Transmembrane Protein-free Membranes Fuse into Xenopus Nuclear Envelope and Promote Assembly of Functional Pores*§

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Post-mitotic reassembly of nuclear envelope (NE) and the endoplasmic reticulum (ER) has been reconstituted in a cell-free system based on interphase Xenopus egg extract. To evaluate the relative contributions of cytosolic and transmembrane proteins in NE and ER assembly, we replaced a part of native membrane vesicles with ones either functionally impaired by trypsin or N-ethylmaleimide treatments or with protein-free liposomes. Although neither impaired membrane vesicles nor liposomes formed ER and nuclear membrane, they both supported assembly reactions by fusing with native membrane vesicles. At membrane concentrations insufficient to generate full-sized functional nuclei, addition of liposomes and their fusion with membrane vesicles resulted in an extensive expansion of NE, further chromatin decondensation, restoration of the functionality, and spatial distribution of the nuclear pore complexes (NPCs), and, absent newly delivered transmembrane proteins, an increase in NPC numbers. This rescue of the nuclear assembly by liposomes was inhibited by wheat germ agglutinin and thus required active nuclear transport, similarly to the assembly of full-sized functional NE with membrane vesicles. Mechanism of fusion between liposomes and between liposomes and membrane vesicles was investigated using lipid mixing assay. This fusion required interphase cytosol and, like fusion between native membrane vesicles, was inhibited by guanosine 5'-3-O-[(thio)triphosphate], soluble N-ethylmaleimide-sensitive factor attachment protein, and N-ethylmaleimide. Our findings suggest that interphase cytosol contains proteins that mediate the fusion stage of ER and NE reassembly, emphasize an unexpected tolerance of nuclear assembly to changes in concentrations of transmembrane proteins, and reveal the existence of a feedback mechanism that couples NE expansion with NPC assembly.

The nuclear envelope (NE)² prevents free diffusion of macromolecules between the nucleus and cytoplasm and therefore

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2 The abbreviations used are: NE, nuclear envelope; DOPC, dioleoyl phosphatidylcholine; ER, endoplasmic reticulum; NBD-DOPE, 7-nitrobenz-2-oxa-1,3-diazole-dioleoyl phosphatidylethanolamine; NPC, nuclear pore complex; MV, membrane vesicle; MR proteins, exclusively membrane-re-siding proteins; R18, octadecyl rhodamine B chloride; Rd-DOPE, N-lissamine rhodamine B sulfonyl-dioleoyl phosphatidylethanolamine; WGA, wheat germ agglutinin; GTPγS, guanosine 5'-3-O-[(thio)triphosphate]; ATPγS, adenosine 5'-O-(thiotriphosphate); NEM, N-ethylmaleimide; BSA, bovine serum albumin; DIO, 3,3'-dioctadecyloxacarbocyanine perchlorate; EE, egg equivalent; Ab, antibody; PC, phosphatidylcholine; NSF, N-ethylmaleimide-sensitive factor; PE, phosphatidylethanolamine; SNAP, soluble NSF attachment protein receptor; SNAP, soluble NSF attachment protein; PLC, phospholipase C.
membranes, or both in cytosol and at the membranes remain elusive (11, 14, 15, 27). Because MVs fuse and form an ER network in a protein-free buffer supplemented with GTP (28), the proteins required for these rearrangements are either transmembrane proteins or cytosolic proteins tightly associated with the membranes. It has been also proposed that ER and NE assembly involves NSF- and SNARE-mediated fusion (14). On the other hand, membrane targeting to the chromatin and GTP-dependent lipid mixing at the chromatin surface at the early stages of NE assembly do not require transmembrane proteins as demonstrated in an in vitro system in which MVs were replaced by phosphatidylcholine (PC) liposomes with associated cytosolic proteins (29).

In this study of ER and NE assembly in the *Xenopus* egg reconstitution system, we explored the relative contributions of cytosolic proteins (defined here as proteins that are present either only in cytosol or both in cytosol and, as peripheral proteins, at the membranes) and exclusively membrane-residing (MR) proteins, such as transmembrane proteins. To identify the functions that do not require MR proteins to be present on each of the membranes involved, we replaced some of the MVs with MR proteins functionally impaired by trypsin or N-ethylmaleimide (NEM) treatments or with protein-free PC liposomes. We found that functionally impaired MVs and liposomes in the presence of interphase but not mitotic cytosol undergo fusion with each other and native MVs and participate in the formation of the tubular ER network. Like fusion between native MVs, this cytosol-dependent fusion involving membranes without functional MR proteins was inhibited by nonhydrolyzable forms of GTP, by NEM pretreatment of cytosol, and by addition of soluble NSF-attachment protein (α-SNAP), an inhibitor of SNARE machinery (14, 30).

In NE assembly, vesicle-liposome fusion allowed us to add membrane material without adding transmembrane proteins. Co-incubation of interphase cytosol and chromatin with MVs in concentrations lowered relative to the standard MV concentrations in the NE assembly reconstitution system resulted in formation of nuclei that were smaller than the control ones, had a decreased number of NPCs, and failed to actively replicate DNA and import substrates. Addition of functionally impaired MVs or liposomes to the reaction mixture and their cytosol-dependent fusion to native MVs compensated for the shortage of membrane material and increased the sizes of the nuclei. Furthermore, the liposome-rescued nuclei appeared similar to the control ones in ultrastructure, active nuclear transport, and DNA replication. The recovery required nuclear transport and involved an increase in the number of functional NPCs. Thus, although MR proteins at MVs were required for generating the characteristic morphology of the branched ER network and of fully extended and functional NE, cytosol conferred on liposomes the ability to fuse and, in the presence of native MVs, to participate in formation of ER and NE. In contrast to nuclei formed at a lowered concentration of MVs, liposome-rescued nuclei with most of the membrane material provided by liposomes had normal function and spatial distribution of NPC. Our findings emphasize the mutual dependence of NPC assembly and NE expansion and suggest that interphase cytosol contains proteins that mediate the fusion stage of the envelope growth.

