

GTP Hydrolysis Is Required for Vesicle Fusion during Nuclear Envelope Assembly In Vitro

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Abstract. Nuclear envelope assembly was studied in vitro using extracts from *Xenopus* eggs. Nuclear-specific vesicles bound to demembrated sperm chromatin but did not fuse in the absence of cytosol. Addition of cytosol stimulated vesicle fusion, pore complex assembly, and eventual nuclear envelope growth. Vesicle binding and fusion were assayed by light and electron microscopy. Addition of ATP and GTP to bound vesicles caused limited vesicle fusion, but enclosure of the chromatin was not observed. This result suggested that nondialyzable soluble components were required for nuclear vesicle fusion. GTP γ S and guanylyl imidodiphosphate significantly inhibited vesicle fusion but had no effect on vesicle binding to chromatin. Preincubation of membranes with 1 mM

GTP γ S or GTP did not impair vesicle binding or fusion when assayed with fresh cytosol. However, preincubation of membranes with GTP γ S plus cytosol caused irreversible inhibition of fusion. The soluble factor mediating the inhibition by GTP γ S, which we named GTP-dependent soluble factor (GSF), was titratable and was depleted from cytosol by incubation with excess membranes plus GTP γ S, suggesting a stoichiometric interaction between GSF and a membrane component in the presence of GTP γ S. In preliminary experiments, cytosol depleted of GSF remained active for fusion of chromatin-bound vesicles, suggesting that GSF may not be required for the fusion reaction itself. We propose that GTP hydrolysis is required at a step before the fusion of nuclear vesicles.

THE nucleus, ER, and Golgi complex disassemble during mitosis in most higher eukaryotes (Fry, 1976; Zeligs and Wollman, 1979; Lucocq et al., 1987). Membranes derived from each organelle are dispersed throughout the cell until the end of mitosis, when presumably specific sets of membranes and vesicles begin fusing to reassemble the organelles. The signals that control vesicle targeting and fusion during organelle assembly are unknown, but may be related to those that regulate vesicle activity during interphase (Warren, 1985). Rothman and colleagues have described ordered pathways for the formation of fusion-competent ER and Golgi vesicles based upon the in vitro inhibitory effects of ATP depletion and compounds such as GTP γ S and *N*-ethyl-maleimide (Beckers et al., 1989; Orci et al., 1989; Rothman and Orci, 1990). GTP γ S, in particular, was shown to inhibit uncoating of ER- and Golgi-derived transport vesicles (Beckers and Balch, 1989; Melançon et al., 1987). A family of small *ras*-like GTP-binding proteins that includes yeast Sec4 and Ypt1 and mammalian rab proteins is proposed to regulate the specificity of vesicle fusion during transport through the secretory and endocytic pathways (Bourne, 1988; Goud et al., 1988; Bacon et al., 1989; Baker et al., 1990; Mayorga et al., 1989; Plutner et al., 1990; for reviews see Balch, 1989; Hall, 1990). This proposed role is supported by evidence that individual members of the family are localized to specific membrane compartments within the cell

(Chavrier et al., 1990; Gorvel et al., 1991; Goud et al., 1990; Fischer v. Mollard et al., 1990). No rab proteins have yet been localized to the nuclear envelope or implicated in postmitotic reassembly of organelles. However, rat liver nuclei appear to contain GTP-binding proteins as determined by ³⁵S-GTP γ S blots (Rubins et al., 1990), and a 28-kD GTP-binding protein that is specific to the nuclear envelope has been identified by cross-linking studies (Seydel and Gerace, 1991).

To study vesicle targeting and fusion during nuclear envelope assembly we have used extracts from the eggs of the frog, *Xenopus laevis* (for review see Newport and Forbes, 1987). These extracts were originally described by Lohka and Masui (1983). Each *Xenopus* egg contains components sufficient to assemble over 4,000 nuclei, including histone and nonhistone chromosomal proteins, lamins, pore complex components, and nuclear membrane vesicles. The crude cytoplasm from lysed eggs can be fractionated to separate the membranes and cytosol; each fraction is stable to freezing (Wilson and Newport, 1988). Extracts active for nuclear envelope assembly are reconstituted by mixing membranes and cytosol with demembrated sperm chromatin (Lohka and Masui, 1983), naked DNA (Newport et al., 1985; Newport, 1987), or chromosomes (shown using CHO cell lysates; Burke and Gerace, 1986).

Nuclei assembled in vitro using the *Xenopus* extracts are

structurally normal; two membrane bilayers enclose the decondensed chromatin, a nuclear lamina (containing lamin L_{III}) is organized under the inner membrane, and pore complexes span both membranes (Newport et al., 1990; for reviews see Newport and Forbes, 1987; Lohka, 1988). Furthermore, numerous studies have demonstrated that the in vitro-assembled nuclei are functional for (a) chromatin assembly (Laskey et al., 1977; Newport, 1987), (b) ATP-dependent import of nuclear proteins through pore complexes (Newmeyer et al., 1986; Newmeyer and Forbes, 1988), (c) DNA replication, which requires an intact nuclear envelope (Blow and Laskey, 1986; Blow and Watson, 1987), and (d) mitotic disassembly in extracts that contain active cyclin/p34^{cdc2} kinase (Miake-Lye and Kirschner, 1985; Lohka and Maller, 1985; Newport and Spann, 1987; for reviews see Nurse, 1990; Pines and Hunter, 1990).

Vesicles that assemble the nuclear envelope in vitro are a functionally distinct subset of the membrane fraction. Previous work showed that only 20% of the vesicles carrying ER markers (BiP and glucosidase II) are incorporated into growing nuclear envelopes; the remaining vesicles are intact but unable to support nuclear envelope growth (Wilson and Newport, 1988). These results suggest that vesicles derived by mitotic disassembly of the nuclear envelope carry targeting information that is nuclear specific. Vigers and Lohka (1991) have found that nuclear-specific vesicles can be subdivided into two classes that differ in sensitivity to *N*-ethylmaleimide and salt extraction; only the vesicles sensitive to high salt, termed NEP-B, exhibit chromatin-binding activity.

In the present study, we have used a rapid light microscopic assay to investigate the role of cytosolic factors required for (a) the binding of nuclear-specific vesicles to chromatin and (b) nuclear vesicle fusion. Our findings with respect to vesicle binding confirm the results of Pfaller et al. (1991) who demonstrated that soluble components are not required for binding in defined buffers that contain ATP. We demonstrate that nuclear vesicle fusion, on the other hand, is mediated by cytosolic components and requires GTP hydrolysis.

Materials and Methods

Buffers and Reagents

Buffer X: 200 mM sucrose, 7 mM $MgCl_2$, 80 mM KCl, 15 mM NaCl, 5 mM EDTA, 15 mM Pipes, pH 7.4. Egg lysis buffer for nuclear assembly extracts: 250 mM sucrose, 2.5 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 50 μ g/ml cycloheximide (Calbiochem-Behring Corp., San Diego, CA), 5 μ g/ml cytochalasin B (prevents actin gelation; Sigma Chemical Co., St. Louis, MO), 10 μ g/ml each aprotinin and leupeptin (Sigma Chemical Co.). Membrane wash buffer (MWB): 250 mM sucrose, 50 mM KCl, 2.5 mM $MgCl_2$, 50 mM Hepes, pH 8.0, 1 mM DTT, 0.5 mM ATP, 1 μ g/ml each aprotinin and leupeptin. Hoechst dihexyloxycarbocyanine (DHCC) buffer: buffer X with 20 μ g/ml bisbenzimidazole DNA dye (Hoechst 33258; Calbiochem-Behring Corp.) and 3.7% formaldehyde. 20 μ g/ml lipid dye 3,3'-dihexyloxycarbocyanine iodide (DHCC, Calbiochem-Behring Corp.) was added to the buffer just before use. Dialysis buffer: 100 mM sucrose, 50 mM KCl, 2.5 mM $MgCl_2$, 50 mM Hepes, pH 7.5, 1 mM DTT, 1 μ g/ml each aprotinin and leupeptin. Nucleotides and analogs: lithium salts of GTP, GTP γ S guanylyl imidodiphosphate (GMP-PNP), and ATP γ S (Boehringer Mannheim Corp., Indianapolis, IN) 10-mM stock solutions were stored at -80°C . $AlCl_3$ and KF were obtained from Sigma Chemical Co.

1. *Abbreviations used in this paper:* ARF, ADP-ribosylation factor; DHCC, dihexyloxycarbocyanine; GMP-PNP, guanylyl imidodiphosphate; GSF, GTP-dependent soluble factor; MWB, membrane wash buffer.

Preparation of Sperm Chromatin

Demembrated sperm chromatin consists of *Xenopus* sperm treated with lyssolecithin to remove the plasma membrane and the nuclear membrane without affecting the highly condensed chromatin. Chromatin was prepared as described, using buffer X (Lohka and Masui, 1983). Chromatin was stored at -80°C at a concentration of 40,000/ μ l. For detailed protocol, see Newmeyer and Wilson (1991).

