large number of proteins in Xenopus egg cytosol, the loss of individual bands after immunodepletion could not be detected.)

WGA-depleted Xenopus cytosol was prepared by two sequential 1 h incubations (4°C, with mixing) using one half the cytosol volume of WGA-Sepharose beads (E. Y. Laboratories, San Mateo, CA). If the WGA-depleted cytosol was to be used to reconstitute nuclei, the beads were first pre-equilibrated as described for antibody-Sepharose above. Alternatively, if the WGA-depleted cytosol was to be used for the pre-equilibration of beads, cytosol was added directly to buffer-washed WGA-Sepharose.

Xenopus WGA-binding proteins (XE) were isolated by incubation of freshly made cytosol with one-tenth volume of WGA-Sepharose (1 h, 4°C). The beads were then washed three times with 10 bead vol of ELB and the WGA-binding proteins were eluted with 1 bead vol of buffer containing the competing sugar (HSB; 125 mM GlcNAc and 2 mM TCT in ELB). Recovery was typically 50%, consequently XE was treated as a 5× stock of the WGA-binding proteins.

To assay for nuclear import in control and p97-depleted nuclei, nuclear reconstitution was carried out as follows. A 60 μl assembly reaction typically contained 36 μl of control-, p97-depleted, or WGA-depleted cytosol, 12 μl XE or HSB, 1× washed Xenopus membranes, an ATP regeneration system (6 μl; 16.7 mM creatine phosphate, 1.67 mM ATP and 4 μg/ml creatine phosphokinase, final concentration) and demembranated sperm chromatin (1,000/μl final). For reactions with WGA-depleted cytosol, glycogen (20 mg/ml) was included to facilitate nuclear formation (Hartl et al., 1994). Reactions were incubated at RT for approximately 2 h or until nuclei had formed in the WGA-depleted controls. Nuclear formation was assessed by exclusion of 150 kD FITC-labeled dextran (0.8 mg/ml; Sigma Chem. Co., St. Louis, MO). Once nuclei were formed, RITC-labeled transport substrate was added (SV-40 TAg nuclear localization signal cross-linked in multiple copies to human serum albumin; Newmeyer and Forbes, 1988). Immediately and at 30 min intervals thereafter, a 6-μl aliquot was removed from the reaction and fixed by the addition of paraformaldehyde (1.8 μl; 3.7% final concentration). Samples were held in the dark at 4°C for later quantitation.

To quantitate nuclear size and nuclear protein import, paraformaldehyde-fixed aliquots were mixed with 150-kD FITC-dextran and the fluorescent DNA dye, Hoechst 33258. Nuclei were observed with a Zeiss Photomicroscope III using a 63× planapochromat objective and CCD camera with frame integrator (Newmeyer and Forbes, 1990). Only nuclei which excluded the FITC-dextran were quantitated for import (a fraction of nuclei were broken during processing of the slide). The rhodamine transport signal was integrated (usually 16× or 32× depending upon intensity) and captured using an image analysis program (NIH Image 1.49). The perimeter of each nucleus was circled and both the cross-sectional area (pixels) and the average fluorescent intensity within the nucleus (luminance/pixel, on a linear scale of 1 to 255) were determined. Intensity values between 25 and 200 were considered to be in the linear range. Nuclei with intensity values above or below these limits were requantitated at a different integration. All values were then adjusted to the equivalent of 16× integration for comparison. Nuclei were photographed using T-MAX P3200 film. All photomicrographs of rhodamine fluorescence were taken under identical conditions to accurately indicate relative intensities.

Immunofluorescence Assays

Xenopus A6 kidney epithelial cells were cultured at 25°C in 60% L15 media with 5% FBS and 1% fungibact. For immunofluorescence using anti-p97 antibodies, cells grown on coverslips were permeabilized for 5 min on ice with 0.2% Triton X-100 in transport buffer (TB; 20 mM Hepes, pH 7.3, 110 mM KOAc, 5 mM NaOAc, 1 mM EGTA 2 mM $Mg[OAc]_2$, and 2 mM DTT). Cells were then fixed for 7 min at RT with 4% paraformaldehyde in TB. Nonspecific sites were blocked by a 1 h incubation with 10% FBS in TB (FBS/TB) and cells were then incubated for 1 h with affinity purified anti-p97 diluted 1:50 in FBS/TB, followed by a secondary RITC labeled goat anti-rabbit antibody (1:50 in FBS/TB; Boehringer-Mannheim Biochemicals, Indianapolis, IN). Coverslips were washed, blotted, and mounted with 90% glycerol in 10 mM Tris-Cl, pH 8.3, containing 10 mM paraphenylenediamine. DNA was visualized by staining with Hoechst 33258. Samples were observed using a Zeiss Photomicroscope III with a 100× planapochromat objective and a CCD camera with frame integrator. The rhodamine fluorescence signal was integrated 64× and photographed using a Sony videographic printer and thermo-sensitive paper.

To localize p97 in reconstituted nuclei, 25 μl standard reconstitution reactions containing Xenopus egg cytosol, 1× Xenopus membranes, an ATP regeneration system, and 1–2,000 sperm chromatin per μl were incubated for 1 h. After nuclei had formed, the reactions were incubated 15 min on ice, and then diluted into 1 ml ELB with 2 mM ethylenglycol*bis*(succinimidylsuccinate) (EGS; Pierce, Rockford, IL; 10 μl from a 200 mM stock in DMSO) and fixed for 15 min at RT. The fixed, reconstituted nuclei were layered over 25% sucrose in ELB and centrifuged onto polylysine-coated coverslips. The nuclei were fixed on the coverslips for 10 min at RT with 4% paraformaldehyde in PBS and were then permeabilized with 0.1% Triton X-100 (in PBS, 100 mM glycine). After rinsing in PBS, non-specific sites were blocked by incubation with 5% FBS in PBS (1 h), and nuclei were then incubated with affinity purified anti-p97 (1:50 in 5% FBS; 1 h). RITC-labeled goat anti-rabbit IgG (1:100 in 5% FBS) was used as the secondary antibody. The coverslips were washed and mounted as above. Rhodamine fluorescence was integrated 16× and photographed as described above.

To assay for RPA binding to chromatin, nuclear reconstitution reactions from which Xenopus membranes had been omitted were incubated for 1 h. The reactions were diluted into ELB, centrifuged onto polylysine-treated coverslips, and fixed as described above. After nonspecific sites were blocked with 5% FBS, coverslips were inverted onto polyclonal rabbit anti-RPA serum (Fang and Newport, 1993; 1:100 in 5% FBS). After 1 h incubation, coverslips were washed and then incubated with FITC-labeled goat anti-rabbit IgG (Cappel, Durham, NC; 1:100, 1 h). Following a final wash, coverslips were mounted and observed as described above. The fluorescein signal was integrated 16×.

DNA Replication Assays

To assay chromosomal DNA replication, nuclear assembly reactions were set up as described above for nuclear import, except that 2–4 μCi of α^{32}P-dCTP (3000 Ci/mmol; ICN, Irvine, CA) was included in the 60 μl reaction. To assess replication of single stranded DNA templates, sperm chromatin was replaced by 3 ng/μl ss M13 DNA. Replication assays were incubated at RT for either 3 or 5 h. Before the start of the incubation and at 1 h intervals thereafter a 5 μl aliquot was withdrawn from each reaction and added to 5 μl of replication sample buffer (80 mM Tris-Cl, pH 8.0, 8 mM EDTA, 0.13% phosphoric acid, 10% Ficoll, 5% SDS, 0.2% bromphenol blue; Smythe and Newport, 1991). Samples were then treated with proteinase K (1 mg/ml final) for 2 h at 37°C, followed by electrophoresis through a 0.8% agarose gel in 1× TAE (40 mM Tris, 20 mM HOAc, and 1 mM EDTA). Gels were dried and exposed to film. The rat WGA-binding proteins (rat eluate, RE) were isolated from RLN by Mega 10 (Pierce, Rockford, IL) extraction and WGA-Sepharose binding as described in Finlay and Forbes, 1990.