**EXPERIMENTAL PROCEDURES**

**Liposome Preparation**—All lipids were purchased from Avanti Polar Lipids. Lipid stocks were dissolved in benzol/ methanol mixture (95:5, v/v) and stored at −20 °C under argon. To prepare unilamellar liposomes from dioleoyl phosphatidylcholine (DOPC), from DOPC and rhodamine-dioleoyl phosphatidylethanolamine (Rd-DOPE) (95:5 mol %), or from DOPC and 7-nitrobenz-2-oxa-1,3-diazole-dioleoyl phosphatidylethanolamine (NBD-DOPE) (99:1 mol %) corresponding lipids were mixed and rapidly frozen in liquid nitrogen. Solvents were removed under vacuum overnight. Resulting lipid powder was dissolved in a membrane wash buffer (MWB): 250 mM sucrose, 50 mM KCl, 2.5 mM MgCl2, 50 mM Hepes-NaOH, pH 7.5, to a final concentration of 10 mg/ml. Lipid suspension was extruded through double-stacked 100-nm filters (GE Water and Process Technologies) using Lipex extruder (Northern Lipids Inc., Canada). DOPC/NBD-DOPE liposomes with encapsulated dextran were prepared as above, except dried lipids were dissolved in 50 mM Hepes, 100 mM KCl, pH 7.4 buffer, containing 1 mM 70-kDa rhodamine B isothiocyanate-dextran (Sigma). Loaded liposomes were separated from the free dextran by size exclusion on Superose™12 prep grade (Amersham Biosciences) column.

**NE and ER Assembly**—Components of the *X. laevis* in vitro NE reconstitution system were prepared as described previously (31). In brief, female frogs were injected with 500 units/ml human chorionic gonadotropin (Sigma), and eggs were collected after 18 h. Dejellied eggs were crushed by centrifugation at 12,000 × g (9,000 rpm, 12 min; SW28, Beckman), and crude nuclear extract was obtained. This extract was further fractionated by centrifugation at 200,000 × g (55,000 rpm, 2 h; rotor TLC-55, Beckman) into cytosolic light membrane and heavy membrane (enriched in mitochondria) fractions. Membrane-free nuclear assembly extract (referred to as “interphase cytosol”) and light membrane fraction (referred to as “MV”) were stored at −70 °C. To test for the presence of membrane-residing proteins in our cytosolic preparation, we carried out Western blot analysis for the following proteins: p78, reticulon RTN4, Nup210 (supplemental Fig. S1), and ribophorin (see Ref. 29), and as expected, we found these proteins in our MV but not in the cytosol. Mitotic cytosol was prepared as above except EGTA and β-glycerophosphate (Calbiochem) were added before crushing the eggs (31).

Control nuclei were formed by mixing 20 μl of interphase cytosol (~20 mg/ml total proteins (BCA protein assay kit, Pierce)), 2 μl of MVs (~20 mg/ml of total proteins (BCA protein assay kit, Pierce)), ~10 μm lipids, as determined by an assay described previously (32), and demembranated sperm chromatin (~3,000 sperms/μl) in the presence of the ATP-regeneration system (1 mM ATP (Roche Applied Science), 10 μM creatine phosphate (Calbiochem), and 50 μg/ml creatine kinase (Calbiochem)) and incubated for 2 h at 23 °C.

In most experiments, “undeveloped nuclei” were formed as control ones, except MVs were diluted 10 times (0.1 egg equivalent (EE)) (5) in MWB. To form “rescued nuclei,” we supplemented 2 μl of 0.1 EE of MVs with 5 μl of 10 mg/ml DOPC.
In some experiments at the end of the incubation membranes added to the samples 30 min before analysis under microscope. Diego), and fluorescein-12-UTP (Roche Applied Science) were (kind gift of Douglass J. Forbes, University of California, San Diego), and fluorescein-12-UTP (Roche Applied Science) were added to the samples 30 min before analysis under microscope. In some experiments at the end of the incubation membranes were stained by adding 0.2 μl of 1 mg/ml 3,3’-diodoctadecylox-acarbocyanine perchlorate (DiO; Invitrogen) or octadecyl rhodamine B chloride (R18; Invitrogen).

Cytosol-independent ER reconstitution was performed in high salt buffer (HSB): 250 mM sucrose, 200 mM KCl, 2.5 mM MgCl₂, 50 mM Hepes-KOH, pH 7.5, as described in Ref. 28 except HSB was supplemented with both ATP-regeneration system and 2 mM GTP (Roche Applied Science).

Quantification of Nuclear Pore Complexes—For direct immunofluorescence with Alexa 488-labeled monoclonal 414 antibody against NPC (Covance), labeled nuclei were fixed with 3.5% paraformaldehyde and 1% glutaraldehyde and subsequently centrifuged (15 min, 10,000 rpm; rotor TY.JS-4.2; Beckman) through a cushion of 30% (w/v) sucrose in MWB onto poly-L-lysine-coated coverslips. Similar results were obtained if Ab414 was applied after fixation. Fluorescence was measured with ImageJ software (National Institutes of Health).