Preparation of Nuclear Assembly Extracts

Extracts were prepared as described (Wilson and Newport, 1988; Newmeyer and Wilson, 1991) from unactivated *Xenopus* eggs. In brief, dejellied and packed eggs were lysed by centrifugation at 10,000 g for 12 min. The crude cytoplasm was fractionated by ultracentrifugation (model TL100, Beckman Instruments, Carlsbad, CA) at 200,000 g (TLS-55 rotor, Beckman Instruments; 1 h, 4°C). The soluble fraction, or cytosol, was recentrifuged at 200,000 g for 25 min to remove residual membranes, and supplemented with an ATP regenerating system (1 mM ATP, 50 μ g/ml creatine phosphokinase, 10 mM creatine phosphate). Aliquots were frozen in liquid nitrogen and stored at -80°C . The membrane fraction was washed by dilution with 10 vol of MWB, pelleted onto a 1.3-M sucrose cushion at 26,600 g (TLS-55 rotor, 15 min, 4°C), resuspended in MWB containing 0.5 M sucrose, frozen in liquid nitrogen, and stored at -80°C . The resuspended membrane volume was $\sim 10\%$ of the corresponding cytosol volume, and the final protein concentration of both the cytosolic and membrane fractions ranged from 25 to 35 mg/ml. Frozen-thawed extract components were used in all experiments.

Preparation of Mitotic Cytosol

Mitotic cytosol was prepared as described by Newport and Spann (1987) with the addition of 30 μ g/ml each aprotinin and leupeptin in the final lysis buffer rinse, followed by ultracentrifugation at 200,000 g (as above) to remove mitotic membranes. Aliquots were frozen in liquid nitrogen and stored at -80°C .

Purification of Nucleoplasmin

Nucleoplasmin was purified from heated cytosol as described (Newmeyer and Wilson, 1991) using a phenyl-Sepharose column. The pooled fractions containing nucleoplasmin were dialyzed, concentrated to 0.13 mg protein per ml, aliquoted, and stored frozen at -80°C . The swelling effect of nucleoplasmin on chromatin (Lohka and Masui, 1984; Philpott et al., 1991; W. Dunphy, personal communication) can be duplicated using polyglutamic acid (Pfaller et al., 1991), indicating that the size increase is due to an ionic effect of the negatively charged regions of nucleoplasmin.

Nuclear Assembly Assays

Assays were performed as described by Wilson and Newport (1988). Frozen cytosol and membrane aliquots were rapidly thawed by hand and mixed with demembrated sperm chromatin. A typical reaction consisted of 20 μ l cytosol, 2 μ l membranes, and 1 μ l chromatin (@ 40,000/ μ l). Reactions were incubated at room temperature (23°C) for up to 4 h. Aliquots were removed at different times, diluted 1:1 with Hoechst/DHCC buffer, and examined by light microscopy. The time of initial nuclear envelope fusion as scored by light microscopy, 20–40 min, is consistent for a given extract but varies between preparations.

Assays for Vesicle Binding to Chromatin and Vesicle Fusion

Sperm chromatin (1 μ l) was swollen using 3 μ l MWB and 2 μ l nucleoplasmin for 10 min at 23°C . To assay binding, 2 μ l of membranes was added and allowed to bind for 30 min with or without added GTP γ S or GTP (see Results). Binding was observed by light microscopy of aliquots diluted 1:5 in Hoechst/DHCC buffer. To assay vesicle fusion (or mitotic release), 20 μ l of cytosol or MWB (or mitotic cytosol) was added to the binding reaction and incubated at 23°C for up to 4 h. To separate chromatin with bound vesicles from the reaction, samples (up to 40 μ l) were layered onto 100 μ l of 1.3 M sucrose and centrifuged 3 min in a horizontal centrifuge (Eppendorf Inc., Fremont, CA) at 13,000 g. The pelleted chromatin was resuspended into either cytosol or MWB.

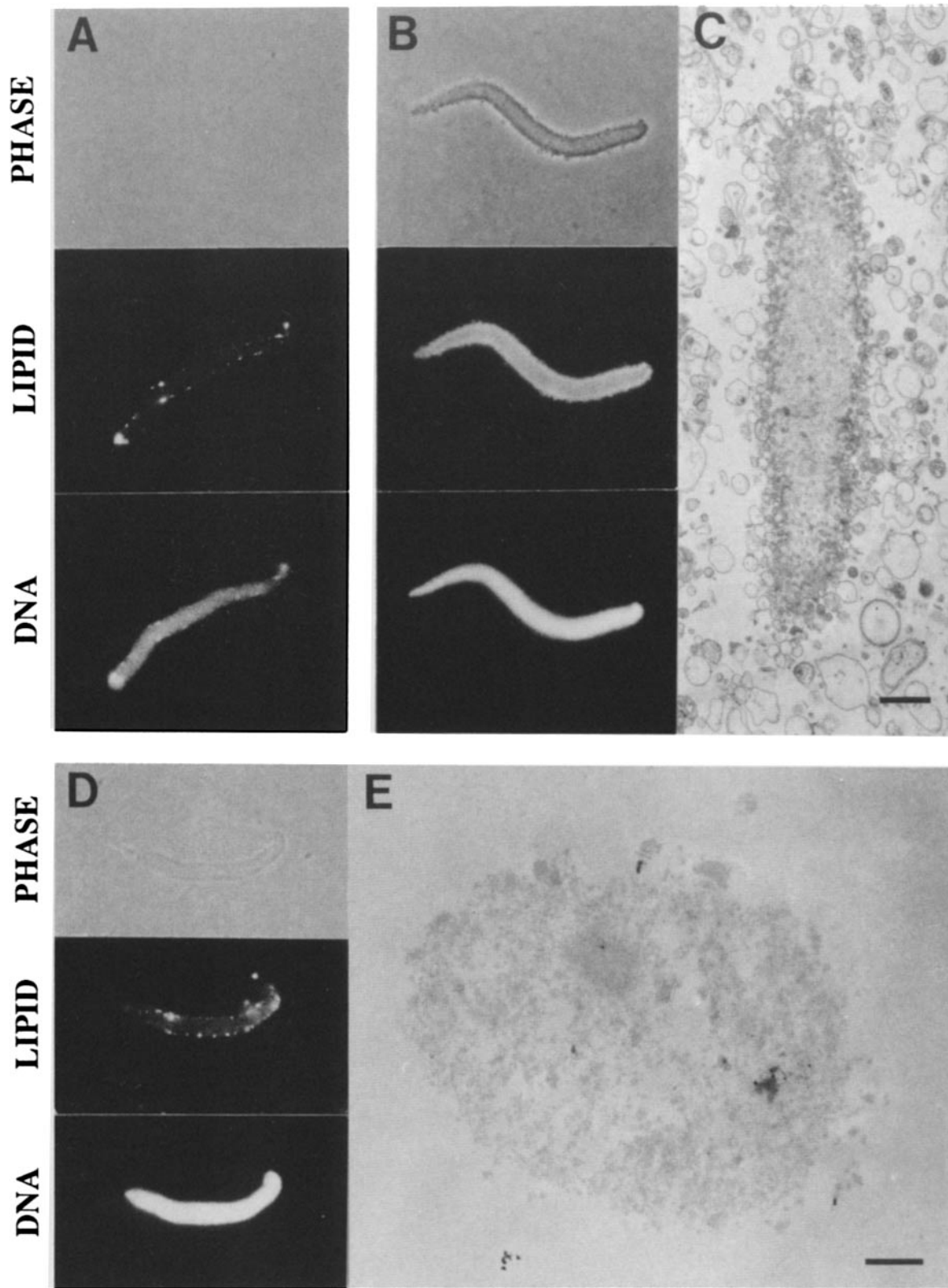


Figure 1. Membrane binding to chromatin. (A) Demembrated sperm chromatin was incubated with purified nucleoplasmin for 10 min at 23°C to swell the chromatin. Each swelling reaction contained 3 μ l MWB and 0.26 μ g nucleoplasmin (2 μ l) per 40,000 chromatin (1 μ l). (B and C) Vesicle binding to chromatin is shown by light and electron microscopy 30 min after addition of membranes (2 μ l) to swelled chromatin. (D and E) No vesicle binding was detected by light or electron microscopy after incubation of swelled chromatin with cytosol for 30 min at 23°C, indicating that our cytosol is depleted of membranes. PHASE, phase contrast. LIPID, stained with DHCC. DNA, fluorescence of DNA stain Hoechst 33258. Note the presence of contaminating DHCC stain on the chromatin in the absence of membranes or cytosol (A). This may be due to incomplete removal of membranes during chromatin preparation or to nonspecific sticking of DHCC to the chromatin. Bars, 1 μ m.