Results

Isolation of p97 and Production of Anti-p97 Antisera

Xenopus eggs contain three major GlcNAc-modified glycoproteins, p200, p97, and p60, and a number of minor glycoproteins (Fig. 1, lane *2*; Finlay and Forbes, 1990). When a low-speed extract of eggs is further fractionated into high-speed cytosolic, membrane vesicle, and pellet fractions, the three major GlcNAc-bearing proteins are present in the cytosol. To obtain purified p97, the above glycoproteins were first isolated on WGA-Sepharose, followed by extensive washing, and then separated on a preparative polyacrylamide gel (as in Fig. 1, lane *1*). The p97 band was excised and injected into rabbits to prepare polyclonal anti-p97 antiserum, which was subsequently affinity purified. Affinity purified anti-p97 antiserum recognized a single major band in Xenopus egg cytosol (Fig. 1, lane *4*), in the isolated WGA-binding glycoproteins from Xenopus cytosol (Fig. 1, lane *5*), and in Xenopus A6 tissue culture cells (lane *6*). The anti-p97 antiserum failed to recognize any p97 in the membrane fraction

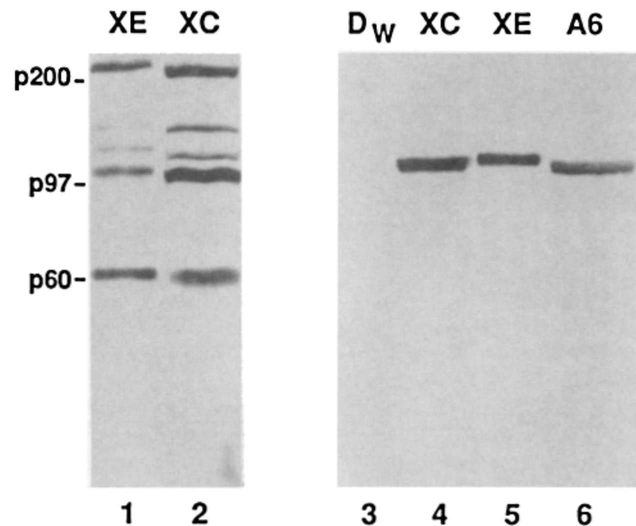

Figure 1. Polyclonal antiserum recognizes p97. Total Xenopus cytosol (*XC*) and isolated Xenopus WGA-binding proteins (Xenopus eluate, *XE*) were separated by 10% SDS-PAGE, transferred to nitrocellulose and probed with ^{125}I-WGA (lanes *1* and *2*). The positions of the three major WGA-binding proteins are indicated. The slight apparent shift in size seen between bands in the XC and XE results from the great difference between total protein concentration in these samples. Affinity purified, polyclonal antiserum raised against p97 (lanes *3–6*) recognizes a single protein of the correct size in both Xenopus cytosol and eluate, as well as in a total cell lysate of cultured Xenopus A6 cells (*A6*). The p97 protein is absent in cytosol depleted of the WGA-binding proteins (D_W).

of an egg extract (not shown; see also Fig. 6) or in cytosol that had been depleted with WGA-Sepharose (Fig. 1, lane *3*).

p97 Is a Homolog of the GLFG Family of Yeast Nuclear Pore Proteins

To obtain sequence information on p97, the protein was isolated in a manner similar to that for antibody production and treated with the endopeptidase Lys C to produce peptide fragments. The peptide mixture was fractionated by HPLC with further chromatography when necessary to resolve mixed peptides. Distinct peaks were chosen for sequencing. Three informative peptide sequences were obtained (Fig. 2 *a*). Peptide 1 contained the sequence SLEELRLEDYQANRK. This sequence, when compared to a protein sequence data bank, matched a sequence in the NH_2-terminal region of the yeast GLFG pore protein, NUP116, in 11/15 positions (Fig. 2 *b*; Wente et al., 1992; Wimmer et al., 1992). Peptide 2, GPQNPVGAPAGTGLFGTIAAYF, contained a GLFG repeat common to the family of yeast pore proteins which includes NUP116 (Fig. 2 *a*). The amino acids surrounding the GLFG sequence were uncharged as in the yeast GLFG family. Peptide 3, QGAQFVDYRPESG(S)(C)V, also resembled a sequence present in NUP116 and included five amino acid identities (*underlined*, Fig. 2 *c*). This latter peptide when scored for similarity between the p97 and NSP116 sequence gave a weighted score (where similar amino acids contribute in addition to identities) of 10.4, indicating significant homology (Schwartz and Dayhoff, 1978; Doolittle, 1987). The peptide 3 homology mapped to a COOH-terminal region of NUP116, which also has homology to the other yeast GLFG-containing proteins, NUP100 and NUP145 (Fig. 2 *d*; Wente et al., 1992; Wimmer et al., 1992; Fabre et al., 1994; Wente and Blobel, 1994). The weighted

Figure 2. Peptide sequences from Xenopus p97 show homology to members of the yeast GLFG family of nucleoporins. p97 was gel purified and digested with the endopeptidase Lys-C. Individual peptides were isolated by HPLC and sequenced by automated Edman degradation. Three distinct sequences were obtained. (*A*) The sequences of Xenopus p97 peptides 1, 2, and 3 are shown. The GLFG motif found in peptide 2 is underlined. Parentheses indicate amino acids about which there was uncertainty. (*B*) Sequence comparison between p97 peptide 1 and yeast pore protein NUP116 (Wente et al., 1992). The starting amino acid position in the NUP116 sequence is indicated at the left. Amino acid identities are darkly shaded. The peptide 1 sequence homology was identified using the BLAST program (Altschul et al., 1990). (*C*) Sequence comparison between p97 peptide 3 and NUP116. The homology score for each pair of amino acids is shown below and the total weighted score for the matched peptides (where, in addition to identities, similar amino acids contribute) is shown at the right, exclusive of (S) and (C) (Schwartz and Dayhoff, 1978). The score inclusive of all 16 amino acids is given in parentheses. A score greater than 10 is considered highly significant (Doolittle, R., personal communication). Amino acid identities are darkly shaded and similarities are lightly shaded. (*D*) The region of NUP116 shown in C is compared to related regions of NUP100 (Wente et al., 1992) and NUP145 (Wente and Blobel, 1994; Fabre et al., 1994). Amino acid identities and similarities to p97 are shaded as above. The homology score for comparison of each yeast sequence with Xenopus p97 is shown at the right. Amino acid positions are indicated at left. (*E*) Schematic representation of the domain structures of the GLFG nucleoporin family. Alignment of the Xenopus p97 peptides based on sequence homology is indicated. The exact positioning of peptide 2 within the GLFG domain is uncertain and is indicated in parentheses.

score for comparison of peptide 3 with NUP100 was 9.6 and for NUP145, 10. Thus, p97 contains: (*a*) a sequence highly similar to a unique region of NUP116; (*b*) at least one GLFG motif; and (*c*) a sequence most similar to NUP116 but related to NUP100 and NUP145, both of which resemble NUP116 in this COOH-terminal region (Fig. 2 *e*).

Polyclonal antisera were raised to peptides 1 and 3 and used to confirm that these sequences were indeed derived from the Xenopus glycoprotein p97. Both anti-peptide 1 and 3 antisera react with p97 when used on blots of the Xenopus egg GlcNAc-bearing proteins (Fig. 3, lanes *3* and *4*). Anti-peptide 1 also reacts with additional proteins when used to probe total Xenopus cytosol (Fig. 3, lane *7*). These proteins are not GlcNAc bearing and we do not yet know whether they are pore proteins.

To examine the localization of p97, the anti-p97 polyclonal antiserum was used to stain nuclei which had been assembled by combining sperm chromatin, Xenopus egg cytosol, and the membrane vesicular fraction of Xenopus eggs. The affinity purified anti-p97 antiserum gave a nuclear rim stain

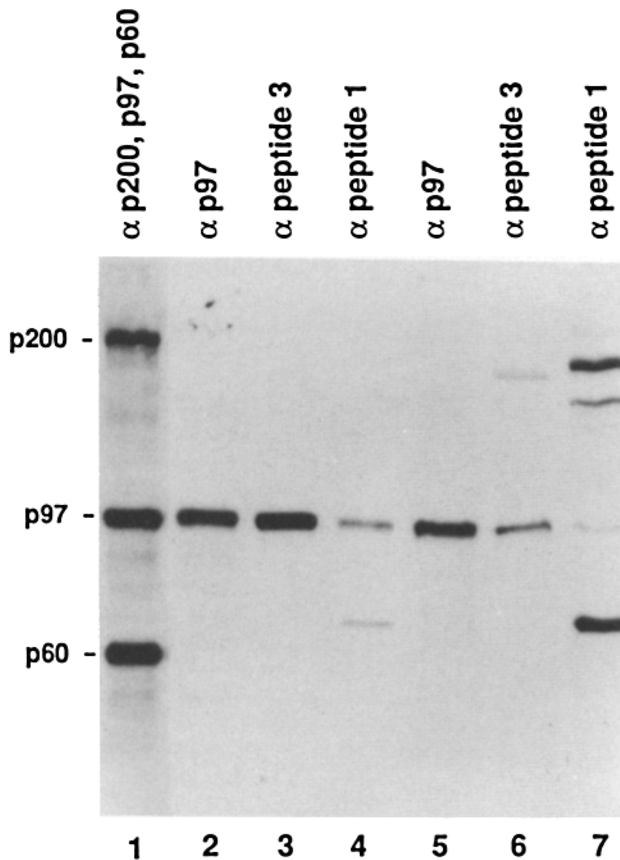

Figure 3. Antisera prepared against peptides 1 and 3 recognize p97. Peptides 1 and 3 were synthesized as multiple antigen peptide (MAP) conjugates and injected into rabbits. Antisera were affinity purified on a column of the appropriate peptide coupled to Affigel 10. Xenopus eluate (lanes *1–4*) or Xenopus cytosol (lanes *5–7*) was resolved by 8% SDS-PAGE and transferred to nitrocellulose. Strips of the blot were probed with anti-peptide 1 (1:75), anti-peptide 3 (1:300), affinity purified anti-p97 (1:4,000), or with a mixture of affinity purified antibodies to p200, p97, and p60. The proteins were detected with HRP-conjugated secondary antibody and a chemiluminescent substrate.

on the reconstituted nuclei (Fig. 4 *b*). Under certain fixation conditions there was also a significant amount of intranuclear staining (Fig. 4 *b*), which we failed to see with anti-p200 or anti-p60 antisera (Macaulay, C., unpublished results). This staining could represent unincorporated p97 or, alternately, incorporation of p97 into other structures in addition to pores. When the anti-peptide antisera were used in immunofluorescence on reconstituted nuclei, they too gave a strong nuclear rim stain and an intranuclear stain (not shown), identical to the polyclonal anti-p97 antisera stain in Fig. 4 *b*. These results support a nuclear pore location for p97, with an additional intranuclear localization, but also established that during in vitro nuclear reconstitution cytosolic p97 can be assembled into the nuclear envelope.