MV Labeling and Treatments—For lipid-mixing assay, MVs were labeled with R18. 10 μl of MVs and R18 (0.03 μg final concentration) were mixed in MWB (to final volume of 100 μl) and incubated for 10 min on ice. Column chromatography (Poly-Prep columns, Bio-Rad; 10-μm agarose beads; Agarose Bead Technologies) was used to remove unbound dye. Fluorescence of R18 in labeled MVs was quenched ~2 times. To label MVs with DiO, we incubated 20 ml of MVs with 0.5 μl of 5% (w/v) dye in 100 μl of MWB for 10 min on ice. Labeled MVs were separated from free dye by centrifugation at 15,000 rpm (rotor TLC 55; Beckman) for 20 min through a cushion of 30% (w/v) sucrose in MWB. Then MVs were resuspended in a small volume of MWB. Trypsin- (Sigma) and NEM-treated (Calbiochem) MVs were prepared as described in Refs. 5, 33, respectively.

Lipid Mixing Assay—To monitor lipid mixing, R18-labeled and unlabeled MVs (1:10 ratio), labeled (DOPC/Rd-DOPC) liposomes and unlabeled (DOPC) liposomes (1:10 ratio), or labeled (DOPC/Rd-DOPC) liposomes and unlabeled MVs (1:10 ratio) were mixed in 200 μl of HSB or cytosol (pre-warmed to room temperature) in the presence of ATP-regeneration system and 2 mM GTP. An increase in the fluorescence at excitation and emission wavelengths of 550 and 590 nm, respectively, was recorded for 1 h at room temperature with Spectra Max Gemini XS plate reader fluorimeter (Molecular Devices). The percentage of lipid mixing was calculated as 100 × (F - F₀)/ (Fₘₐₓ - F₀), where F₀ is the initial level of the fluorescence, and Fₘₐₓ is the maximum dequenching of the fluorescent probe measured by solubilizing membranes with 0.03% (final) Triton X-100 (Fluka) at the end of each experiment.

Inhibitors of Nuclear Assembly and Membrane Fusion—Active nuclear transport was blocked with 50 μg/ml wheat germ agglutinin (WGA; Invitrogen). Fusion stages of nucleus assembly were blocked by supplementing cytosol with 1 mM of either ATPγS or GTPγS (Roche Applied Science), or 100 μM (final) lauroyl lysophosphatidylcholine, or 1.5 μM α-SNAP. α-SNAP plasmid DNA was kindly provided by Hemmo Meyer (Institute of Biochemistry, ETH Zurich, Switzerland). Recombinant α-SNAP was purified by Protein One (Bethesda, MD). The same concentrations of ATPγS, GTPγS, lysophosphatidylcholine, and α-SNAP were used in lipid mixing assays. HSB buffer and cytosol were supplemented with these inhibitors before adding MVs or liposomes.

Fluorescence Microscopy—Samples were observed using Axiocert 135 microscope (Zeiss, Germany) with ×100/1.3 Plan-NeoFluar objective (Zeiss, Germany) and ORCA II camera (Hamamatsu, Japan). Fluorescence recovery after photobleaching experiments was carried out using ×100/1.4 Plan-Apochromat (Zeiss, Germany) objective on LSM510 (Zeiss, Germany) confocal microscope.

Transmission Electron Microscopy—Nuclear pellets were fixed at room temperature in 2.5% glutaraldehyde made in 0.13 M sodium cacodylate buffer, pH 7.4, and post-fixed in 2% osmium tetroxide. The pellets were then processed into Embed-812 epoxy (Electron Microscopy Sciences) via increasing concentrations of ethanol followed by propylene oxide and an infiltration series of resin. The epoxy resin was polymerized for 20 h in an oven set at 60 °C. Thin sections were prepared on a Reichert-Jung Ultracut-ultramicrotome (95 nm thick). The grids were post-stained with uranyl acetate and lead citrate and examined in a JEOL 1010 transmission electron microscope operating at 80 kV.

RESULTS

Functionally Impaired MVs Join Native MVs in ER and NE Assembly in the Presence of Cytosol—Trypsin or NEM treatments of MVs have been shown to inhibit the assembly of ER (10) and NE (5, 33, 34). As expected, although intact MVs form characteristic ER networks both in cytosol and in HSB (10), trypsin-treated MVs formed ER neither in HSB nor in cytosol (not shown).

Surprisingly, trypsin-treated fluorescent lipid-labeled MVs joined untreated MVs in ER formation (seen as the development of labeled ER networks) in the presence of cytosol but not in HSB (Fig. 1A). Similarly, although trypsin-treated MVs did not form NE on their own, these MVs supported the formation of nuclei in a reaction mixture containing chromatin, cytosol, and untreated MVs (Fig. 2A). Trypsin-treated R18-labeled MVs
Functionally impaired MVs participate in ER formation by native MVs only in the presence of cytosol. R18-labeled trypsin- (A) or NEM-treated (B) MVs (MVs*, red) were incubated with DIO-labeled untreated MVs (green) in HSB or cytosol (top and bottom panels, respectively). ER formation was analyzed by fluorescent microscopy after 1 h. Scale bar, 10 μm.

FIGURE 1. Functionally impaired MVs participate in ER formation by native MVs only in the presence of cytosol. R18-labeled trypsin- (A) or NEM-treated (B) MVs (MVs*, red) were incubated with DIO-labeled untreated MVs (green) in HSB or cytosol (top and bottom panels, respectively). ER formation was analyzed by fluorescent microscopy after 1 h. Scale bar, 10 μm.

co-localized with untreated DIO-labeled MVs and produced nuclei with bright and smooth fluorescent rims, suggesting that trypsin-treated MVs had become a part of the NE. Similar experiments were carried out on MVs treated with NEM and produced similar results; cytosol restored the ability of MVs to participate in ER (Fig. 1B) and NE (Fig. 2B) formation. To summarize, cytosol restores the ability of functionally impaired MVs to participate in the formation of ER and NE.