A **LIPID** **PHASE**

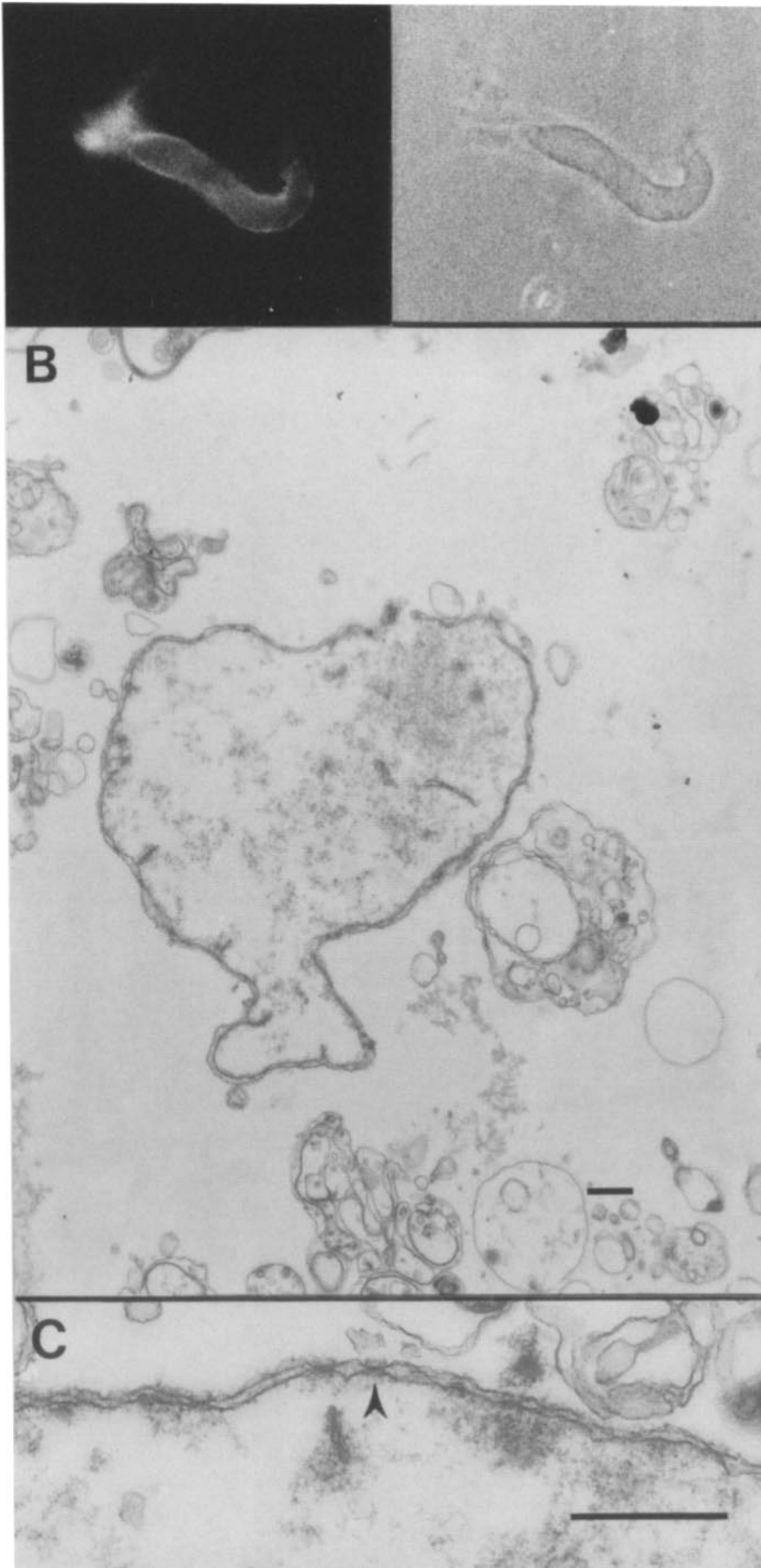


Figure 2. Early vesicle fusion. Membranes were incubated with chromatin to allow binding as in Fig. 1. The sample was then supplemented with cytosol, observed by light microscopy until fusion was scored positive (40 min), and immediately photographed (A) and processed for EM (B and C). Arrowhead in C indicates nuclear pore complex. Bars, 500 nm.

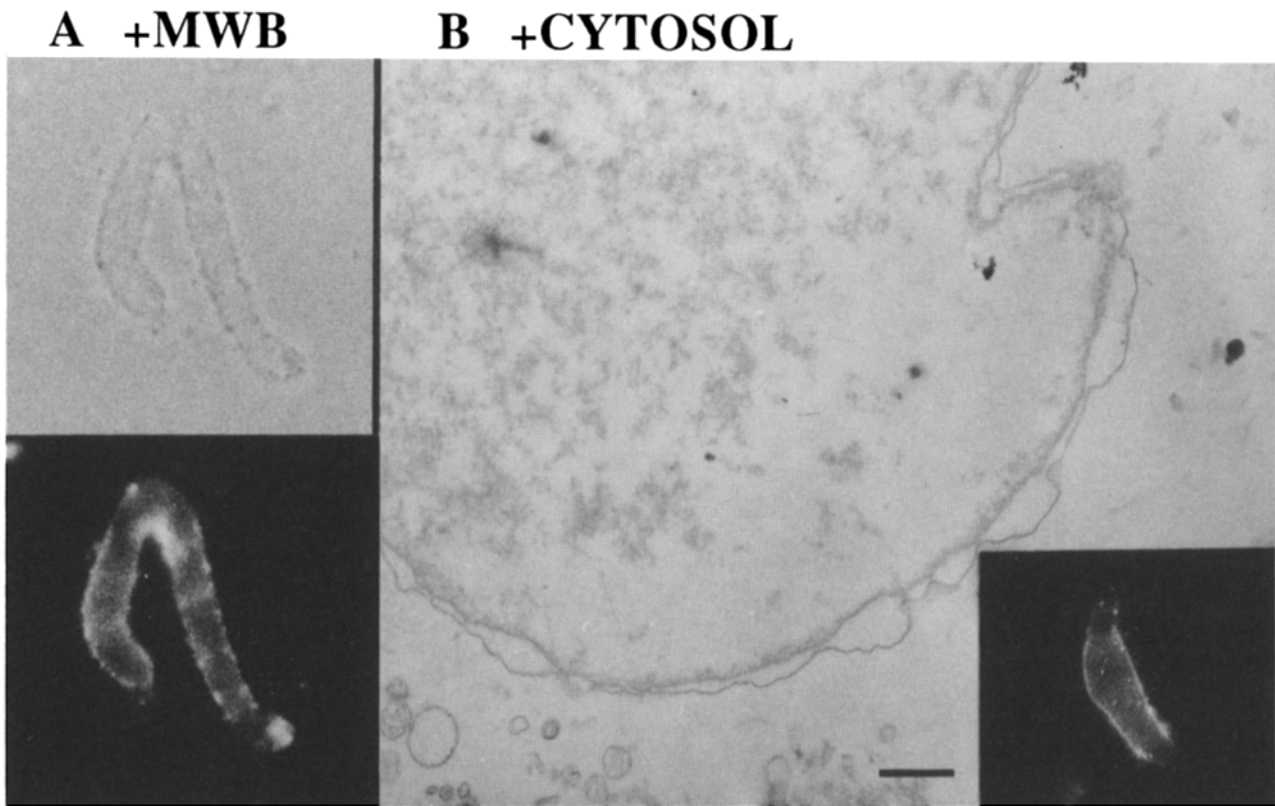


Figure 3. Bound vesicles are the precursors of the nuclear envelope. Membranes and swollen chromatin were incubated for 30 min; the chromatin was then centrifuged through 1.3 M sucrose to remove unbound membranes. The chromatin pellet was resuspended in either (A) MWB or (B) cytosol. In MWB the vesicles remained bound but did not fuse for at least 3 h (A; photographs taken 1 h after MWB addition). In the presence of cytosol, however, the vesicles fused to enclose the chromatin within 1 h, at which time the sample was fixed for EM (B). (B, inset) Photograph of fused sample 3 h after addition of cytosol. Bar, 500 nm.

Pretreatment of Membranes with GTP γ S

Thawed membranes were mixed with a 2–10-fold excess of cytosol (see titration results) or MWB and 1 mM GTP γ S or GTP. Samples were preincubated at 23°C for 30 min, diluted with at least an equal volume MWB containing GTP γ S or GTP, and centrifuged at 20,000 *g* (TLS-55 rotor, 15 min, 4°C) to pellet the membranes. Note that these conditions will pellet membranes if the total volume is small (<150 μ l) and the solution is dilute. The membranes were resuspended to their original volume in MWB. 2 μ l of treated membranes was added to 1 μ l of swollen chromatin and allowed to bind for 30 min; then 20 μ l fresh cytosol was added to assay for fusion.

In the experiments shown in Fig. 11, cytosol was preincubated with or without an equal volume of membranes and 1 mM GTP γ S, centrifuged at 26,600 *g* to remove membranes, microdialyzed to remove excess GTP γ S, and then assayed for both GTP-dependent soluble factor (GSF) activity and fusion activity. In these cases the cytosol was not diluted before removing membranes by centrifugation.

Thin Layer Chromatography

9- μ l samples of an assembly reaction containing 2 μ l chromatin, 4 μ l membranes, 30 μ l cytosol, and 2 μ Ci [γ -³²P]ATP were diluted 10-fold and centrifuged to remove membranes (as above). 1 μ l of each sample was spotted onto 1 cm \times 10 cm strips of a 20-cm polyethyleneimine-cellulose plate. The plate was developed for 12 min in 1 M sodium formate, 0.5 M LiCl, pH 3.4 (Kornberg et al., 1978). The plate was dried and autoradiographed to determine the locations and relative intensities of ATP and P_i.

Dialysis of Cytosol

Dialysis was performed using Spectra/por dialysis tubing (12–14-kD cutoff; Spectrum Medical Industries, Inc., Los Angeles, CA) at 4°C. Small volumes (50–100 μ l) of thawed cytosol were dialyzed into 25 ml dialysis buffer for 2 h using a microdialyzer (Pierce Chem. Co., Rockford, IL).

Larger volumes of fresh cytosol (2 ml) were dialyzed into 2 liters dialysis buffer (three changes of 700 ml each) for 7 h; aliquots were frozen in liquid nitrogen and stored at –80°C. Dialyzed cytosol was replenished with an ATP regenerating system before use. Cytosol volume did not change during dialysis.

Calculation of Nuclear Envelope Surface Area

Measurements of the diameter (or length and width) of at least 10 nuclei per sample were made using the ocular micrometer; surface areas were calculated assuming shapes of cylinders, ellipsoids, or spheres for the nuclei (obvious by observation). The mean surface area was plotted at each time point, and the curves were fit using linear or polynomial regression. For each graph, surface areas at the zero time point coincided. All points within a graph are from the same experiment except in Fig. 10.