When immunofluorescence was performed on Xenopus A6 tissue culture cells, the anti-p97 antiserum gave a punctate nuclear rim pattern with intranuclear staining (Fig. 4, *d* and *f*). This intranuclear staining was not observed with anti-p200 or anti-p60 antisera (not shown). The intranuclear staining decreased greatly in prophase nuclei, where the nuclear rim of such cells was the sole stained entity and appeared brighter than that found in interphase cells (Fig. 4 *f, middle nucleus*). No staining of the metaphase chromosomes was observed with the anti-p97 polyclonal antisera (Fig. 4 *f, right-hand cell*), but a bright nuclear rim stain reappeared at telophase (not shown). In all the above anti-p97 immunofluorescence staining, the nuclear rim stain was often punctate (see Fig. 4, *b*, *d*, and *f*), consistent with a nuclear pore staining pattern.

To determine whether further evidence of similarity between p97 and the yeast GLFG pore proteins could be obtained, anti-p97 antiserum was tested for cross-reactivity with other species. On Western blots of a total yeast cell lysate, the anti-p97 polyclonal antiserum recognized five proteins of ∼116, 100, 65, 54, and 49 kD (Fig. 5 *B*, lane *2*). All were also recognized by the anti-GLFG monoclonal antibody, mAb 192, as was an intensely stained protein of 35 kD which does not co-enrich with nuclei (Fig. 5 *A*, lane *2*; Wente et al., 1992). The yeast protein showing the strongest cross-reactivity with anti-p97 was that of 116 kD, presumably NUP116. It is likely that the anti-97 antiserum which was raised to the complete Xenopus p97 protein contains both anti-GLFG antibodies and antibodies specific to p97 unique sequences, and thus reacts with the whole family of yeast GLFG nucleoporins. The high reactivity to NUP116 may indicate the presence of a greater number of GLFG sequences than the other yeast GLFG pore proteins, a greater similarity in the unique regions of the p97 and NUP116, or simply a higher concentration of NUP116 in yeast. (The anti-peptide antisera failed to react with the yeast proteins; data not shown). Both the anti-p97 polyclonal antiserum and the anti-GLFG mAb 192 when tested on rat liver nuclei cross-reacted with a 97 kD or slightly smaller protein (Fig. 5, *A* and *B*, lane *1*). This protein was also recognized by anti-peptide 1 serum (see Fig. 9 *C*). Interestingly the anti-GLFG mAb recognized a doublet of >200 kD in Xenopus cytosol, indicating that p97 may not be the only GLFG protein in Xenopus (Fig. 5 *A*, lane *3*). However, this protein doublet is not depleted by anti-p97 antibodies (Fig. 5 *A*, lane *4*), and thus is not related to the defects in p97-depleted nuclei that are addressed below. In summary, from the immunofluorescence pattern, the incorporation into recon-

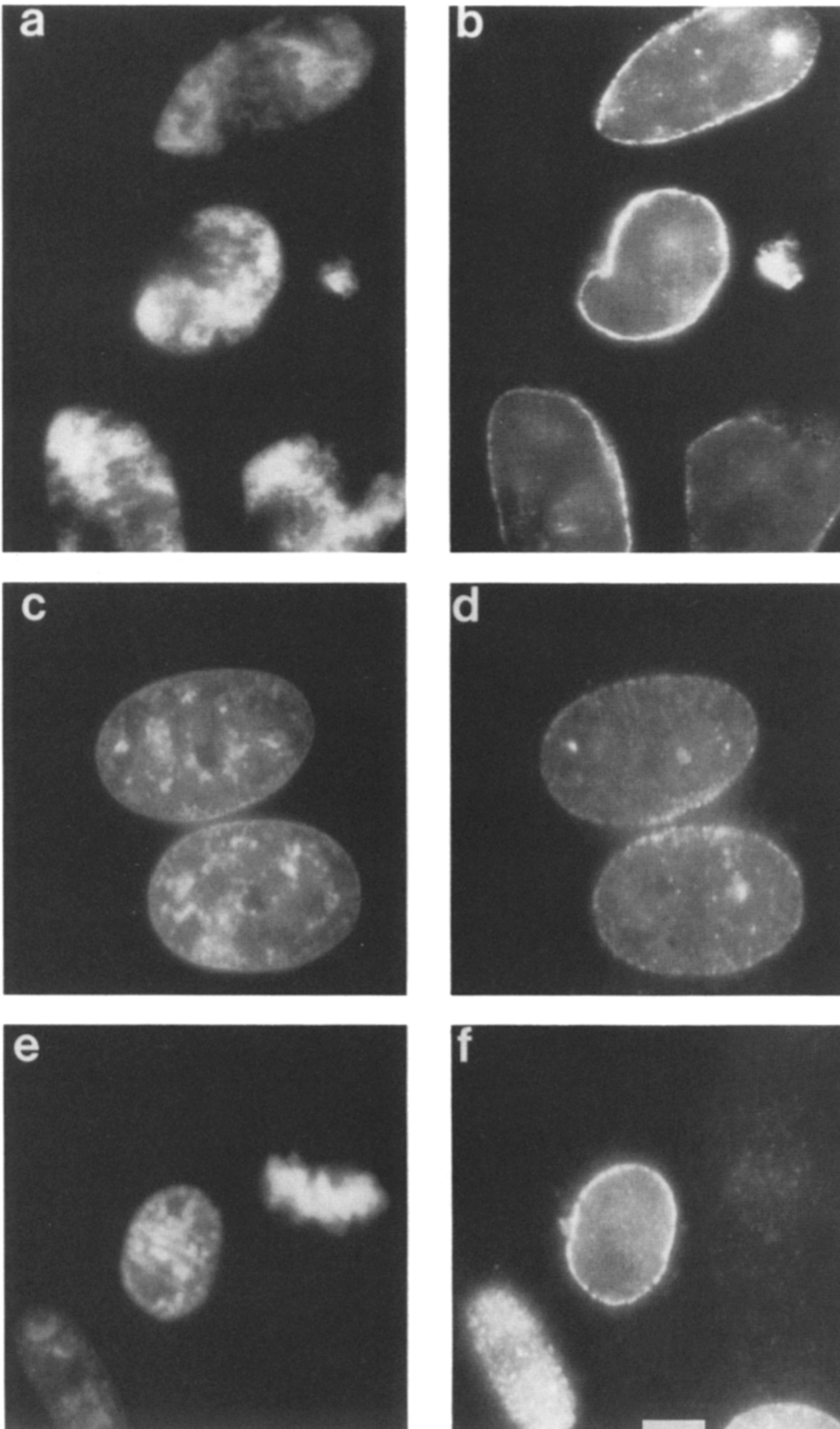

Figure 4. Immunofluorescence assay localizes p97 to both nuclear pores and the interior of the nucleus. In vitro reconstituted nuclei (*a* and *b*) or cultured Xenopus A6 cells (*c–f*) were fixed and permeabilized as described in Materials and Methods. Affinity purified anti-p97 (1:50), followed by RITC-labeled secondary antibody, was used to establish the distribution of p97 (*b*, *d*, and *f*). Hoechst 33258 was used to stain the DNA of reconstituted nuclei or cells (*a*, *c*, and *e*). Panels *a* and *b* are shown at 1,500× magnification. Panels *c–f* are shown at 2,400× magnification. The immunofluorescence reveals intranuclear p97 as well as a punctate rim stain characteristic of nuclear pore proteins (*b* and *d*), the intensity of which increases noticeably at prophase (*middle cell*, *f*). During metaphase, p97 is dispersed throughout the cell (*right-most cell*, *f*). Bars: (*a* and *b*) 0.667 μm; (*c–f*) 0.416 μm.