Liposomes Participate in ER Formation—Although trypsin and NEM treatments functionally impair MVs, treated vesicles still carry MR proteins. To test whether these proteins are required for cytosol-dependent functional recovery of MVs, we replaced MVs with liposomes. We used liposomes from dioleyl-PC, which does not promote nonspecific protein-independent fusion between vesicles (35). Our analysis of the lipid composition of MVs indicated that these vesicles, along with PC, contain significant amounts of PE (data not shown; see also Ref. 36). To minimize nonspecific fusion (35) in our liposomes, we did not include PE and had no more than 5% mol of Rd-DOPE.

ER network formed in the presence of MVs, liposomes, and cytosol demonstrated co-localization of fluorescent tags of MVs and liposomes (Fig. 3). In contrast, in HSB, ER did not carry fluorescent probe of the liposomes. Thus liposomes, like functionally impaired MVs, participated in ER formation only in the presence of cytosolic factors. In the absence of MVs and, thus of tubule-forming proteins such as reticulon Rtn4a/NogoA and DP1/Yop1p (28, 37), liposomes did not yield the characteristic ER morphology of a network of membrane tubules. To summarize, in the presence of cytosol, liposomes acquire the ability to participate in ER formation.

Protein-free Liposomes Rescue NE Formation—As described earlier (3), 2-h co-incubation of chromatin and standard amount of intact MVs referred to as 1 egg equivalent (EE), i.e. concentration of MVs in the crude nuclear assembly extract (5), in the presence of interphase cytosol yielded round nuclei (Fig. 4, A and B, top panels). Smooth rims of these nuclei contained NPC, as evidenced by their labeling with NPC-specific antibody Ab414. The nuclei assembled in these control experiments excluded 70-kDa dextran, had decondensed chromatin, actively imported a substrate containing a nuclear localization signal, and carried out DNA replication. We will refer to such nuclei as functional.

In contrast to intact MVs, and similarly to trypsin- and NEM-treated MVs, liposomes on their own did not form functional nuclei (Fig. 4A, bottom panel) (29). Can liposomes support nucleus formation when amounts of MVs in reaction mixture are lowered? It has been shown that MV concentration determines the size of nuclei (5). Lowering the MV concentration 10 times versus the standard 1 EE led to a 3-fold decrease in the mean cross-section area of the nuclei (Fig. 4C). Like control nuclei, these small nuclei had a smooth NE decorated with NPCs, as evidenced by their labeling with NPC-specific antibody Ab414. The nuclei assembled in these control experiments excluded 70-kDa dextran, had decondensed chromatin, actively imported a substrate containing a nuclear localization signal, and carried out DNA replication. We will refer to such nuclei as functional.

The formation of NE at a 0.1 EE concentration of MV was rescued by addition of liposomes. The concentration of lipid in liposomes in these experiments was 2.5 times higher than the concentration of lipid in 1 EE of MVs. Addition of liposomes increased the cross-section area of the resulting nuclei in comparison with the undeveloped nuclei (Fig. 4C). The liposome-dependent recovery of the nucleus size was only partial, and nuclei were still smaller than the control ones. However, DNA replication and active nuclear transport (Fig. 4, A and B, 2nd panel) were restored, and the extent of chromatin decondensation was similar to that in control nuclei (Fig. 4A).

The similarity between rescued nuclei and control ones was confirmed by electron microscopy (Fig. 5). Like the NE of control nuclei, the NE of rescued nuclei consisted of two continuous membranes with a visible space between them bridged by multiple NPCs (Fig. 5, bottom panels). The NEs of undeveloped nuclei were also embedded with NPCs, but in contrast to control and rescued nuclei enclosed chromatin was only partially decondensed (Fig. 5, top panels).

As with the assembly of control nuclei from Xenopus egg extracts (18), liposome-dependent rescue of the assembly did not involve microtubules, as evidenced by its insensitivity to the
microtubule-depolymerizing reagent nocodazole (0.2 mM; data not shown). Furthermore, because our cytosol preparations did not contain ribosomes and included the inhibitor of protein synthesis cycloheximide (31), it is unlikely that cytosol-dependent fusion between liposomes and between liposomes and MVs involved insertion of newly synthesized transmembrane proteins into liposomal bilayers.

Mitotic cytosol supported neither binding nor fusion of liposomes at the chromatin surface (supplemental Fig. S2). In brief, liposomes compensated for the shortage of MVs, allowing the cytosol-dependent formation of functional nuclei.

**Rescue of NE Formation with Liposomes Involves Their Cytosol-dependent Fusion**

—Cytosol-dependent participation of impaired MVs and protein-free liposomes in ER and NE formation suggest that cytosol confers upon membranes the ability to fuse. Because of the importance of this conclusion, we verified it using several different experimental approaches.

First, the fluorescent rim of the rescued nuclei that formed in the presence of Rd-DOPE-labeled liposomes might represent bound liposomes. To verify that lipids delivered by the liposomes became a part of the NE of the rescued nuclei, we used fluorescence recovery after photobleaching assay. We bleached a patch at the NE surface and examined lateral mobility of the Rd-DOPE molecules along the surface of rescued nuclei (Fig. 6A). Very rapid and complete fluorescence recovery indicated that lipids freely diffuse over distances greatly exceeding the ~100-nm diameter of our liposomes. Thus, liposomal lipids were distributed in NE bilayers rather than limited in their diffusion, as expected for bound liposomes.