Microscopy

For light microscopy, we used a Nikon Microphot (Nikon Inc., Melville, NY) equipped with epifluorescence optics suitable for DHCC (fluorescein channel) and Hoechst (UV). Photographs were taken with an FS-35WA camera (Nikon Inc.) using either a Fluor 40 \times objective (Nikon Inc., Figs. 1, A and D, and 3 A) or a PlanApo 60 \times objective (Nikon Inc., all others). Electron micrographs of thin sections (90 nm) were made on a TEM10 (Carl Zeiss, Inc., Thornwood, NY) at 60 kV as described by Wilson and Newport (1988) except that the agarose was omitted.

Results

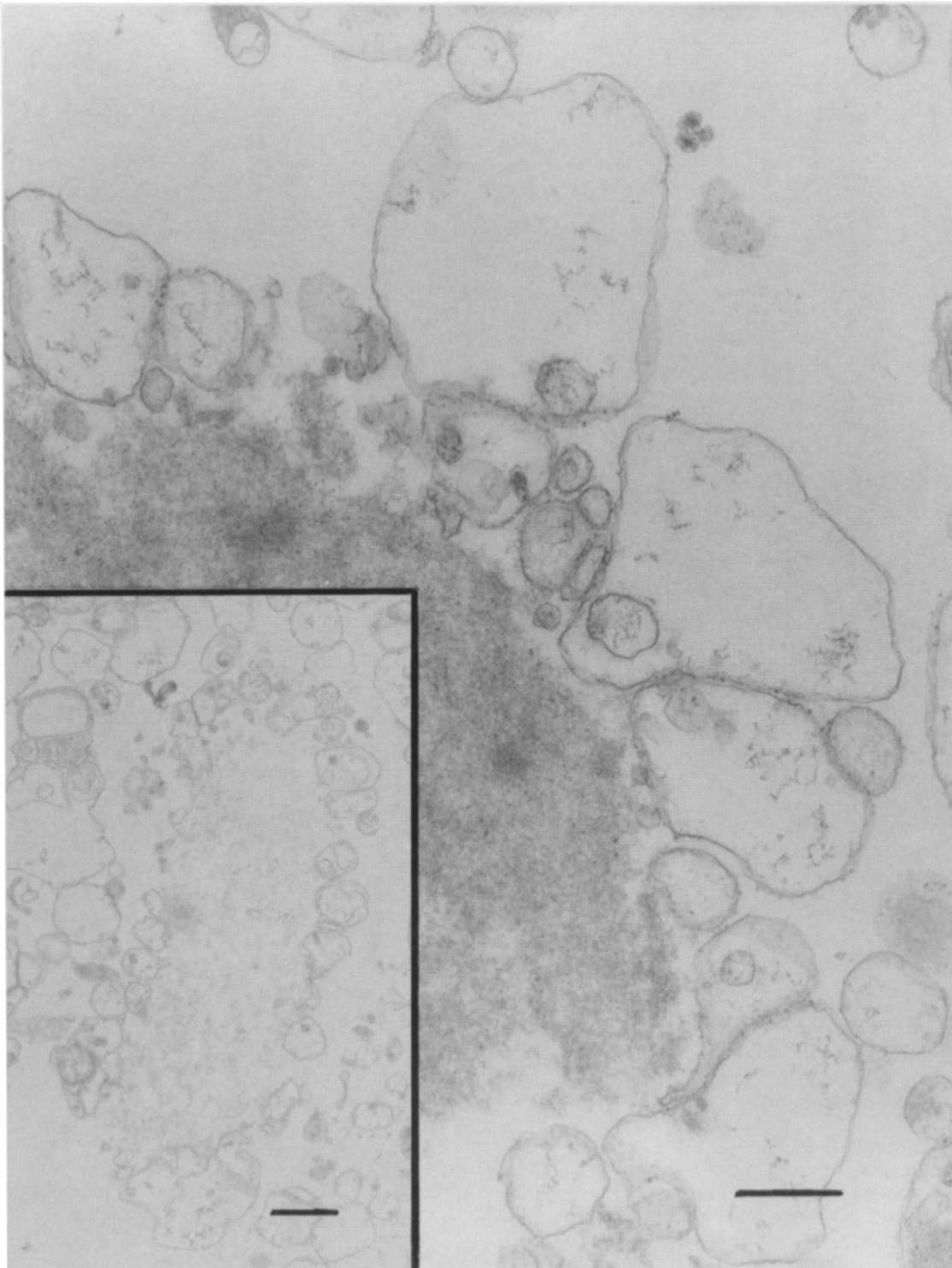
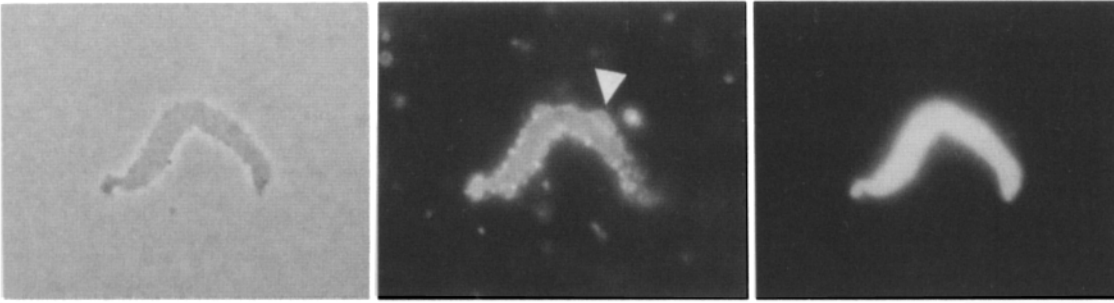
Binding of Nuclear Vesicles to Chromatin

To identify and study the components that mediate nuclear

PHASE

LIPID

DNA



envelope assembly, we needed assays for two distinct vesicle activities: vesicle binding to chromatin, and vesicle-vesicle fusion. To assay for vesicle binding to chromatin, demembrated sperm chromatin (Lohka and Masui, 1983) was first swollen for 10 min (Fig. 1 *A*; see Materials and Methods). The increased size of the chromatin permitted vesicle binding to be assayed easily by light microscopy (Fig. 1 *B*). The binding of membranes to chromatin was detected as an irregular thick layer by phase contrast and by using a fluorescent lipid dye, DHCC. By EM (Fig. 1 *C*) the membranes that bound to chromatin consisted mostly of vesicles 130–200 nm in diameter that were filled with electron-dense material. Electron-dense vesicles were observed previously (Lohka and Masui, 1984) and may correspond to the NEP-B vesicles described by Vigers and Lohka (1991; see Discussion). A small proportion of the bound vesicles were larger and unfilled, and could be NEP-A vesicles (Vigers and Lohka, 1991). No membrane binding was detected by light microscopy (Fig. 1 *D*) or by EM (Fig. 1 *E*) when cytosol was incubated with chromatin, confirming that our cytosol was depleted of membranes during fractionation.

Bound vesicles were released from chromatin within 60 min after addition of extracts containing active cyclin/p34^{cdc2} kinase (see Fig. 5 *C*). Vesicle binding was thus mitotically reversible, as one would predict for nuclear-specific vesicles.

Vesicles Fuse upon Addition of Cytosol

Fusion of nuclear vesicles was stimulated by the addition of cytosol. 40 min after adding cytosol to bound vesicles, the phase (and lipid dye) boundary changed from a thick, rough pattern to a thin, smooth line (Fig. 2 *A*). As confirmed by EM, this change was due to the fusion of vesicles on the surface of the chromatin to form a double bilayer envelope that contained nuclear pore complexes (Fig. 2, *B* and *C*; *arrowhead* indicates a pore complex). Concomitant with the fusion, the nuclei changed shape (more ellipsoidal, less snake-like), had begun to grow (seen as slight chromatin decondensation in the electron micrograph), and were competent for the import of rhodamine-labeled nuclear proteins (data not shown). Nuclear envelope growth and chromatin decondensation continued for several hours after addition of cytosol (see Figs. 5 and 8). In controls incubated with MWB rather than cytosol, no vesicle fusion was observed for over 3 h (data not shown). These results confirmed that cytosolic factors were required for vesicle fusion (see Lohka and Masui, 1984) and showed that we could reliably assay both vesicle binding and vesicle fusion (chromatin enclosure) by light microscopy.

The Bound Vesicles Are the Precursors of the Nuclear Envelope

As shown in Fig. 3 *A*, chromatin that was pelleted through 1.3 M sucrose retained its bound vesicles. These bound vesi-

cles fused after addition of cytosol. Fusion was detected within 1 h, at which time aliquots were removed and fixed for EM (Fig. 3 *B*); the remaining fused samples were photographed after 3 h (Fig. 3 *B*, *inset*). Nuclear envelope growth was not detected even after 3 h, as expected, since centrifugation through sucrose removed unbound membranes from the reaction. Thus vesicles that bound to chromatin were also competent to fuse. We inferred that our membrane fraction contains vesicles with both chromatin-binding and fusion-stimulating activities (see Discussion).

Cytosolic Proteins Are Required for Nuclear Vesicle Fusion and Chromatin Enclosure

Previous studies showed that nuclear envelope assembly *in vitro* requires ATP (Newmeyer et al., 1986; Burke and Gerace, 1986; Vigers and Lohka, 1991). To determine whether the cytosol contributed factors other than nucleotides that were required for fusion, we added ATP and GTP (1 mM each) to chromatin-bound vesicles. After a 160-min incubation with ATP and GTP, we observed thick vesicle binding and some blebbing of the membranes (Fig. 4, *arrowhead* shows a bleb). We interpreted the blebs as evidence that a limited amount of fusion was stimulated by adding ATP and GTP in the absence of cytosol (compare sizes of vesicles in Fig. 4 with those in Fig. 1 *C*). However, this limited fusion was not sufficient to enclose the chromatin within a membrane, since the samples did not change during prolonged incubations (up to 3 h). We concluded that cytosolic proteins are required for the extensive vesicle fusion that is necessary for nuclear envelope formation.