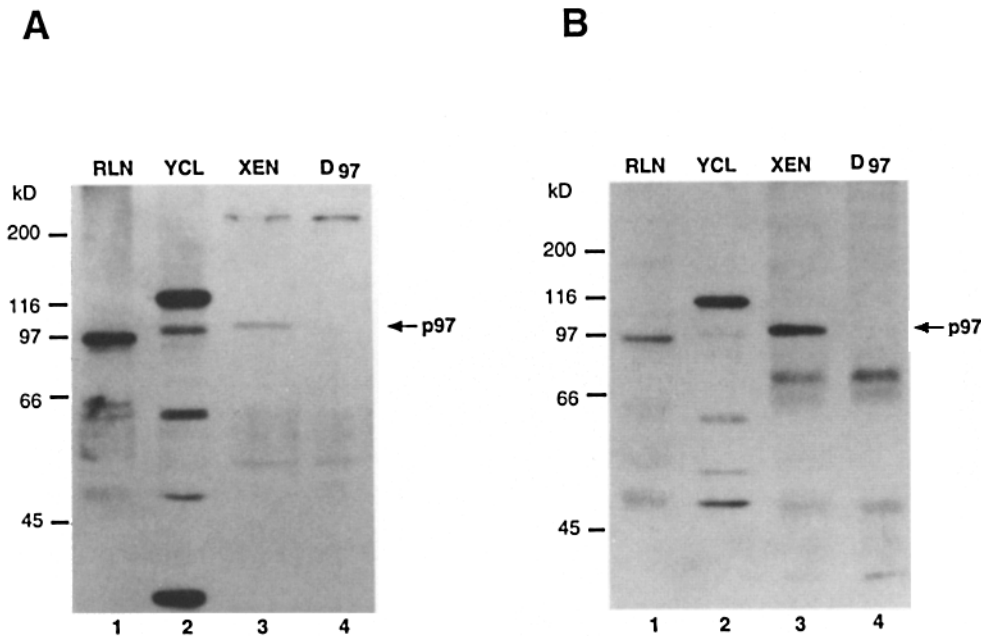

Figure 5. Anti-p97 and monoclonal antibody 192 (anti-GLFG domain) recognize the same family of proteins in multiple species. To determine cross-reactivity of anti-p97 and mAb 192 with different species, rat liver nuclei (*RLN*), total yeast cell lysate (*YCL*), Xenopus cytosol (*XC*), and Xenopus cytosol immunodepleted of p97 (D_{97}) were immunoblotted with either mAb192 (*A*) or affinity purified anti-p97 (*B*). (*A*) For immunoblotting with mAb192, nuclease-treated rat liver nuclei (1.5×10^6), yeast cell lysate (1.7×10^5 cells), and 0.5 μl each of cytosol and p97-depleted cytosol were resolved by 8% SDS-PAGE and transferred to PVDF. The blot was probed by overnight incubation with mAb192 (1:100) followed by rabbit anti-mouse IgM, goat anti-rabbit IgG conjugated to HRP, and development with a chemiluminescent substrate. (*B*) For immunoblotting with anti-p97, nuclease-treated rat liver nuclei (1.5×10^6), yeast cell lysate (3.4×10^5 cells), and 0.25 μl each of cytosol and p97-depleted cytosol were electrophoresed and transferred as above. The blot was probed by overnight incubation with anti-p97 (1:1000) followed by goat anti-rabbit IgG conjugated to HRP and development with a chemiluminescent substrate. The positions of protein size markers (Bio-Rad Laboratories, Richmond, CA) are shown. Both blots were exposed to film for 1 min.

stituted nuclei, and the sequence similarity with yeast GLFG pore proteins as shown both by crossreactive antisera and peptide sequencing, we conclude that p97 has the characteristics of a nuclear pore protein with homologs in both yeast and rat.

p97-deficient Nuclei Remain Competent for Nuclear Import but Do Not Grow Over Time

With clear indications that p97 is a nuclear pore protein we wished to determine whether nuclei lacking p97 are capable of import. For this, nuclear reconstitution extracts were prepared from which p97 had been specifically immunodepleted. Crude Xenopus egg cytosol was separated by high-speed centrifugation into soluble cytosol, a membrane vesicular fraction, and a gelatinous pellet. The cytosol, which contained the p200, p97, and p60 GlcNAc-bearing proteins (Finlay and Forbes, 1990; see also Fig. 1, lane 2) was split into aliquots. One aliquot was immunodepleted with affinity purified anti-p97 antibodies bound to protein A-Sepharose. An identical aliquot was mock depleted with nonspecific rabbit IgG protein A-Sepharose. For use as a negative control, an additional aliquot was depleted of all three proteins using WGA-Sepharose. To check for immunodepletion of p97, the proteins of the various cytosols were electrophoresed, transferred to nitrocellulose, and probed with a mixture of anti-200, anti-97, and anti-60 antibodies. As can be seen, p97 has been specifically removed from the p97-depleted cytosol, while p200 and p60 remain relatively unchanged in amount (Fig. 6, lane *3*). Even when eight times as much p97-depleted cytosol was examined, no p97 could be detected (Fig. 6, lane *8*). Control-depleted cytosol contained all three glycoproteins (Fig. 6, lane *4*), while WGA-depleted cytosol lacked all three (Fig. 6, lane *2*). Thus, affinity purified anti-p97 polyclonal antiserum is clearly capable of reacting specifically with the native soluble form of p97 in Xenopus egg cytosol and is able to fully immunodeplete it from the cytosol.

To reconstitute p97-deficient nuclei, p97-depleted cytosol was mixed with demembranated sperm chromatin and membrane vesicles, and nuclei depleted of p97, termed D_{97} nuclei, were formed. In a similar manner nuclei were reconstituted in control-depleted cytosol (D_C nuclei), WGA-depleted cytosol (D_W nuclei), and in p97-depleted cytosol which had been supplemented with a mixture of the isolated GlcNAc-bearing proteins (D_{97} + XE nuclei; Fig. 6, lane *9*). To assess transport, nuclei were allowed to form for a given amount of time, usually two hours, at which time rhodamine-labeled transport substrate was added. Aliquots from each reaction were withdrawn at various times and examined in the fluorescence microscope for nuclear size and protein import. Individual nuclei were captured by video imaging and quantitated for nuclear size (cross-sectional surface area) and nuclear transport (fluorescence intensity/pixel; Newmeyer and Forbes, 1990). Fig. 7 shows typical nuclei reconstituted in D_C and D_{97} cytosol. Mock-depleted nuclei (D_C) grow to large size and clearly import the transport substrate. D_{97} nuclei, which lack p97, are small and remain at that size throughout the period of import (Fig. 7). The p97-deficient nuclei are slightly but reproducibly larger than WGA-depleted nuclei (Fig. 8 *A*). The D_{97} size defect could be completely prevented by the addition of

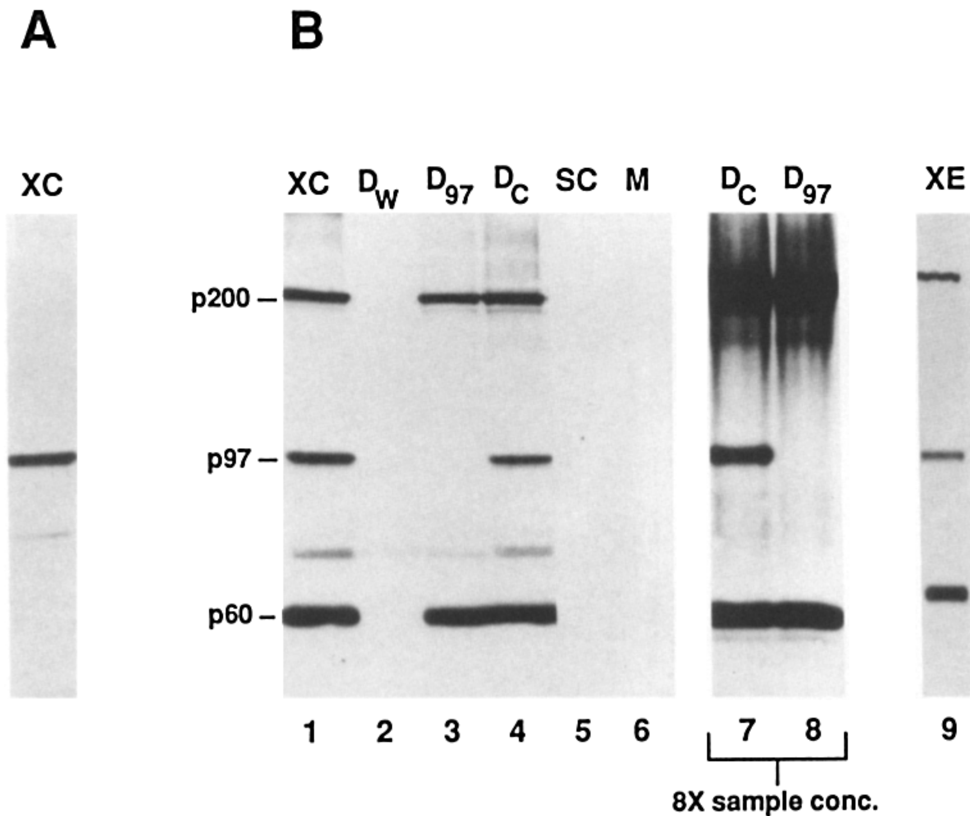

Figure 6. p97 can be immunodepleted from Xenopus egg cytosol. Xenopus cytosol was immunodepleted by two sequential incubations with affinity purified anti–p97 bound to protein A-Sepharose (D_{97}) but not by similar treatment with non-specific rabbit IgG-protein A-Sepharose (D_C). Complete Xenopus cytosol (*XC*) and cytosol depleted of all WGA-binding proteins (D_W) are shown as controls. (*A*) 3 μl of complete cytosol was resolved by 8% SDS-PAGE, transferred to PVDF, and immunoblotted with anti–p97 antiserum alone (1:1,000). (*B*) To demonstrate specific immunodepletion of p97 from Xenopus cytosol, proteins from immunodepleted and control cytosols were separated by 8% SDS-PAGE and transferred to PVDF. The blots were probed with a mixture of affinity purified antibodies to the three major WGA-binding proteins, anti–p200, anti–p97, and anti-p60. 3 μl of each type of cytosol (lanes *1–4*) as well as demembranated sperm chromatin and washed membranes (*SC* and *M*, lanes *5* and *6*; in amounts proportional to 3 μl of cytosol based upon their relative concentrations in a nuclear reconstitution reaction) were loaded on the gels. To confirm that p97 was fully immunodepleted, eight times the volume of D_C and D_{97} cytosol used in lanes *3* and *4* was concentrated on WGA-Sepharose, electrophoresed, and immunoblotted (lanes *7* and *8*). Even in these enriched samples, no trace of p97 can be observed in the D97 cytosol (lane *8*). Isolated WGA-binding proteins (Xenopus eluate, *XE*, lane *9*) can be seen to contain p200, p97, and p60.