Lipids of liposomes might be delivered into NE either by MV-liposome fusion or by lipid-transfer proteins. To clarify the mechanism of this delivery, we prepared liposomes with encapsulated 70-kDa rhodamine B isothiocyanate-dextran as a content probe and with 5% mol NBD-DOPE as a lipid probe. Dextran-loaded liposomes were included in the control nuclei formation mix, and lipid and content probes were co-localized in the NE of the mature nuclei (Fig. 6B and supplemental Fig. S3). In control experiments, free dextran did not enter the perinuclear space. The loading of the perinuclear space with fluorescent dextran in the experiment with dextran-loaded liposomes is readily explained by...
FIGURE 4. Shortage of MVs that results in formation of undeveloped nuclei can be compensated for by liposomes yielding functional nuclei. To assemble the nuclei, chromatin was incubated with cytosol in the presence of 1.0 EE of MVs (control nuclei), 0.1 EE of MVs (undeveloped nuclei), a mixture of 0.1 EE of MVs with liposomes (LPs) (rescued nuclei) and liposomes only. A, R18-labeled MVs (red; top two panels) and rhodamine PE-tagged liposomes (red; bottom two panels) were used to visualize NE membrane. NPCs and DNA were labeled without fixation with anti-NPC monoclonal antibody Ab414-Alexa 488 (green) and with Hoechst (blue), respectively. To check DNA replication activity, fluorescein-12-UTP (green) was applied to assembled nuclei. B, fluorescein-labeled import substrate BSA-nuclear localization signal (BSA-NLS; green) was applied to assembled nuclei to characterize active nuclear transport. Complete sealing of the NE was established by exclusion of 70-kDa rhodamine B isothiocyanate dextran (red) and the lack of labeling with concanavalin A-Alexa 488 (green). Nuclei were analyzed after a 2-h incubation without fixation. Scale bar, 10 μm. C, at lowered concentration of MVs, liposomes increased the size of assembled nuclei. Cross-section areas of 150 nonfixed nuclei for each kind of preparations (control, undeveloped, rescued) were analyzed. Statistical significance was evaluated by unpaired two-tailed Student’s t test.
MV-liposome fusion but not by an action of lipid-transfer proteins.

The lipidosome-dependent increase in the cross-section area of rescued nuclei (Fig. 4C) suggests that the NE of these nuclei contain lipids of both liposomes and MVs. Indeed, when we used unlabeled liposomes to rescue nuclei formed by DiO-labeled MVs, the fluorescent rims of the rescued nuclei were 3-fold less bright than the rims of undeveloped nuclei, indicating that almost two-thirds of the lipids in NE of rescued nuclei originated from liposomes (supplemental Fig. S4). To further verify the ability of cytosolic factors to support membrane fusion, we explored fusogenic properties of MVs and liposomes in suspension.

**Cytosol-dependent Fusion of Functionally Impaired MVs and Liposomes in Suspension**—To directly assay the merger between MV bilayers, we mixed unlabeled MVs with MVs labeled with R18 at a self-quenching concentration. Lipid mixing between labeled and unlabeled MVs resulted in dilution of the fluorescent lipid and thus in an increase in the fluorescence emission signal. As expected, co-incubation of labeled and unlabeled MVs either in the presence of interphase cytosol or in HSB resulted in lipid mixing (Fig. 6C). Note that whereas MVs fused in HSB, the rate of lipid mixing was always faster in cytosol. Trypsin-treated MVs did not fuse in HSB but fused in the presence of cytosol (Fig. 6D) indicating that cytosolic factors substituted for or replaced trypsin-cleaved components of the fusion machinery at MVs.

In the experiments with liposomes, we used unlabeled DOPC liposomes and DOPC liposomes labeled with a self-quenching concentration of Rh-DOPC. Cytosol promoted lipid mixing between labeled and unlabeled liposomes, detected as an increase in the fluorescence (Fig. 6E). No lipid mixing between liposomes was observed in HSB or in the presence of heat-inactivated or mitotic cytosol. Lipid mixing between MVs and liposomes was also observed only in the presence of cytosol but not in HSB (Fig. 6F). We then verified that fluorescent dequenching in MV-liposome and MV-MV lipid mixing assays reflect membrane fusion by the experiments (supplemental Fig. S5), in which fluorescence increase in both assays was suppressed by fusion-inhibiting lipid lysophosphatidylcholine (reviewed in Ref. 39).

Both MV-MV and liposome-liposome fusion were inhibited by NEM allowing us to explore the interplay between cytosolic proteins and proteins at MVs (Fig. 7, A and B). NEM-treated MVs did not fuse in the buffer, but in the presence of cytosol lipid mixing between NEM-treated MVs was restored (Fig. 7A). For untreated MVs, NEM treatment of cytosol did not affect lipid mixing (Fig. 7B) confirming that under normal conditions the vesicles carry all required components of the fusion machinery. In contrast, liposome-liposome fusion was not supported by NEM-treated cytosol (Fig. 7B). Thus, NEM treatment inactivates both membrane-associated proteins involved in MV-MV fusion and cytosolic proteins involved in liposome-liposome fusion, and the latter can replace the former or substitute for them. In control experiments, NEM quenched with dithiothreitol did not inhibit fusion. To summarize, interphase cytosol supports fusion between liposomes, between functionally impaired MVs, and between either liposomes or impaired MVs and untreated MVs both at the chromatin surface and in suspension.

**Rescue of Nuclei by Liposomes Involves GTP and ATP Hydrolysis**—Nucleus formation is an energy-dependent process (40, 41). In particular, membrane fusion requires GTP hydrolysis, and addition of GTPγS at the onset of a reaction completely blocks the formation of nuclei (40). Similarly, we found GTPγS to inhibit lipid mixing between MVs (Fig. 6, C and D) and liposome-liposome and MV-liposome fusion in cytosol (Fig. 6, E and F). GTPγS also blocked liposome-induced rescue of NE formation, leaving chromatin in its snake-like shape (supplemental Fig. S6). In these experiments, the surface of chromatin with associated liposomes looked shaggy rather than smooth, and no NPCs were detected, as confirmed by the lack of labeling with Ab414. ATPγS also inhibited NE assembly (data not shown and see Ref. 11) and cytosol-dependent fusion between liposomes (Fig. 6E).