GTP γ S Inhibits Nuclear Vesicle Fusion

Addition of 1 mM GTP γ S, a hydrolysis-resistant GTP analogue, inhibited the nuclear assembly reaction at an early stage (not shown), suggesting that GTP hydrolysis was required for nuclear envelope assembly. Control reactions supplemented with 1 mM GTP assembled normal nuclei (Fig. 5 *E*). Lower concentrations of GTP γ S did not fully inhibit assembly.

To determine which stage of assembly was affected, GTP γ S was included in the assays for vesicle binding to chromatin and vesicle fusion. We observed robust binding of nuclear vesicles to chromatin in the presence of 1 mM GTP γ S (Fig. 5 *A*) or 1 mM GTP (Fig. 5 *B*). Vesicle binding in the presence of GTP γ S was completely reversible upon addition of mitotic cytosol (Fig. 5 *C*). We concluded that GTP γ S did not inhibit specific vesicle binding to chromatin. This is consistent with Pfaller et al. (1991) who include GTP γ S in their binding reactions.

In contrast, vesicles bound in the presence or absence of GTP γ S were unable to fuse upon addition of cytosol containing 1 mM GTP γ S (Fig. 5 *D*), indicating that vesicle fusion was sensitive to GTP γ S. GTP (1 mM) had no effect on fusion (Fig. 5 *E*). Inhibition of vesicle fusion by GTP γ S was

Figure 4. Cytosolic factors in addition to ATP and GTP are required for vesicle fusion. Vesicles were incubated with swelled chromatin for 30 min at 23°C, and then supplemented with 1 mM (each) ATP and GTP. (*Top*) After 3 h, no membrane enclosure of the chromatin was detected by light microscopy. However, small membranous blebs (*arrowhead*) were observed at all time points, indicating that limited fusion occurred. Samples were photographed 160 min after nucleotide addition. (*Bottom*) Identical samples were fixed for EM 40 min after addition of ATP and GTP. The panel shows large bound vesicles (blebs) but no enclosure of the chromatin. Bar, 500 nm. (*Inset*) A different view also shows large bound vesicles; note the scarcity of the small dense vesicles observed in Fig. 1 *C*. Bar, 1 μ m.

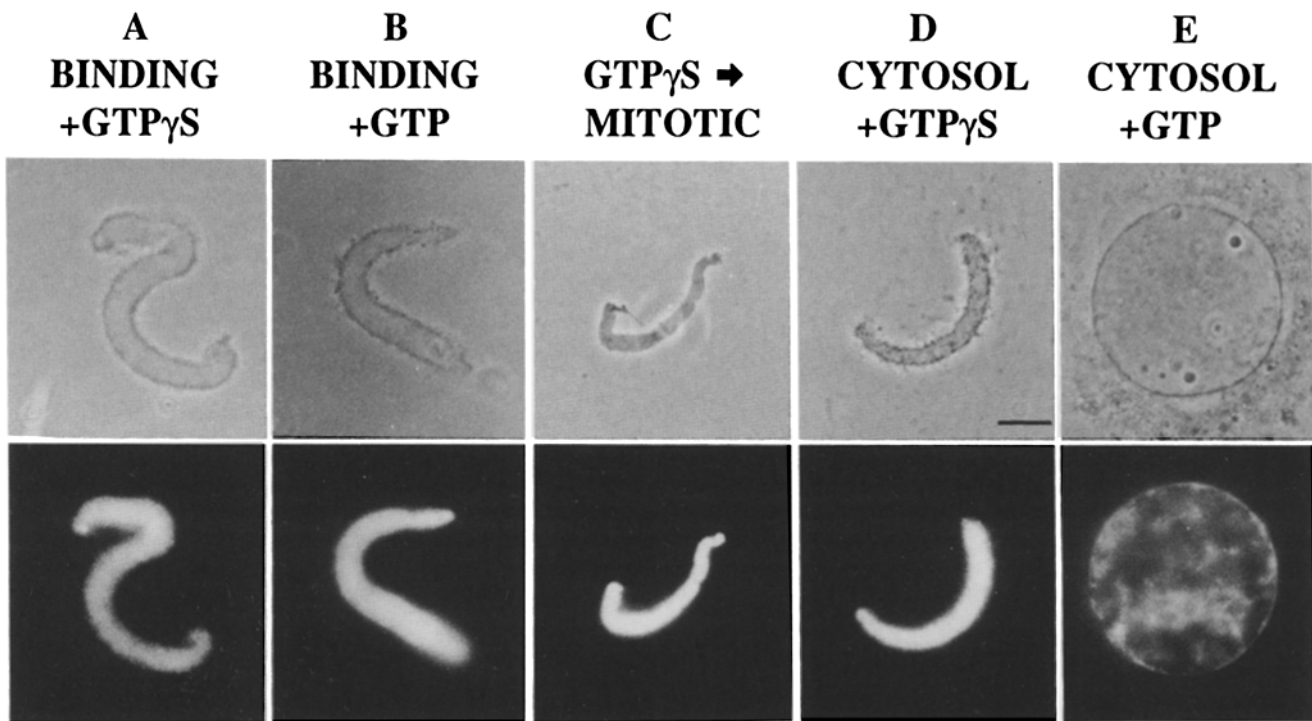


Figure 5. Vesicle fusion is inhibited by $GTP\gamma S$. Vesicle binding to chromatin was assayed in the presence of (A) 1 mM $GTP\gamma S$ or (B) 1 mM GTP. Neither nucleotide interfered with binding. The reactions were photographed 30 min after addition of membranes to chromatin. (C) Vesicle binding to chromatin in the presence of $GTP\gamma S$ was mitotically reversible, as shown by vesicle release within 1 h after the addition of 20 μl mitotic cytosol. (D) Cytosol containing 1 mM $GTP\gamma S$ was added to a vesicle binding reaction (containing $GTP\gamma S$). Vesicle binding remained constant in the presence of cytosol and $GTP\gamma S$, but no fusion was observed for at least 3 h. (E) Addition of GTP (1 mM) and cytosol did not inhibit fusion; normal growth and DNA decondensation were observed. Photographs in D and E were taken 135 min after the addition of cytosol. Bar, 10 μm .

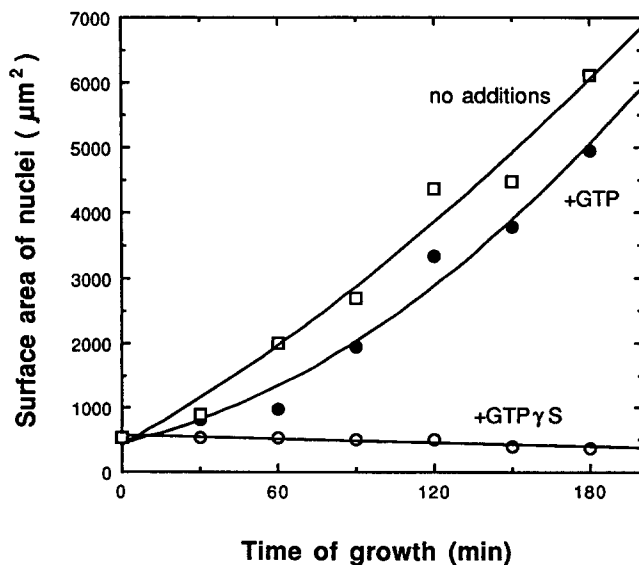


Figure 6. $GTP\gamma S$ inhibits membrane fusion. Inhibition of fusion by $GTP\gamma S$ was quantitated assuming that the rate of fusion is directly proportional to nuclear envelope growth. Aliquots of each nuclear assembly reaction (1 μl chromatin, 2 μl membranes, and 20 μl cytosol) were examined at 30 min intervals. The mean surface area of at least 10 nuclei per sample is shown. (\circ) 1 mM $GTP\gamma S$ included in assembly reaction; (\bullet) 1 mM GTP included in assembly reaction; (\square) no additions to assembly reaction.

quantitated by assuming that the rate and extent of nuclear envelope growth are directly proportional to the rate of fusion in the presence of excess membranes (Wilson and Newport, 1988). Nuclear envelope (or chromatin) surface areas were measured during assembly reactions containing 1 mM GTP or $GTP\gamma S$. Fig. 6 shows that fusion was completely inhibited by $GTP\gamma S$ for at least 3 h.

Inhibition of Fusion Is Guanine Nucleotide Specific

To determine whether $GTP\gamma S$ inhibited fusion directly, we tested a second GTP analogue. GMP-PNP is nonhydrolyzable and cannot be used as a substrate for protein thiophosphorylation. Fig. 7 shows that GMP-PNP inhibited vesicle fusion, although 3 mM GMP-PNP was required for maximum inhibition. This is consistent with previous reports that 80–100 times more GMP-PNP than $GTP\gamma S$ is required to inhibit intra-Golgi transport and ER-Golgi transport (Melançon et al., 1987; Beckers and Balch, 1989).

ATP levels in the extracts were stable for at least 3 h in 1 mM $GTP\gamma S$ or 1 mM GMP-PNP as determined by thin layer chromatography (data not shown). We therefore concluded that the inhibition of fusion by $GTP\gamma S$ was not an artifact of protein thiophosphorylation or reduced ATP levels.