a mixture of isolated p200, p97, and p60 proteins at the start of the nuclear reconstitution reaction (D_{97} + XE; Fig. 7 and 8 *A*). Surprisingly, we found that D_{97} nuclei show substantial nuclear import. Some variation was observed from experiment to experiment and the transport data from two experiments are presented in Fig. 8 (*B* and *C*) to show this range. In Fig. 8 *B* (the most typical result), D_{97} nuclei transported in a manner similar to the control nuclei, whereas the WGA-depleted nuclei were extremely reduced in transport as previously reported (Finlay and Forbes, 1990; Finlay et al., 1991). In Fig. 8 *C*, D_{97} nuclei are reduced in transport less than twofold with respect to the D_C nuclei, except at the 30 min time point. The D_{97} nuclei were still approximately eightfold higher in import than were the D_W nuclei at 2 h. We conclude that even though p97 has all the aspects of a nuclear pore protein, its absence from the pore fails to significantly impair nuclear protein import of a standard transport substrate. It is also possible to see in Fig. 7, the binding of the transport substrate to coiled bodies (Bauer et al., 1994) in both the D_C and the D_{97} + XE nuclei. Although such binding is not visible in those D_{97} nuclei in Fig. 7, being obscured by the highly concentrated transport substrate, we did frequently see coiled body staining in D_{97} nuclei at times when transport had not proceeded so far (not shown). This argues that the proteins which form the coiled body are also capable of assembling in p97-depleted nuclei.

p97-deficient Nuclei Fail to Replicate Their Chromosomal DNA

The large effect of p97 depletion on nuclear size in p97-deficient nuclei prompted us to examine these nuclei for other functional defects. It is known that nuclei assembled around sperm chromatin in undepleted Xenopus egg extracts undergo replication of the enclosed chromosomal DNA (Blow and Lasey, 1986; Newport, 1987). The replication of chromosomal DNA commences only after the added sperm chromatin becomes enclosed in a nuclear envelope, i.e., replication has an absolute requirement for an intact nuclear envelope. Replication proceeds until one complete round of replication has occurred and then ceases (for review see Almouzni and Wolffe, 1993. To determine whether p97-deficient nuclei can replicate, sperm chromatin was added to mock-depleted (D_c), anti–p97 depleted (D_{97}), and WGA-depleted (D_W) cytosol, as well as to cytosol to which the isolated Glc-NAc-modified proteins p200, p97, and p60 were added (+XE). Membranes and ^{32}P-dCTP were added to the reconstitution reaction at t = 0. Aliquots were withdrawn after 0, 1, 2, 3, 4, and 5 h of incubation and tested for incorporation of radioactive nucleotide into the chromosomal DNA. Visual assay in the fluorescence microscope indicated that intact nuclei had formed by 1 h in the D_{97}, D_{97} + XE, and control extracts, and by 2 h in the D_W ex-

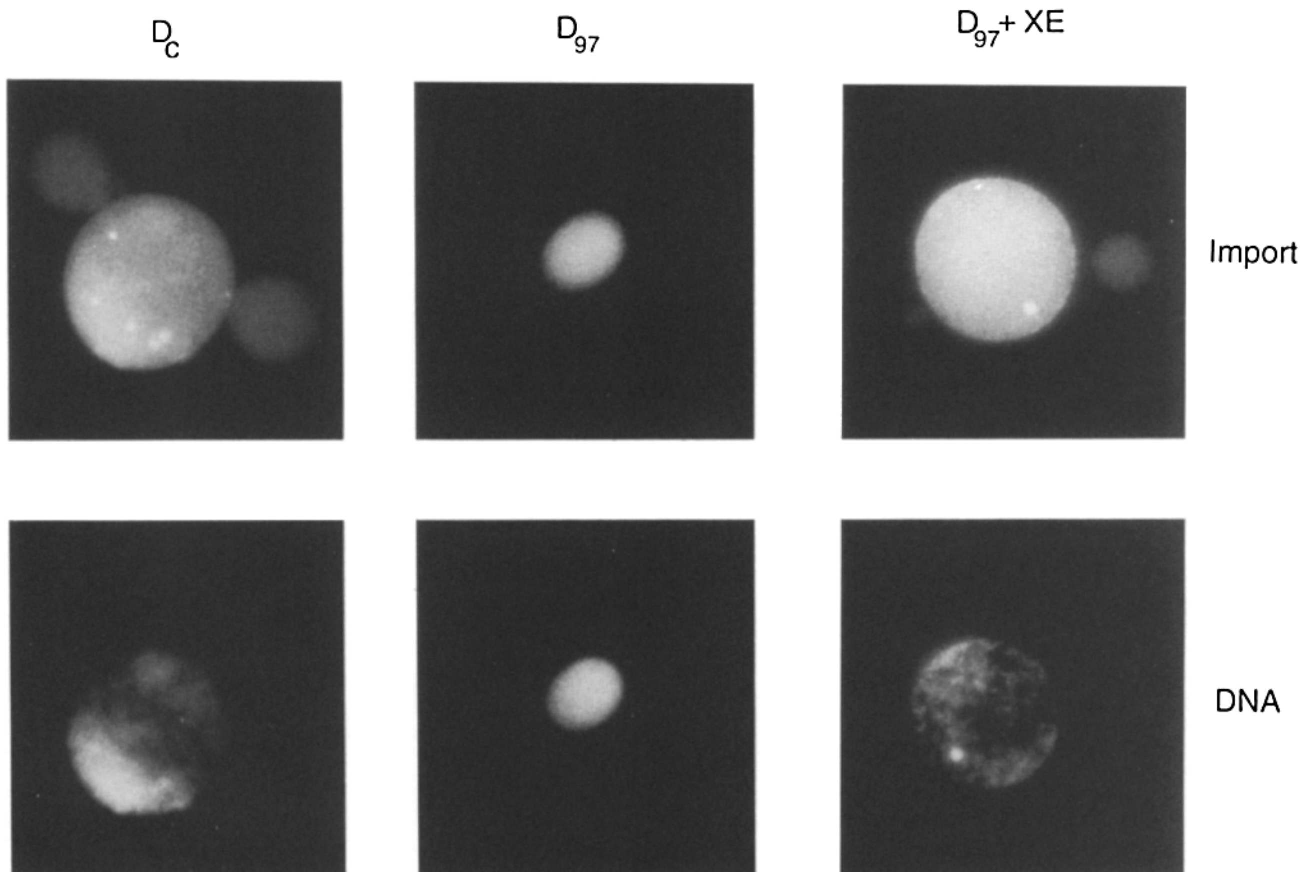

Figure 7. Nuclei form in p97-depleted Xenopus egg cytosol and import a nuclear-targeted protein. Xenopus cytosol was immunodepleted using either anti–p97 antibodies (D_{97}) or non-specific rabbit IgG (D_C) bound to protein A–Sepharose. As a control, cytosol was also depleted using WGA-Sepharose (not shown). To reconstitute nuclei in depleted cytosol, 36 μl of cytosol were mixed with 12 μl of either buffer or isolated Xenopus WGA-binding proteins (*XE*), 1× washed Xenopus membranes, an ATP regeneration system, and sperm chromatin (1,000/μl) in a final volume of 60 μl. Nuclei were allowed to form for 2 h or until formation of nuclei in WGA-depleted cytosol was completed. A fluorescent transport substrate (TRITC-SS-HSA) was then added, and import was allowed to proceed for 2 h. At 30-min intervals, aliquots were collected and fixed with 3.7% paraformaldehyde (final concentration). Import was assayed by observation of the nuclei in the rhodamine channel of a fluorescence microscope. The nuclear DNA was visualized with the fluorescent dye, Hoechst 33258. After two hours of import, photomicrographs were taken under identical conditions to indicate the relative fluorescent intensities. Control nuclei were large, often containing additional blebs of nuclear envelope, and showed high levels of import (D_C). p97-deficient nuclei showed similar import but remained small (D_{97}). This size defect did not occur if isolated WGA-binding proteins were included with the p97-depleted cytosol at the time of nuclear formation (D_{97} + *XE*).

tract, which has always proved slower in nuclear formation. As can be seen in Fig. 9 *A*, mock-depleted nuclei (D_C) showed high levels of DNA replication, commencing between 1 and 2 h. Replication was sensitive to the inhibitor aphidicolin and dependent upon the presence of membrane (data not shown). In contrast, nuclei lacking all three glycoproteins completely failed to replicate their DNA, even by the 5 h time point (D_W). Surprisingly, p97-deficient nuclei (D_{97}) also were almost completely defective for replication. The defects in replication of both the D_{97} and D_W nuclei could be prevented if the isolated XE glycoproteins were included in the nuclear formation assays at $t = 0'$ (Fig. 9 *A*, D_{97} + XE, D_W + XE). Thus, p97-depleted nuclei have a severe replication defect and this defect can be readily reversed when a mixture of proteins containing Xenopus p97 is added to the nuclear reconstitution mixture.