**MV-Liposome Fusion and Formation of Rescued Nuclei Are Inhibited by α-SNAP**—NE and ER assembly have been reported to involve SNARE machinery and to be inhibited by excess of wild-type α-SNAP, the NSF-adapter that blocks available SNAREs (14, 30). We reproduced these findings and found exogenous α-SNAP to also inhibit lipid mixing between MVs in HSB and cytosol-dependent fusion between MVs and liposomes (Fig. 7C). The same concentration of α-SNAP that inhibited NE assembly, MV-MV and MV-liposome fusion inhibited rescue of NE assembly with liposomes (Fig. 7E). We developed an experimental design allowing us to isolate undeveloped nuclei. First, chromatin was incubated with cytosol and 1 EE of MVs for 30 min to allow enclosure of chromatin in NE (9). We separated these pre-formed nuclei from the ER network that feeds NE assembly (18) by pelleting them through the sucrose cushion. The nuclei were resuspended in a portion of fresh cytosol with either fresh MVs (1 EE) or liposomes and yielded control or rescued nuclei, respectively (Fig. 7, D and E, top panels). We observed no further NE assembly when undeveloped nuclei were resuspended with either fresh MVs (1 EE) or liposomes in the α-SNAP-supplemented cytosol (Fig. 7, D and E, bottom panels). Incubation with cytosol supplemented with...
neither MVs nor liposomes yielded undeveloped nuclei (Fig. 7F and Fig. 8C). The inhibitory activity of the NSF adapter suggests that cytosol-dependent fusion between MVs and liposomes in NE rescue involves NSF/α-SNAP/SNARE machinery.

Rescue of Nuclei Involves Active Nuclear Transport—Maturation and swelling of nuclei require nuclear transport (9). To investigate the role of active nuclear transport in the assembly of rescued nuclei, we irreversibly blocked nuclear transport with WGA (42), which binds to N-acetylgalactosamine residues at the nucleoporins (43). Addition of WGA at the beginning of rescue reaction blocked NE assembly (data not shown), as was reported previously for standard NE reconstitution reaction (44).

To clarify whether WGA affects NE rescue by blocking active transport or by blocking liposome fusion, we used the same experimental design as in Fig. 7 allowing us to isolate undeveloped nuclei. As described above, chromatin was first incubated with cytosol and 1 EE of MVs for 30 min. Then WGA was added for 10 min, and nuclei were pelleted down. Pre-formed nuclei were resuspended in the fresh portion of cytosol supplemented with either fresh MVs (1 EE) or liposomes (Fig. 8, A and B, respectively; top panels). Neither the MVs nor the liposomes rescued nucleus formation.

When WGA application was omitted, full-sized nuclei were formed only in the presence of fresh MVs (Fig. 8A, bottom panel); in the presence of liposomes nuclei were smaller but were nuclear transport competent (Fig. 8B, bottom panel). Thus liposome-dependent rescue of NE assembly proceeds even in the absence of intact nucleus-associated ER structures suggesting that liposomes could fuse directly with NE. Without addition of fresh MVs or liposomes, the nuclei remained both small and functionally inactive (Fig. 8C).

Addition of fluorescent liposomes to WGA-treated nuclei produced bright smooth NE rims (Fig. 8B), suggesting that WGA did not block vesicle fusion. This conclusion was strengthened by spectrofluorometry experiments in which we found WGA to cause no significant changes in lipid mixing between liposomes and MVs (Fig. 8D). Thus, WGA inhibits liposome-dependent nucleus growth by blocking active nuclear transport rather than by blocking vesicle fusion. Even the addition of fresh MVs did not lift the WGA blockage of nuclei growth. These findings indicated that the nuclear trans-
port inhibitor WGA, known to inhibit NE assembly under standard conditions (45), also irreversibly blocks both MV- and liposome-dependent transition from undeveloped to mature nuclei. In brief, like assembly of control nuclei, liposome-dependent formation of rescued nuclei depends on active nuclear transport.

**Liposomes Restore Normal Distribution of NPCs**—Undeveloped nuclei carried NPCs but did not demonstrate nuclear transport (Fig. 4). To explore the nature of this deficiency, we analyzed the distribution and density of NPCs at the surface of control, rescued, and undeveloped nuclei using direct immunofluorescence with monoclonal Ab414 antibody conjugated with Alexa 488. This antibody reacts with a number of late nucleoporins of the NPC and thus is often used to identify assembled NPCs (43). Nuclei stained with Ab414 displayed bright fluorescence on the periphery (Fig. 4A). Focusing on the nucleus surface revealed the spotty fluorescence of NE (Fig. 4A), which is thought to represent staining of individual NPCs (43). In agreement with earlier reports (45), the distribution of NPCs at the surfaces of control nuclei appeared to be uniform. In contrast, at the surfaces of undeveloped nuclei, bright patches rather than distinct dots of individual NPCs were observed. Although undeveloped nuclei presented an uneven pattern of fluorescence, suggesting NPC clustering, rescued nuclei had a pattern of NPC distribution similar to that of control nuclei. We also noticed that in contrast to the smooth surfaces of control and rescued nuclei, undeveloped ones had wrinkled surfaces.