Inhibition by AIF_4^- has been used as a rigorous test for direct involvement of a GTP-binding protein in other systems (Gilman, 1987; Melançon et al., 1987). However, we found that AIF_4^- (5 mM fluoride and up to 1 mM aluminum) had

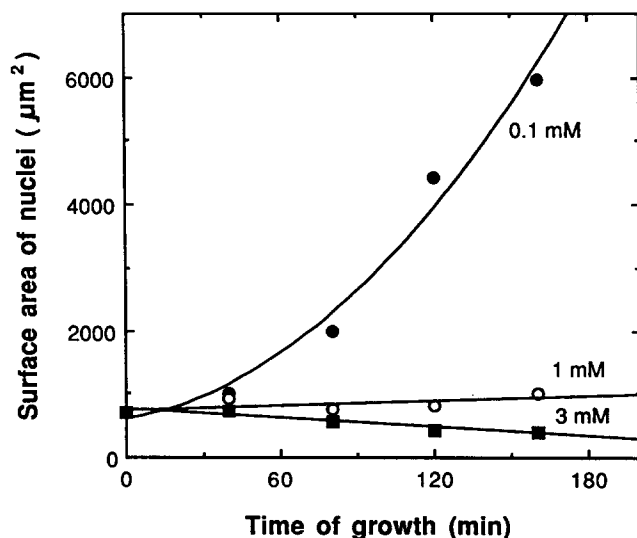


Figure 7. GMP-PNP inhibits vesicle fusion. Addition of 3 mM GMP-PNP (■) to a nuclear assembly reaction completely inhibited vesicle fusion and nuclear envelope growth for at least 160 min. Addition of 1 mM GMP-PNP (○) delayed vesicle fusion until 80 min after cytosol addition and inhibited the extent of nuclear envelope growth at least sixfold after 160 min. Reactions that contained 0.1 mM GMP-PNP (●) were indistinguishable from the no-addition control (not shown).

no effect on vesicle binding or fusion (Fig. 8). (Chromatin decondensation was inhibited by both AIF_4^- and 1 mM AlCl_3 .) Controls treated with 5 mM fluoride alone exhibited normal binding, fusion, and decondensation. The lack of inhibition by AIF_4^- directly showed that a heterotrimeric G protein was not involved in nuclear vesicle fusion. However, since several small GTP-binding proteins are insensitive to AIF_4^- (Kahn, 1991), this class of GTP-binding proteins may be involved in nuclear vesicle fusion.

Membranes Are Inhibited for Fusion by Preincubation with $\text{GTP}\gamma\text{S}$ and Cytosol

To determine which fraction contained the protein inhibited by $\text{GTP}\gamma\text{S}$ we preincubated membranes with 1 mM $\text{GTP}\gamma\text{S}$ or GTP before assaying for fusion. After pretreatment, membranes were pelleted and assayed using fresh cytosol. Fusion of the $\text{GTP}\gamma\text{S}$ -pretreated membranes was normal (Fig. 9 *a*). Similarly, pretreatment of cytosol with 1 mM $\text{GTP}\gamma\text{S}$, followed by dialysis to remove excess $\text{GTP}\gamma\text{S}$, did not inhibit fusion of fresh membranes (not shown).

In contrast, after preincubation of membranes with $\text{GTP}\gamma\text{S}$ in the presence of cytosol, the reisolated membranes bound to chromatin but fusion was significantly delayed (Fig. 9, *c* and *d*). The inhibition of membrane fusion by pretreatment with $\text{GTP}\gamma\text{S}$ and cytosol was not an artifact of proteolysis, since control membranes pretreated with cytosol and 1 mM GTP were not impaired for fusion or nuclear envelope growth (Fig. 9, *b* and *d*). Two observations suggested that the soluble factor that mediated inhibition by $\text{GTP}\gamma\text{S}$ was a protein: inhibition did not occur when the preincubation was carried out on ice for 30 min, and the soluble factor was not removed from the cytosol by dialysis (data not shown). We concluded that a soluble protein, designated the GTP-dependent soluble

factor, GSF, associated with a membrane component(s) in the presence of $\text{GTP}\gamma\text{S}$ or GTP, and that subsequent hydrolysis of GTP was required for nuclear-specific vesicle fusion.

GSF May Bind Stoichiometrically to Membranes

If GSF binds to membranes in the presence of $\text{GTP}\gamma\text{S}$, then we should be able to titrate the inhibition by altering the amount of cytosol in the preincubation step. Membranes were preincubated with 1 mM $\text{GTP}\gamma\text{S}$ and varying amounts of cytosol for 30 min at 23°C. The membranes were then reisolated, bound to chromatin, and assayed for fusion in the presence of fresh cytosol. The time of initial fusion strongly depended on the amount of cytosol present in the preincubation step (Fig. 10). Membranes preincubated in $\text{GTP}\gamma\text{S}$ without cytosol fused as usual, 40 min after addition of fresh cytosol. Preincubation with $\text{GTP}\gamma\text{S}$ and small amounts of cytosol delayed fusion by 45–60 min, and preincubation with $\text{GTP}\gamma\text{S}$ and larger amounts of cytosol inhibited fusion irreversibly (at least 3 h). Irreversible inhibition is shown on the graph as a plateau. The amount of cytosol required for irreversible inhibition was determined empirically for each set of extracts, since membranes from different preparations vary in concentration. Fig. 10 shows that titration was reproducible; experiments done on different days with the same extract fit a single curve. These experiments raised the interesting possibility that GSF was binding stoichiometrically to membranes during preincubation with $\text{GTP}\gamma\text{S}$.

Depletion of GSF from Cytosol Does Not Inhibit Fusion

If the titration result were valid, we predicted that cytosol would be depleted of GSF by preincubation with excess membranes plus $\text{GTP}\gamma\text{S}$. If, on the other hand, GSF acted enzymatically in the presence of $\text{GTP}\gamma\text{S}$ or GTP (for example, as a GTP-dependent protein kinase), we expected GSF to remain in the cytosol. To test these predictions, cytosol was preincubated with excess membranes and 1 mM $\text{GTP}\gamma\text{S}$, and the membranes were removed by centrifugation. As a control, cytosol was preincubated with $\text{GTP}\gamma\text{S}$ in the absence of membranes. The cytosols were then dialyzed to remove the $\text{GTP}\gamma\text{S}$ and tested in two ways.

First, each cytosol was assayed for GSF. For this experiment, each cytosol was incubated with a small amount of membranes plus 1 mM $\text{GTP}\gamma\text{S}$. The membranes were then reisolated, bound to chromatin, and tested for fusion with fresh cytosol. Fig. 11 *A* shows that the treated cytosol ("depleted"; originally exposed to excess membranes plus $\text{GTP}\gamma\text{S}$) was unable to inhibit new membranes for fusion after preincubation with $\text{GTP}\gamma\text{S}$. This suggested that GSF was depleted from the treated cytosol within the limits of sensitivity of the assay. The control cytosol (originally exposed to $\text{GTP}\gamma\text{S}$ but no membranes) was able to inhibit new membranes for fusion; enclosure of the nuclear envelope was delayed until 2 h, and growth was inhibited at least threefold after 3 h (Fig. 11 *B*). This inhibition of fusion indicated that the control cytosol still contained GSF. We concluded that GSF function probably requires stoichiometric association with a vesicle component.

Second, the depleted and control cytosols were tested for fusion of fresh membranes. Both cytosols stimulated fusion and nuclear envelope growth (data not shown). If GSF was