Previously, we had shown that nuclei lacking all three glycoproteins were negative for nuclear import (Finlay and Forbes, 1990). This defect was found to be mimicked exactly by the immunodepletion of the pore protein p60 (Finlay et al., 1991). The p60 immunodepletion transport defect could be reversed by the inclusion of XE glycoproteins or, alternately, by GlcNAc-bearing glycoproteins extracted from rat liver nuclear envelopes, which contain large amounts of the rat p60 homologue, p62 (Finlay and Forbes, 1990). To determine whether the replication defect of p97-depleted nuclei could be reversed by the 97-kD immunologically related rat homologue observed in Fig. 5 (*A* and *B*, lane *1*), rat liver nuclei were prepared and extracted with the detergent Mega 10. The extracted nuclear envelope proteins were applied in batch to WGA-Sepharose and the rat WGA-binding proteins (RE) were eluted from the Sepharose with N-acetylglucosamine and trichitotriose (Finlay and Forbes, 1990). The eluted rat proteins were electrophoresed and immunoblotted with antiserum to p97 peptide 1 and could be seen to contain the rat homolog of p97 (Fig. 9 *C*). When rat GlcNAc-modified glycoproteins were added to a p97-depleted extract and nuclei were formed and assayed for chromosomal repli-

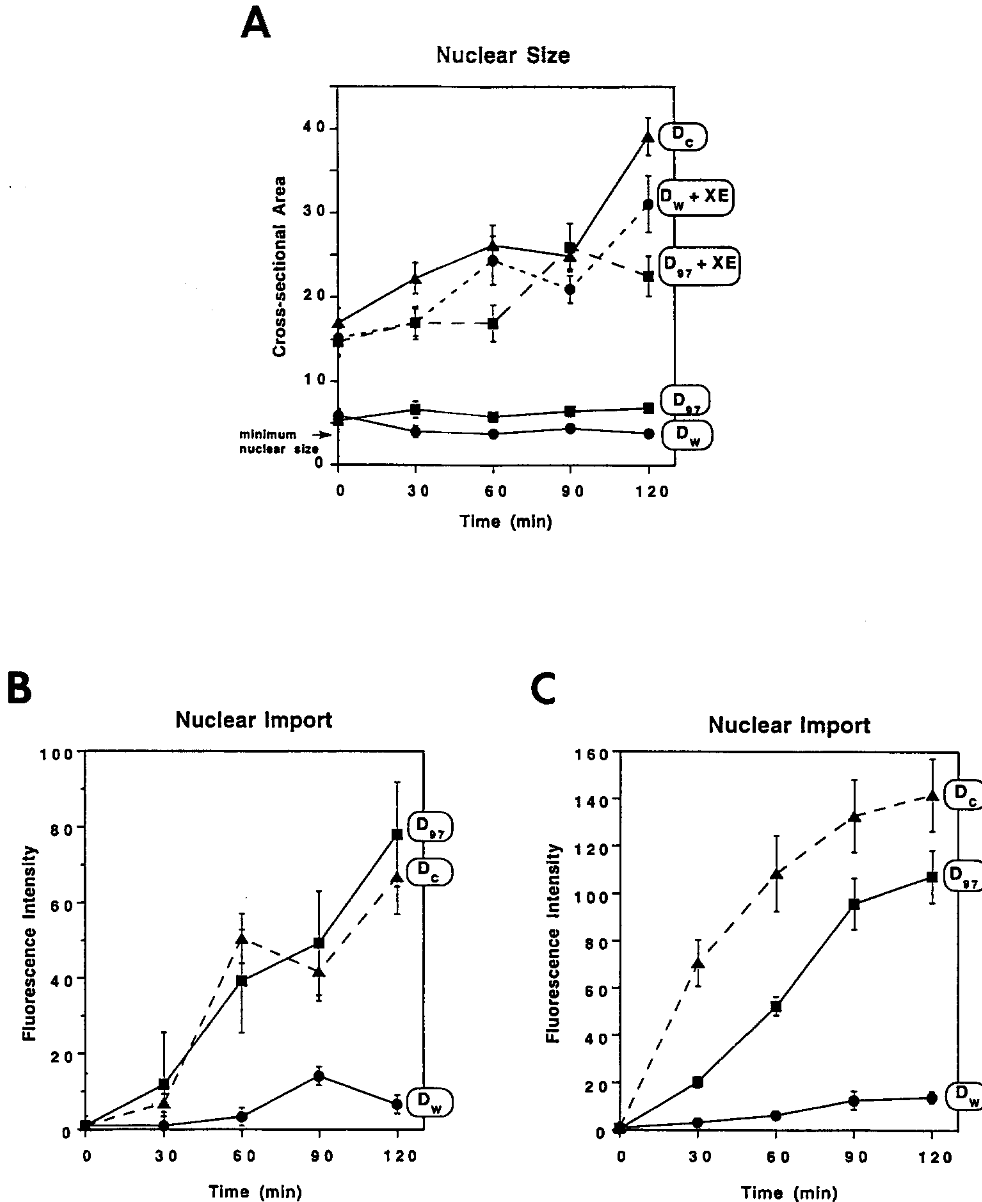

Figure 8. p97-deficient nuclei are competent for nuclear protein import but do not grow over time. Nuclear reconstitution reactions were incubated and aliquots collected and fixed as described in Fig. 7. (*A*) Nuclear size, measured as cross-sectional area (pixels), was determined over 2 h. t = 0 min represents the time at which nuclear import substrate was added to the reactions and sample collection began. Nuclei were observed using a 63× objective with a CCD camera, frame grabber, and video monitor attached to a computer utilizing the Image analysis program (NIH). Each data point represents the average of approximately 20 nuclei and error bars indicate the standard error of the mean. (*B* and *C*) Nuclear import, measured as the average fluorescence intensity (luminance/pixel) on an arbitrary scale of 1–255, was quantitated in reconstituted nuclei. Each data point is the average of approximately 20 nuclei and error bars indicate the standard error of the mean (SEM). *B* and *C* depict the results of two independent experiments. The data in *B* was gathered from the same nuclei analyzed in *A*. In *B*, the rhodamine fluorescence was integrated at 32× for all nuclei. In *C*, since a higher level of import substrate was added, the range of import in the different types of nuclei could not all be quantitated at 32× and therefore was quantitated at different integration values and normalized to 16×.

cation, the rat glycoproteins substantially reversed the replication defect (Fig. 9 *B*, D_{97} + RE). We conclude that rat nuclei contain a protein that is functionally homologous to Xenopus p97, and is capable of substituting for p97 in assembling replication competent nuclei.

A formal possibility was that p97 is enzymatically required for all replication in Xenopus extracts. To determine if this was so, i.e., whether all replication is inactivated in p97-depleted extracts, single stranded M13 DNA was added to the D_C and D_{97} extracts used above (in the absence of chromatin and membranes) and continuously labeled for 0, 1, 2, and 3 h. Clearly, no difference in replication is observed between the D_{97} and control extracts (Fig. 9 *D*). We conclude that the absence of p97 from nuclei severely inhibits replication of their chromosomal DNA, but that p97 is not required for extra-nuclear, single-stranded DNA replication.

Recently it has become clear that a very early step leading up to replication is the formation of pre-replication centers (pre-RCs). These pre-RCs are thought to be sites at which numerous pre-initiation complexes from different chromosomal sites aggregate into a physical entity. Pre-RCs can be identified visually by the binding of anti-replication factor A (RPA) antibody (Adachi and Laemmli, 1992). Approximately 200 such pre-replication centers form on sperm chromatin shortly after addition of condensed sperm chromatin to Xenopus egg cytosol (Adachi and Laemmli, 1992, 1994). Upon assembly of a nuclear envelope, replication commences and the pre-RCs disappear as replication and RPA localization extend along the chromatin. To ascertain whether the lack of replication in p97-deficient nuclei results from an inability to bind RPA at sites of pre-replication, we added sperm chromatin in the absence of membranes to p97-