To compare the density of NPCs in different nuclei, we analyzed average per pixel fluorescence of the nucleus-associated Ab414. Analysis of nonfixed nuclei showed that rescued NE had the highest level of fluorescence and, accordingly, the highest NPC density among all types of nuclei (not shown). The nonuniform shape of rescued and undeveloped nuclei and their relatively small size in comparison with control ones might have an impact on mean fluorescence intensity. To minimize the effects of nucleus morphology on the measured density of NPCs, nuclei were fixed and flattened on the coverslip (Fig. 4B). The density of NPCs turned out to be similar between control, undeveloped, and rescued nuclei (Fig. 4C). Taking into account the differences in the cross-section areas of NE of rescued, control, and
undeveloped nuclei (Fig. 4C and supplemental Fig. S4), this finding indicated that rescued nuclei had larger numbers of NPCs than undeveloped nuclei but still much less pores than the control ones (Fig. 9D). In brief, restoration of active nuclear transport by addition of liposomes is accompanied by restoration of an even distribution of NPCs with no decrease in their average density.

DISCUSSION

In this work, we explored whether fusion steps in ER and NE assembly in the Xenopus nuclear reconstitution system require transmembrane fusion proteins distributed between both fusing membranes. Our experiments argue against this design of the fusion machinery for the Xenopus egg system. We found interphase cytosol to support fusion between MVs and either functionally impaired MVs or liposomes with lipid mixing rates similar to those observed in fusion between intact MVs. Cytosol-dependent fusion under conditions where at least one of the fusing membranes did not carry transmembrane proteins...
shared several important characteristics with fusion between native MVs, including dependence on GTP hydrolysis. Intriguingly, as MV-MV fusion in the buffer, MV-liposome fusion in the interphase cytosol was inhibited by NEM and α-SNAP, both known as inhibitors of SNARE-dependent intracellular fusion (14, 46). These findings substantiate the hypothesis that MV-MV fusion in ER and NE assembly is mediated by NSF/α-SNAP/SNARE machinery (14) and raise an interesting possibility that this machinery involves transmembrane domain-lacking SNAREs that may be present both on the membranes and in the interphase cytosol.

Membrane fusion is required but insufficient to generate the characteristic morphology and function of ER and NE in the Xenopus nuclear reconstitution system. Stabilization of highly curved ER membrane tubules requires the MR protein Rtn4a/NogoA (28, 37), and, not surprisingly, we found impaired MVs and liposomes to be unable to form tubular ER networks in the absence of intact MVs. Similarly, intact MVs were indispensable for GTP-dependent and active nuclear transport-dependent assembly of functional nuclei.

Surprisingly, most of the MVs required for the assembly of functional nuclei might be replaced with liposomes, emphasizing an unexpected tolerance of NE assembly to profound changes in the concentrations of MR proteins and in lipid composition. Our experiments have also identified a novel relation between an expansion of the NE area and assembly and maturation of NPCs.

Cytosolic Proteins Play an Essential Role in Post-mitotic Membrane Remodeling—Intracellular membranes undergo continuous cycles of breaking and resealing of membrane bilayers in fusion and fission. These membrane rearrangements differ in the protein machinery involved, in required rates, and in specificity (47, 48). In contrast to ongoing fusion reactions underlying protein trafficking and secretion, the rearrangements of ER and NE explored in this work are timed to occur post-mitotically, i.e. at a particular stage of the cell cycle. In an earlier work, we reported that proteins of post-mitotic cytosol mediate GTP-dependent lipid mixing between liposomes at the surface of decondensed chromatin but are incapable of generating functional nuclei on their own (29). Here we show that cytosolic proteins confer on liposomes the ability to fuse with MVs, yielding functional NE and tubular ER networks. Interphase cytosol also restores the fusogenic properties of trypsin- or NEM-treated MVs. To reconcile this finding with the fact that intact MVs fuse in the absence of cytosol even after washing with high ionic strength buffer (10), we propose that protein fusogens tightly associated with membranes but lacking transmembrane domains are also present in the interphase cytosol. In this scenario, recovery proceeds by replacement of functionally impaired or, in the case of liposomes, missing fusogenic peripheral membrane proteins with intact copies of the same proteins provided by cytosol. Alternatively, fusogenic cytosolic proteins might replace normal MR components of the fusion machinery with different proteins, thus changing the composition of this machinery. Fusion inhibition by NEM and α-SNAP suggests that cytosol-dependent fusion involves NSF/α-SNAP/SNARE complexes. Although most SNAREs are transmembrane proteins, some are not and can shuttle between cytosolic and membrane-bound forms (34, 49–53). An alternative interpretation would suggest a direct fusogenic role for NSF, a mechanism proposed in Ref. 54 but see Ref. 55).

Our findings suggest that the minimal fusion machinery involved in postmitotic membrane remodeling does not require MR proteins to be present on both fusing membranes. Although it is commonly assumed that protein fusogens have to be anchored in membranes by transmembrane domains, many membrane fission reactions (56, 57), fusion in autophagy (58) and nuclear membrane fusion in yeasts (59), and a mechanism suggested for synaptotagmin-mediated fusion (60) are based on peripheral membrane proteins.

An intriguing mechanism of MV fusion in the sea urchin egg NE reconstitution system has been developed recently (15, 61). These studies report a key role of a specific subpopulation of MVs strongly enriched in membrane-associated cytosolic phospholipase C (PLC) and in its substrate phosphatidylinositol bisphosphate. In the suggested mechanism, GTP-dependent activation of the phospholipase results in generation of fusogenic lipid diacylglycerol and thus induces MV fusion and NE assembly. Although an involvement of cytosolic PLC is consistent with our findings on the importance of proteins that shuttle between cytosol and membrane-associated forms, Xenopus PLC is unlikely to hydrolyze PC in our liposomes. Thus, the applicability of PLC-based mechanisms to our system remains to be explored.