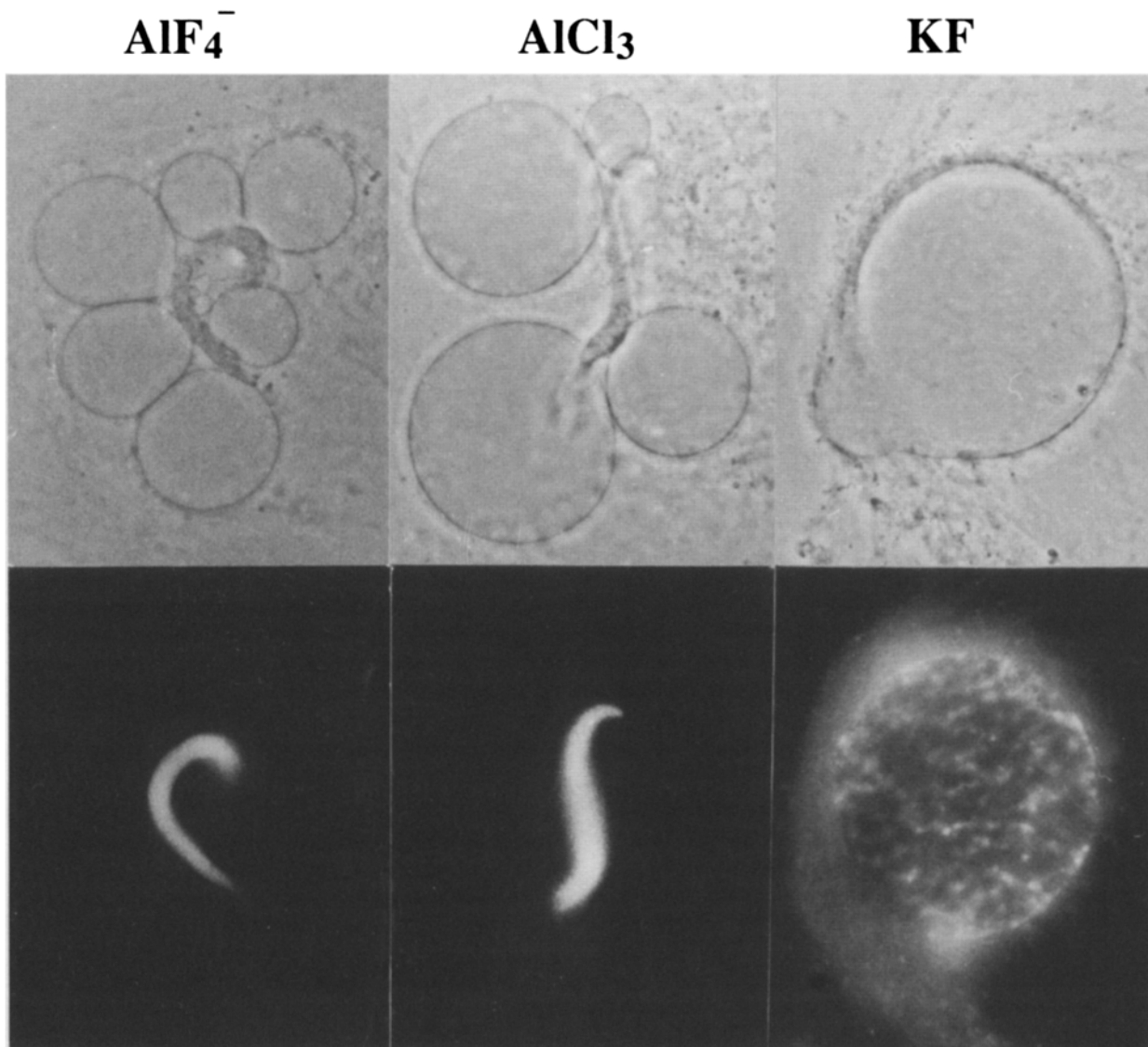


Figure 8. AIF_4^- does not inhibit vesicle fusion. AIF_4^- (5 mM KF and 1 mM AlCl_3) added to an assembly reaction did not inhibit vesicle fusion. Chromatin decondensation was inhibited, however, leading to massive envelope blebbing. 1 mM AlCl_3 alone had the same effect as AIF_4^- on chromatin decondensation. 5 mM KF had no effect on vesicle fusion, chromatin decondensation, or nuclear envelope growth. Photographs were taken after 180 min. (*Top row*) Phase contrast. (*Bottom row*) DNA stained with Hoechst dye.

actually depleted from the cytosol, this result suggested that GSF was not required for the actual fusion event, but rather during a step before fusion.

Discussion

We have described the binding and fusion activities of nuclear-specific vesicles using an *in vitro* assay derived from *Xenopus* egg extracts. We confirmed reports that vesicles bind to chromatin in the absence of cytosol (Pfaller et al., 1991), and have shown that nondialyzable soluble components are required for vesicle fusion (see also Lohka and Masui, 1984; Newport and Dunphy, 1992). Nuclear vesicle fusion was inhibited by $\text{GTP}\gamma\text{S}$; we found that this inhibition of fusion was mediated by a soluble protein that we named GSF. Our results suggested that GSF associates stoichiomet-

rically with vesicles and may regulate a step before the actual membrane fusion event.

Binding and Fusion Activities of Nuclear-specific Vesicles

A recent paper (Vigers and Lohka, 1991) reported two classes of nuclear vesicles derived from *Xenopus* eggs: NEP-A vesicles are sensitive to *N*-ethylmaleimide and exhibit fusion-stimulating activity, whereas NEP-B vesicles, recovered from the cytosol by centrifugation for 4 h at 200,000 g, are sensitive to high salt and exhibit chromatin-binding activity. Although our fractionation procedures are similar, our binding and fusion results differ. Our cytosol, which was routinely spun for 20 min to remove residual membranes (see Materials and Methods), was shown to be free of chromatin-binding vesicles (Fig. 1 E). Our membrane fraction

Pretreatment of membranes with

MWB
+GTP γ S

Cytosol
+GTP

Cytosol
+GTP γ S

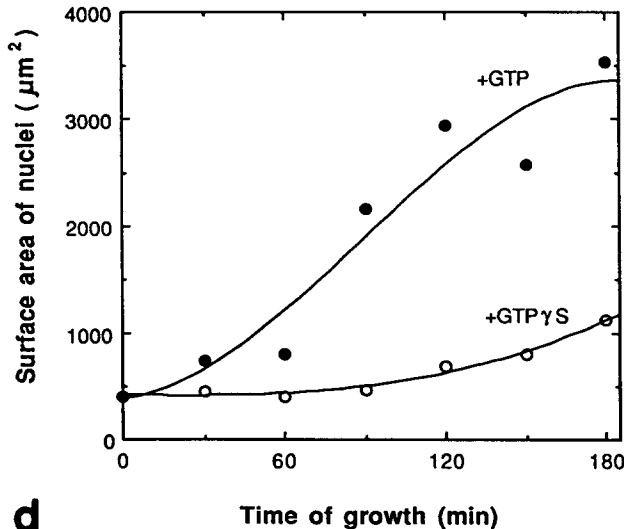
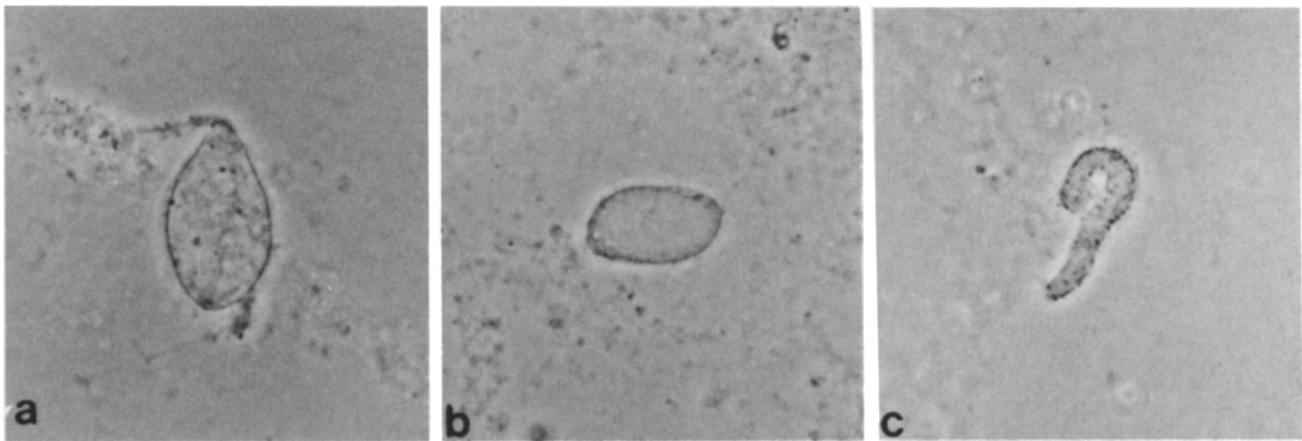


Figure 9. Vesicles are inhibited for fusion by pretreatment with GTP γ S and cytosol. Membranes were pretreated for 30 min at 23°C with (a) MWB and 1 mM GTP γ S, (b) cytosol and 1 mM GTP, or (c) cytosol and 1 mM GTP γ S; the membranes were then pelleted at 20,000 *g* for 15 min and resuspended in MWB. The resuspended membranes were incubated with swelled chromatin for 30 min; binding was robust in all samples (data not shown). Fresh cytosol was then added, and samples were examined by light microscopy every 30 min. The only condition that inhibited vesicle fusion was membrane preincubation with cytosol plus GTP γ S. Photographs were taken 1 h after addition of fresh cytosol. (d) Quantitation of vesicle fusion after pretreatments. Nuclear envelope surface areas were measured as a function of time. Preincubation of membranes with cytosol plus GTP γ S significantly inhibited the rate of fusion.

clearly contained vesicles that bound to chromatin in the absence of cytosol (Figs. 1, B and C, 3, and 5), whereas Vigers and Lohka's equivalent fraction (NEP-A) did not bind to chromatin. In attempts to repeat Vigers and Lohka's membrane fractionation, we found that a significant portion of the NEP-A membranes remain in the supernatant after a 10,000 *g* wash step performed by these authors. We recovered the lost membranes and found that both the NEP-A membranes and the lost membranes bound to chromatin in the absence of cytosol (Boman, A., unpublished observations). Thus we were unable to reconcile our membrane fractionation data with theirs.

Nuclear Vesicle Fusion Involves a GTP-binding Protein

We used nonhydrolyzable GTP analogs to investigate the role of GTP-binding proteins during nuclear envelope as-

sembly. GTP hydrolysis was required for the fusion of nuclear-specific vesicles since addition of GTP γ S or GMP-PNP to nuclear assembly reactions inhibited fusion irreversibly. The following evidence suggested that inhibition was due to a direct effect on a GTP-binding protein(s). First, GMP-PNP does not contain a thiophosphate group that can be exchanged to generate ATP γ S, which might inhibit fusion indirectly. Second, GTP γ S did not interfere with the ATP regenerating system, since ATP levels remained constant as determined by TLC. Finally, the possibility of secondary effects of GTP γ S in the cytosol (such as activation of proteases) was eliminated by pretreating membranes with GSF-depleted cytosol and GTP γ S (see Fig. 11 A); this pretreatment did not inhibit membrane fusion, whereas pretreatment of membranes with cytosol and GTP γ S did inhibit fusion.