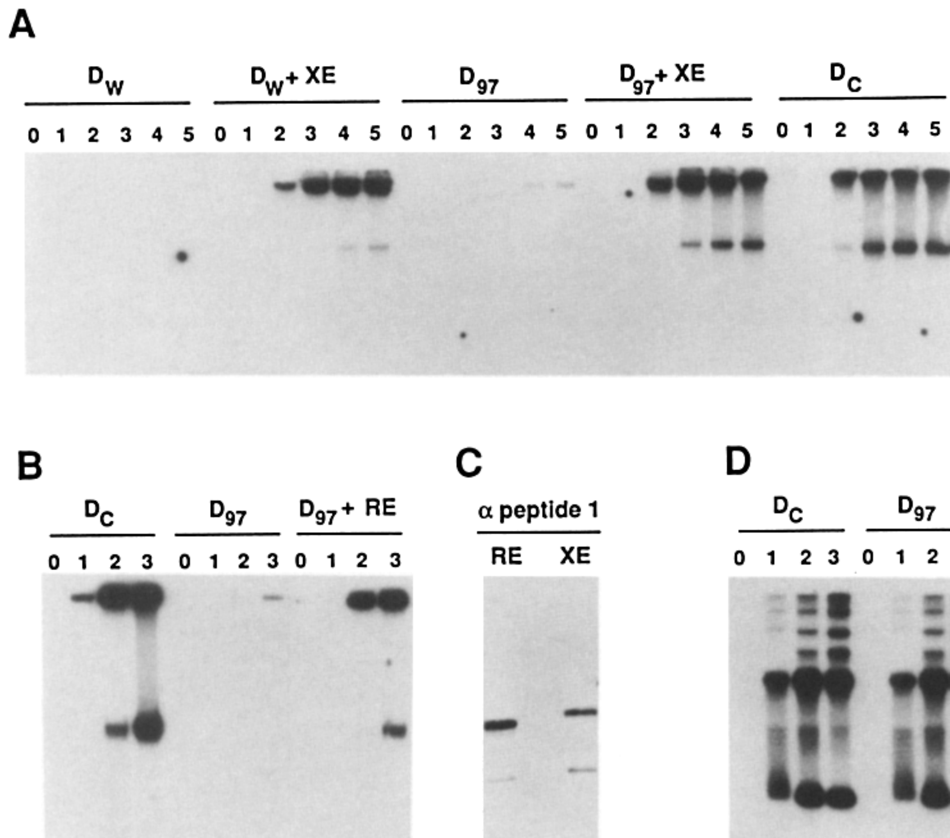

Figure 9. p97-deficient nuclei fail to replicate their chromosomal DNA. (*A*) Nuclei were assembled in D_{97}, D_C, or D_W cytosol with or without the addition of isolated XE, as described in Fig. 7. Radiolabeled α ^{32}P-dCTP was included in the reconstitution reactions to continuously label replicating DNA. At the indicated number of hours after the start of the incubations, aliquots were withdrawn, digested with proteinase K, and analyzed by 0.8% agarose gel electrophoresis and autoradiography. The data shown was obtained from the same depleted and control extracts used in Fig. 8 *C*; identical results were seen with the extracts used in Fig. 8 *B*. (*B*) The ability of WGA-binding proteins isolated from rat liver nuclei (rat eluate, *RE*) to complement the chromosomal DNA replication defect of p97-depleted nuclei was tested. Proteins prepared from 5 × 10^7 rat liver nuclei by detergent extraction and WGA-Sepharose affinity chromatography were added to a 40 μl nuclear reconstitution reaction containing D_{97} cytosol, prepared as described above. Aliquots were removed at the indicated times and analyzed as in *A*. (*C*) The proteins present in RE or XE (each corresponding to one sixth of the amount added to the reactions in *B* were electrophoresed and immunoblotted. The blot was probed with affinity purified p97 peptide 1 antiserum to demonstrate the presence of p97 or the homologous rat protein. (*D*) To assay the replication of single-stranded templates, M13 DNA (3 ng/μl final) along with radiolabeled dCTP were added to reconstitution reactions in the absence of sperm chromatin. Aliquots were withdrawn and analyzed as in *A*. Numbers indicate the hours of incubation before aliquot withdrawal.

depleted cytosol, as well as to mock-depleted cytosol. The sperm chromatin was incubated for 1 h, stained with anti-RPA antiserum, and visualized in the fluorescence microscope. We found that pre-replication centers, as judged by punctate anti-RPA staining, clearly formed on sperm chromatin incubated in the D_{97} cytosol (Fig. 10 *a*), in a manner indistinguishable from that in control extracts (not shown). Thus, we conclude that the absence of p97 from nuclei prevents chromosomal DNA replication at a step subsequent to the formation of the pre-replication centers.

Discussion

Recent work in the field of nuclear import has focused upon defining new components of the nuclear pore complex. This has been a fruitful approach as it has led to the identification of families of sequence-related proteins, as well as groups of physically interacting proteins, which may represent either structural building blocks or functional units of the pore. The first described subset of nuclear pore proteins was the N-glucosaminylated, WGA-binding proteins found in both rat and Xenopus. Of the three major WGA-binding proteins of Xenopus, two have been shown to be pore proteins, one of which (p60) is essential for nuclear import. We report here a study of the third major WGA-binding protein from Xenopus, p97. By immunofluorescence we find that p97 is a nuclear pore protein, but in addition, is localized to the interior of the nucleus. Using immunodepletion of p97 from a nuclear assembly extract derived from Xenopus eggs, we find that p97 is not essential for nuclear protein import. However, in the absence of p97, nuclei neither grow nor replicate their chromosomal DNA. The severe defect in replication was found to occur subsequent to the assembly of pre-replication centers in such nuclei. Thus p97, a pore protein with dual localization affects multiple nuclear functions.

Purification and peptide sequence analysis of Xenopus p97 revealed very informative homologies. Surprisingly, we found p97 to be the first vertebrate member of the GLFG family of nucleoporins. The yeast GLFG nucleoporin family consists of the related proteins, NUP145, NUP116, NUP100, NUP54, NUP49. These proteins each contain many copies of the tetrapeptide, GLFG, and were first identified based upon recognition of the GLFG domain by the monoclonal antibody 192 (Wente et al., 1992). Multiple possible functions have been ascribed to the GLFG nucleoporins in yeast, including mediation of pore-nuclear envelope interactions, RNA export, and less directly, protein import (Wente et al., 1992; Wimmer et al., 1992; Grandi et al., 1992; Schlenstedt et al., 1993; Wente and Blobel, 1994; Fabre et al., 1994).

Xenopus p97 contains at least one GLFG repeat and is rec-

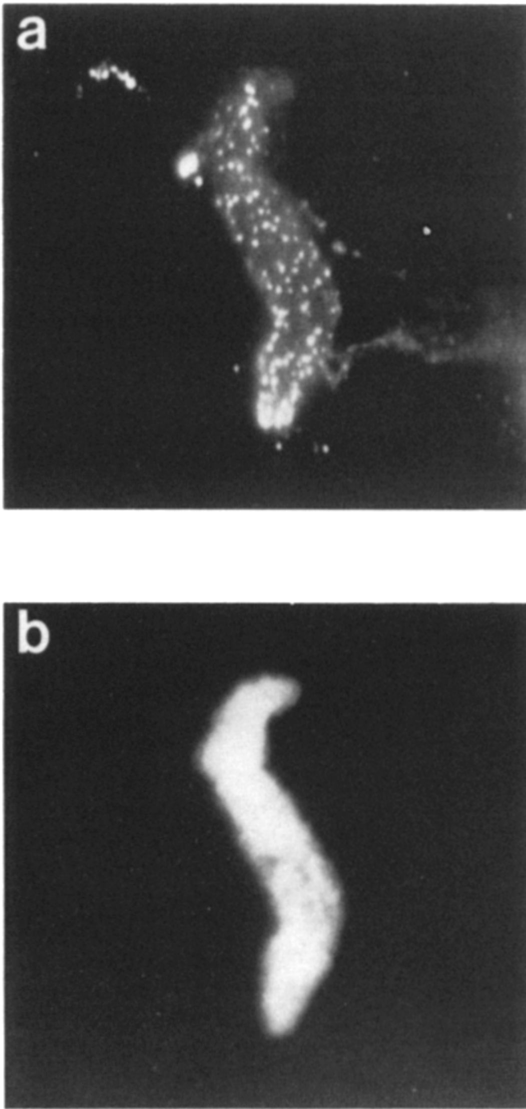

Figure 10. Pre-replication centers form on chromatin in p97-deficient cytosol. Demembranated sperm chromatin were added (1,000/μl final concentration) to D_C or D_{97} cytosol in the absence of membrane and incubated for 1 h. The sperm chromatin was then subjected to immunofluorescence using anti-RPA serum (1:100) and FITC-labeled secondary antibody. A representative sperm chromatin incubated in D_{97} cytosol is shown in *a*, with pre-replication centers identified by anti-RPA staining clearly visible throughout. The DNA of the same sperm chromatin stained with Hoechst 33258 is shown for comparison in *b*. Anti-RPA staining of chromatin incubated in D_C cytosol (not shown) was identical to that in *a*.

ognized by the anti-GLFG family monoclonal, mAb 192. Since a single copy of this motif is insufficient for recognition by mAb 192 (Wente et al., 1992), p97 is likely to contain numerous copies of GLFG. Consistent with membership in the GLFG family, peptide 1 of p97 shows a very high degree of conservation with NUP116, but little similarity to other members of the yeast family. Indeed, although NUP100, NUP116, and NUP145 all have related COOH termini and related GLFG domains, NUP116 has a unique region of 200 amino acids at the NH_2 terminus. It is a sequence within this region that so closely resembles peptide 1 of Xenopus p97. Peptide 3 is most similar to a region in the COOH-terminal domain of NUP116, but also resembles NUP100 and NUP145. Interestingly, this region of homology is found within an RNA binding domain present in NUPs 145, 116, and 100 (Fabre et al., 1994). We are currently investigating the possibility that p97 also binds RNA.

The sequence data suggest that Xenopus p97 is most similar to NUP116. When mAb 192, which recognizes the yeast GLFG nucleoporins is tested in Xenopus, it recognizes p97 but does not give strong indications of a multi-protein family. One additional protein doublet of greater than 200 kD is, however, recognized in Xenopus by mAb 192. Since this doublet is not recognized either by polyclonal anti-p97 serum or by the lectin WGA, the relatedness to p97 appears to be limited. Whether the doublet represents components of the nuclear pore and thus additional members of a vertebrate GLFG nucleoporin family remains to be determined. In any case, this doublet is not absent in p97-depleted nuclei and does not contribute to the defects seen. The presence of multiple GLFG proteins in yeast where Xenopus may have only one or a few may be explained by the observation that the yeast genome frequently encodes closely related and functionally redundant proteins. Consistent with at least partial functional redundancy between NUPs 145, 116, and 110, deletion of the RNA-binding domain from two of these three nucleoporins results in a yeast strain which is only slightly growth impaired, while deletion of all three is lethal (Fabre et al., 1994). As one of a smaller set of proteins in Xenopus, p97 alone might supply much or all of the combined function(s) of the yeast GLFG family.