Recent studies have brought into question whether disassembly and assembly of ER and NE in living cells proceed through a cycle of NE vesiculation and MV fusion (17, 18, 21). An alternative hypothesis developed in these studies suggests that ER remains continuous during mitosis and NE forms by reshaping of the ER network. If this is the case, and MVs in the Xenopus nuclear reconstitution system are generated by fragmentation of intact ER structures during the preparation of the egg extract, the research in this experimental system emphasizes the intrinsic ability of ER membranes in post-mitotic cells to fuse and reassemble. In this scenario, our results characterize the underlying fusion machinery.

A major finding of our study that most of the MVs in the NE assembly mix can be replaced with liposomes can be explained by the heterogeneity of MVs. Different strategies applied during ER preparation have revealed MV fractions with distinct chromatin-binding and fusogenic properties (8, 9). The role of different subpopulations of MVs in NE assembly is likely determined by associated MR and cytosolic proteins and, in analogy to the MVs in sea urchin egg extracts (61), by vesicle lipid composition. One may speculate that MVs that are enriched in integral pore membrane proteins (23, 25, 62) and other MR proteins that control NE assembly (63–67) are the first to bind to chromatin. MVs that bind at later stages mostly serve to provide additional lipid material required for NE growth and may be replaced with liposomes in the presence of cytosolic proteins.

The mechanisms that may control fusion mediated by proteins present in cytosol remain to be explored. Within living cells, in contrast to our reconstitution system, vesicle-sized particles are moved only by active transport machinery, and thus, intracellular fusion reactions are tightly controlled (often by
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Rab GTPases) at the level of bringing membranes into contact prior to fusion (68). Alternatively, protein fusogens present in cytosol, in their membrane-associated form, are stabilized in a pre-fusion state by fusion-preventing interactions with MR proteins.

It is also possible that cytosolic fusogenic proteins are a specific feature of unfertilized Xenopus eggs that are programmed to undergo a series of very fast divisions that within 8 h generate up to 4000 somatic cells (69). The cytosolic fusion machinery might then be degraded after the entering blastula stage like other proteins specific for early embryonic development (70, 71). In our experiments only interphase cytosol supports fusion. Thus, functional fusion proteins may be present in cytosol only at the time of an intensive post-mitotic reorganization of intracellular membranes. We propose that this reorganization involves relatively promiscuous fusion reactions as suggested by an unexpected tolerance of nucleus assembly to concentrations of NE- and ER-specific transmembrane proteins. At other stages of the cell cycle, intracellular fusion reactions that require a higher level of specificity may be controlled by other fusion machineries based on transmembrane proteins. In general, the kind of fusion machinery involved in different fusion processes can reflect a trade-off between the required specificity and breadth.

Interplay between Growth of NE Area and Assembly of Functional NPCs—The Xenopus nuclear reconstitution system has been instrumental in dissecting the mechanisms that control assembly of the complex multiprotein machinery of NPCs, including both integral pore membrane proteins delivered by MVs and soluble nucleoporins that come from the cytosol (20). Our experiments on replacing most of the MVs with liposomes have brought about new insights into NPC assembly.

Although the NPCs of undeveloped nuclei did not support active nuclear transport, pores appeared to be fully assembled and had the surface density similar to those in control and rescued nuclei. The conservation of NPC density for nuclei of different sizes resembles the recently reported conservation of NPC density during nuclear growth yielding a 3-fold increase in NE area (45). However, we found the pores in undeveloped nuclei to be unevenly distributed over NE. Note that changes in the distribution of NPCs do not necessary block nuclear transport, as evidenced by recent reports that incorrect spacing of NPCs in the absence of inner nuclear membrane-associated yeast protein Aqp12 (72) and mammalian Sun1 (73) does not arrest cell growth. However, changes in nuclear organization, including incorrect spacing between NPCs caused by mutation(s) or deletion of lamin protein (74, 75), are associated with several human diseases (76).

One might explain the functional incompetence of NPCs in undeveloped nuclei either by their insufficient numbers due to the insufficient numbers of integral pore membrane proteins or by a shortage of available membrane area. The finding that the MV shortage can be compensated for by liposomes, allowing formation of functional nuclei without addition of integral pore membrane proteins, substantiated the latter mechanism. On the other hand, liposome-induced rescue of the nucleus assembly involves not only the restoration of even distribution and functionality of NPCs but also an increase in their numbers.

Because liposomes have none of the integral pore membrane proteins, this observation indicates that some NPCs in undeveloped nuclei are not completely assembled and therefore cannot be detected by Ab414.

The density of NPC at the surface of Xenopus egg nuclei is very high. Because each pore has an ~0.15-μm external diameter and there are ~50 NPC/μm², ~80% of the total surface area of NE is under NPC (77). The specific mechanisms that control nuclear pore numbers and their changes under different conditions remain to be understood (45, 78). It has been recently reported that some nucleoporins control nuclear envelope formation (23–26). Are there any oppositely directed mechanisms that allow nuclear envelope expansion and availability of membrane to control assembly of functional NPCs? Although NPCs insert into NE before it fully encloses chromatin, transport activity of the pores was detected only after the complete enclosure (22). We hypothesize that NPCs in undeveloped nuclei are in an intermediate assembly stage that follows chromatin enclosure but still precedes functional maturation of the pores. Rescue of nuclear transport by addition of membrane material suggests that the transition from immature to mature pores involves an increase in the NE area to provide certain minimal distances between the pores. While in control nuclei the heterogeneity of MVs allows pore formation to be followed by an increase in the NE area per pore, yielding an even pore distribution, in undeveloped nuclei, lipid deficiency slows down membrane enclosure and disturbs NPC formation.

Importantly, although active nuclear transport is required for growth of the NE area in liposome-induced rescue experiments, sufficient amounts of membrane material are required for assembly of functional NPCs. These unexpected data suggest a novel and intriguing feedback mechanism that connects an expansion of the nucleus envelope with the assembly of functional NPCs.

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