AIF $_4^-$ is a powerful stimulator of signal transducing het-

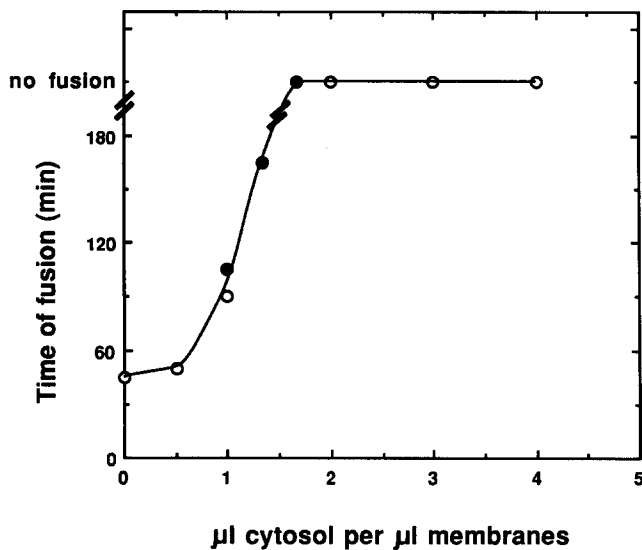


Figure 10. Titration of soluble inhibitory factor by membranes. 4 μ l of membranes were preincubated for 30 min with GTP γ S plus varying amounts of cytosol, pelleted, and resuspended to their original volume with MWB. The resuspended membranes were assayed for binding and fusion in the presence of fresh cytosol. At least 20 nuclei per timepoint were examined by light microscopy to determine when fusion occurred. The graph shows results from two different experiments (indicated by ● ○) using aliquots from the same extract preparation. It is important to note that the samples preincubated with 2:1 or greater ratios of cytosol to membranes were not observed to fuse during the time course of this experiment (180 min), indicated as a plateau labeled "no fusion."

erotrimeric G proteins (Sternweis and Gilman, 1982; Gilman, 1987). It mimics the γ -phosphate of GTP in G proteins to which GDP is bound (Bigay et al., 1985) and locks the G α subunit in its active configuration. Bound GDP is not released from G α under these conditions. However, small GTP-binding proteins are apparently insensitive to AIF $_4^-$. Kahn (1991) recently showed that dissociation of GDP from six small GTP-binding proteins (Rap1, Rab1A, Rab1B, Rab3B, Ha-ras, and ADP-ribosylation factor, [ARF]) is not inhibited by AIF $_4^-$. Furthermore, ARF activities that are stimulated by GTP or GTP γ S, such as its stable association with phospholipids and its ability to serve as a cofactor during cholera toxin-catalyzed ADP-ribosylation of G β , are not stimulated by AIF $_4^-$. Since we found that AIF $_4^-$ did not inhibit fusion in our assay, we deduced that nuclear vesicle fusion may involve a small GTP-binding protein(s), but does not involve a heterotrimeric G protein.

Several GTP-binding proteins have been reported in *Drosophila* nuclei (Berrios et al., 1983) and in rat liver nuclei (Rubins et al., 1990; Seydel and Gerace, 1991). One 28,000-D protein from rat liver cells was demonstrated to be nuclear specific, rather than shared with the ER (Seydel and Gerace, 1991); this protein cofractionates with the pore complex-lamina fraction and is of unknown function, although its small size and lack of oligomerization suggest it is not a classic G protein subunit. We do not yet know if the GTP-binding protein involved in nuclear fusion is related to any of these previously identified nuclear/ER proteins. Furthermore, our results did not reveal whether our GTP-binding protein was

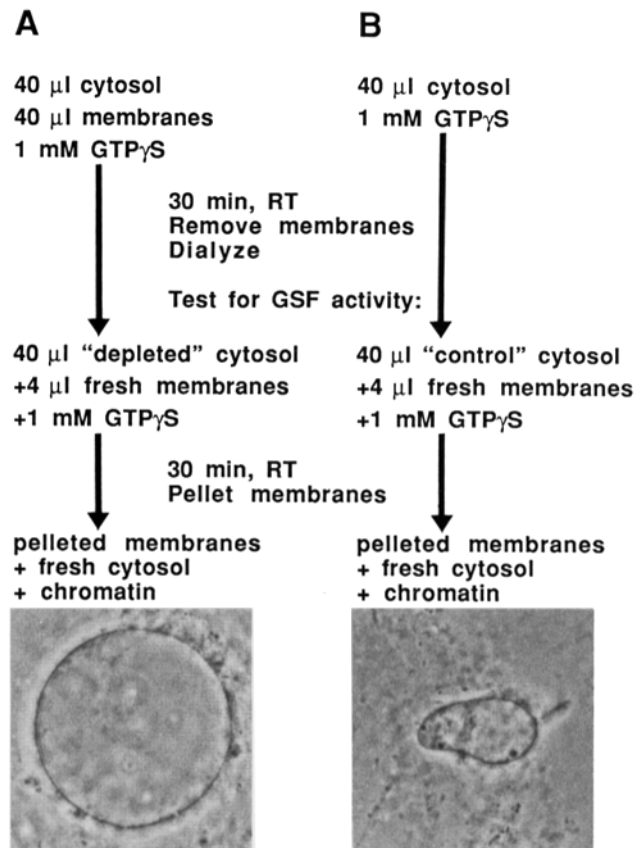


Figure 11. GSF activity can be depleted from cytosol by excess membranes plus GTP γ S. (A) Cytosol was preincubated with excess membranes (1:1 ratio) and 1 mM GTP γ S for 30 min. Membranes were then removed from the depleted cytosol by centrifugation. (B) Control cytosol was preincubated with 1 mM GTP γ S in the absence of membranes, and then centrifuged. Both cytosols were dialyzed to remove excess GTP γ S, and then tested for GSF activity as in Fig. 9. Vesicle fusion and nuclear envelope growth were observed in A, indicating that GSF was indeed removed (within the limits of our assay) from the depleted cytosol. The control cytosol (B) still contained GSF. Phase photographs were taken 180 min after addition of fresh cytosol to pretreated membranes. Both cytosols were still active for fusion in assembly reactions containing fresh membranes (not shown).

soluble or membrane bound, or whether its activity was restricted to vesicles of nuclear origin. These questions are clearly interesting, and efforts to identify the GTP-binding protein(s) are under way.

GSF: a GTP-dependent Soluble Factor Involved in Nuclear Fusion?

Three experiments suggested the existence of a GSF. The first was a membrane poisoning assay, in which pretreatment of vesicles with cytosol plus GTP γ S rendered vesicles non-functional for fusion (Fig. 9). Second, we found that the extent of poisoning (inhibition of fusion) depended upon the amount of cytosol to which the membranes were exposed during pretreatment (Fig. 10). Third, the poisoning factor (GSF) could be depleted from cytosol by preincubation with an excess of membranes and GTP γ S (Fig. 11). We do not

know if GSF is itself a GTP-binding protein. Nevertheless our data are consistent with the idea that GSF associates with nuclear vesicles in the presence of GTP, and that GTP hydrolysis precedes nuclear vesicle fusion.

Could GSF Belong to the RAS Superfamily of Small GTP-binding Proteins?

The Rab and ARF protein families are distinct subgroups of the ras superfamily of small GTP-binding proteins, and both mediate vesicle fusion during secretion (for reviews see Kahn, 1988; Balch, 1989; Hall, 1990). ARF and Ypt1 (a yeast member of the Rab family) are required for transport of vesicles through the Golgi (Stearns et al., 1990). From our limited knowledge of GSF, its properties closely parallel those of GTP-dependent Golgi binding factor (Melançon et al., 1987), a titrable soluble protein that is removed from the cytosol by preincubation with Golgi membranes and GTP γ S, and is not required for fusion. Experiments to test the potential relationship between GSF and the GTP-dependent Golgi binding factor are in progress.

The Role of GTP Hydrolysis

The precise role(s) of GTP hydrolysis in nuclear vesicle fusion is presently unclear. Bourne (1988) proposed that GTP hydrolysis would be used as a molecular switch to ensure unidirectional passage of vesicles through the secretory pathway. Our data suggested but did not prove that GTP hydrolysis regulates a processing step that occurs before fusion. In particular, cytosol depleted of GSF (and dialyzed to remove GTP γ S) remained active for fusion, implying that GSF was not required for fusion per se. It is important to note, however, that our assay (membrane poisoning) may not detect low concentrations of GSF in the depleted cytosol.

We considered two kinds of processing steps that might involve GSF. (a) Uncoating of vesicles. During transport through the secretory pathway, GTP hydrolysis is required to uncoat transport vesicles that are bound to their target Golgi membranes (Melançon et al., 1987; Beckers and Balch, 1989; Orci et al., 1989), and the vesicles must be uncoated before fusion occurs. However, there is only indirect evidence that nuclear vesicles might be coated during mitosis (Newport, 1987), and no evidence that vesicles would remain coated in interphase extracts. (b) Cell cycle regulation of fusion. Warren (1985) proposed that all fusion is inhibited during mitosis, which could account for both organelle disassembly and the lack of secretion and endocytosis (Hesketh et al., 1984; Coleman et al., 1985). We speculate that a GTP hydrolysis step could be negatively regulated during mitosis, and that we have mimicked this regulation using GTP γ S. It is interesting that two small GTP-binding proteins, Rab1 and Rab4, are phosphorylated during mitosis (Bailly et al., 1991; P. vander Sluijs and I. Mellman, personal communication). We clearly need to identify GSF and determine its relationship, if any, to the small GTP-binding proteins, in order to understand the role of GTP hydrolysis during nuclear envelope assembly.

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