In keeping with the possibility that p97 may play multiple roles, the localization of this protein within the nucleus appears to be both complex and dynamic. Immunofluorescence staining reveals a punctate nuclear rim, typical for proteins of the nuclear pore complex. In addition, a significant degree of intranuclear staining is consistently observed with polyclonal antibodies raised against either full length p97 or individual p97 peptides. This pattern is clearly distinguishable from that seen with antisera specific for either Xenopus p60 or p200 which are localized more exclusively to nuclear pores. There are several structures associated with nuclear pores that are known to extend some distance into the interior of the nucleus and these might contribute to a dual rim/intranuclear stain. The nuclear basket is formed of ~100 nm long fibers which project inward from the pore and are joined at their distal end by a ring 50 nm in diameter. The components of the basket remain largely uncharacterized although the 180-kD protein, NUP153, has been proposed to form part of the distal ring (Pante et al., 1994). Interestingly, NUP153 has been additionally localized to an adjacent structure, the intranuclear filaments, which extend from the basket for 600 nm, and possibly further, into the nuclear interior (Cordes et al., 1994). A third intranuclear pore-associated structure is the recently discovered nuclear lattice which links the distal rings of the nuclear pore baskets in a finely woven network (Goldberg and Allen, 1992). The nuclear lattice underlies and is distinct from the nuclear lamina, but its function and protein composition are completely unknown. The dual nature of the p97 localization may result from participation in structures such as these. Our antisera raised to p97 have not yet proven useful in immuno-

electron microscopy, so that a more specific intranuclear localization of p97 will require development of alternate antisera.

As the cell cycle proceeds, the pattern of p97 localization changes. During prophase, before the initiation of nuclear envelope breakdown, we observed a distinct intensification of the punctate nuclear rim fluorescence and a concomitant decrease in the internal nuclear fluorescence. This alteration in the p97 localization pattern during prophase coincides temporally with an observed posttranslational modification of the protein: at the onset of mitosis, p97 along with the nucleoporin p200 becomes hyperphosphorylated (Macaulay et al., 1995). It is known that during mitosis, the nucleus and the nuclear pores disassemble. Mitotic phosphorylation of specific critical pore constituents could constitute the signal for initiation of this process. Indeed, p97 is a substrate in vitro for the mitotic cdc2/cyclin kinase. One explanation of the p97 prophase staining pattern is that mitotic modification of p97 might first result in reorganization of the intranuclear structures of which it forms a part, possibly leading to closer association of p97 with the pore. As mitosis proceeds and the nuclear envelope disassembles, we have observed p97 to disperse throughout the cell, indicating that the next step is complete disassembly of p97-containing pores. In telophase, p97 immunofluorescence was again strongly evident in the reassembling nuclear envelope. Take together, these results indicate that both the nuclear pore and intranuclear structures into which p97 is incorporated are disassembled during the cell cycle. This disassembly is concomitant with p97 mitotic phosphorylation.

Since mutation of members of the yeast GLFG family, either alone or in combination, frequently impairs some aspect of nuclear transport, we asked whether reconstituted nuclei lacking p97 were similarly impaired. Interestingly, in the absence of p97, Xenopus nuclei continued to import a fluorescent nuclear-targeted protein. The internal fluorescence intensity of such nuclei, an indication of the concentration of the substrate within the nucleus, increased at a rate similar to control nuclei. The p97-depleted nuclei were considerably smaller than control nuclei and consequently the total amount of protein internalized was proportionately less, but the intensity was relatively unchanged from the control. This result is not completely without precedent in the yeast system, however, since a mutant strain lacking NUP116 continues to grow, albeit more slowly, and presumably must import nuclear proteins in order to grow. The deletion of NUP116 in combination with mutations in other nonessential pore genes results in a lethal phenotype. It is presumed that lethality results at least in part from an import or export defect. Yeast lacking NUP145 rapidly accumulate polyadenylated RNA in the nucleus, arguing for a possible involvement of NUP145 in RNA export. Only a delayed effect on protein import was observed in NUP145 mutant yeast (Fabre et al., 1994). Given the close relation between NUP116 and NUP145, and the finding that peptide 3 of Xenopus p97 shows homologies to both NUP116 and NUP-145, p97-depleted Xenopus nuclei might be expected to be impaired in RNA export rather than protein import. At present, we have no assay for RNA export in the Xenopus nuclear reconstitution system. A possible future approach to this problem would be the microinjection of anti-p97 antibodies and RNA-coated gold particles into Xenopus oocytes with subsequent assay for an inhibitory effect of the antibody on the export of the RNA-coated gold particles (Dworetzky and Feldherr, 1988).

Although nuclei lacking p97 remained competent for nuclear protein import, they showed two very distinct and reproducible phenotypes. First, p97-deficient nuclei have a clear defect in their chromosomal DNA replication. We initially found that chromosomal DNA replication does not occur in nuclei lacking all three of the WGA-binding proteins (Fig. 9). These results differ from those of Miller and Hanover (1994) who found WGA-depleted nuclei to be competent for replication. We can only conclude that our extracts are more fully depleted of required WGA-binding components. In previous work, we found that WGA-depleted nuclei neither import nuclear proteins nor increase significantly in size after their formation (Finlay and Forbes, 1990). The most likely basis for the lack of replication in nuclei depleted of all WGA binding proteins would be the lack of import of essential factors such as polymerases or lamins, due to the absence of the nucleoporin p60 (Finlay et al., 1990). Unexpectedly, we found that the presence of p97 is also required for chromosomal DNA replication in reconstituted nuclei. Since p97-deficient nuclei retain the ability to import nuclear proteins, it would appear that p97 makes a more direct contribution to replication. This is unlikely to be a role in the actual machinery of replication, since ss M13 DNA templates which do not incorporate into nuclei replicate equally well in control and p97-depleted extracts and, indeed, in WGA-depleted extracts. It was a possibility that replication is delayed in p97-depleted nuclei because a threshold amount of some factor must be accumulated. However, we have monitored replication in these nuclei for an extended period of time (3.5–4 h after nuclear formation) and see only minimal levels of DNA synthesis. We further showed that prereplication centers assemble normally on chromatin in the absence of p97, indicating that the replication defect is downstream of the very early preRC assembly step.

The second distinct phenotype of p97-deficient nuclei is that the nuclei do not significantly increase in size after the initial formation of a closed nuclear envelope. The nuclei remain only slightly larger than WGA-depleted nuclei and substantially smaller than mock-depleted controls. The mechanism of nuclear growth in reconstituted nuclei is not well characterized. Such growth may require: (*a*) the binding and fusion of additional nuclear vesicles to the newly formed envelope; (*b*) the import and incorporation of lamins and other nuclear scaffold proteins into essential structures; (*c*) the uptake of additional nuclear components; and possibly (*d*) chromatin decondensation. We would not expect membrane vesicle fusion to the outer nuclear membrane to be disrupted in p97-depleted nuclei, making the first possibility unlikely. Nuclei depleted of p97 also clearly remain capable of nuclear protein uptake, importing a synthetic transport substrate and proteins of the coiled body. It is possible that the import of an essential factor required for growth is specifically impaired, and strategies to test for this possibility can be devised. Lastly, we find that p97-depleted nuclei frequently exhibit chromosomal decondensation, thus it would appear that an inability to undergo decondensation is unlikely to be the source of the nuclear growth defect. This result also indicates that despite the absence of p97, the import of nuclear factors is sufficient to support chromosomal

rearrangement and decondensation. Interestingly, temperature sensitive mutations within members of the GLFG family in yeast (NUP116, NUP145) have resulted in aberrant nuclear envelope structure and nuclear pore distribution, suggesting that interaction with the nuclear envelope or with an underlying structure may be a conserved function of the GLFG proteins.

The phenotype of p97-depleted nuclei can be explained by several distinct types of models. In one, p97 could be a component of the nuclear pore and also of filaments extending inward from the pore, a localization similar to that seen for the pore protein NUP153. In this model, the filaments might represent a scaffold upon which DNA replication (and possibly the preliminary stages of RNA export) would take place. The absence of nuclear growth might, in this model, be tied to an inability to form such a scaffold or to enlarge it. In a second model, p97 might be a nuclear pore/intranuclear protein that acts as a foundation upon which further nuclear structure is built or as a link between the nuclear pores and such structures. The absence of p97 could remove that foundation and prevent assembly of structures required for growth and chromosomal replication. Lastly, it is possible that p97-depleted nuclei, while competent for import of most proteins, are specifically defective in the import of one or more proteins required for nuclear growth or chromosomal DNA replication. These two complex processes might well be interrelated, such that a defect in one would impact on the other and result in the combined phenotype. In all cases it is possible that one or more additional unidentified proteins is complexed with p97 and is depleted from Xenopus egg cytosol by p97 antibodies. Individual members of such a p97 complex could directly mediate different aspects of the p97 depletion phenotype.

The analysis of nuclear ultrastructure is still in its infancy. The structures present within the nucleus remain, in large part, unknown and the protein composition of potential structures, undiscovered. The studies presented here indicate that p97 will be an interesting and essential protein. Further analysis of mutant forms of p97 and a definition of the proteins with which p97 interacts will be important for future progress in this complex problem.

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