A Rab GTPase Is Required for Homotypic Assembly of the Endoplasmic Reticulum*

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To define the requirements for the homotypic fusion of mammalian endoplasmic reticulum (ER) membranes, we have developed a quantitative in vitro enzyme-linked immunosorbent assay. This assay measures the formation of IgG (H2L2) following the fusion of ER microsomes containing either the heavy or light chain subunits. Guanine nucleotide dissociation inhibitor (GDI), a protein that extracts Rab GTPases in the GDP-bound form from membranes, potently inhibits fusion. Inhibition was not observed using GDI mutants defective in Rab binding. Kinetic analysis of the inhibitory effects of GDI revealed that Rab activation is required immediately preceding or coincident with fusion and that this step is preceded by a priming event requiring a member of the AAA ATPase family. Our results suggest that homotypic fusion of ER membranes requires Rab and that Rab activation is a transient event necessary for the formation of a fusion pore leading to the mixing of luminal contents of ER microsomes.

Regulated fusion is a critical feature of heterotypic membrane interactions involved in vesicular transport of cargo through the exocytic and endocytic pathways (reviewed in Ref. 1) and homotypic events leading to the reassembly of intracellular organelles following their disassembly during mitosis (reviewed in Ref. 2). A number of advances have been made in recent years in recognition of components comprising the targeting/fusion machinery used by vesicles to deliver cargo to subcellular organelles (reviewed in Refs. 3 and 4). In contrast, less is known about the mechanism of homotypic fusion that controls the assembly of these compartments.

To study homotypic fusion, cell-free assays have been developed that measure the fusion of endosomes (5), mitotic Golgi fragments (6, 7), yeast vacuoles (8–10), and ER1 microsomes (11–16). In yeast, homotypic fusion of ER membranes requires Rab and that Rab activation is a transient event necessary for the formation of a fusion pore leading to the mixing of luminal contents of ER microsomes.

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EXPERIMENTAL PROCEDURES

Materials—Chicken anti-mouse IgG horseradish peroxidase was obtained from Chemicon (Temecula, CA). Maleimide reagents (m-maleimido-3-(2-pyridylmaleimidophenyl)-butyrate (sulfo-MBS), succinimidyl 4-(p-maleimidomethyl)butyrate (sulfo-SMPB), and sulfo-succinimidyl 4-ethylmaleimide 4-(N-maleimidomethyl)cyclododecane-1-carboxylate (sulfo-SMCC)) were obtained from Pierce. BSA-PDP was synthesized, and the number of PDP groups introduced quantified as described (14). RhGDI was obtained from G. Bokoch (The Scripps Research Institute, La Jolla, CA); active, purified spl rat liver p97 was provided by G. Warren (Imperial Cancer Research Fund, London, UK); recombinant NSF was obtained from S. Whiteheart (University of Kentucky Medical Center, Lexington, KT).

Cell Lines—Ag8(8) cells were obtained from L. Hendershot (St. Jude Children’s Research Hospital, Memphis, TN). P3 × 63 (Ag8U.1) and P3K (P3U.1) cells were obtained from ATCC (Bethesda, MD).

Preparation of Microsomes and Cytosol—To prepare ER microsomes, cells (1 × 10⁸) were collected at 200 × g for 5 min by centrifugation and then resuspended in 10 ml of 25 mM HEPES-KOH, 125 mM KCl, pH 7.2 (25/125) and washed. The pellet was resuspended in an equal volume in 5% sorbitol, 10 mM HEPES, pH 7.2. Cells were homogenized by two passes through a stainless steel ball bearing homogenizer (27). A post-nuclear supernatant was obtained by centrifuging the crude homogenate at 500 × g for 10 min. Cytosol was prepared as described previously (28).

Membrane Fusion/Assembly Assay—Post-nuclear supernatants from P3U.1 and Ag8(8) cells were incubated at 32 °C for the time indicated under “Results” (generally 15 min) in a total volume of 200 µl containing 25/125 supplemented with 2.5 mM MgOAc, 1 mM ATP and an ATP-regenerating system (4.8 µM creatine phosphate and 5 U/ml creatine phosphate kinase). The assay was terminated by transfer to ice and supplemented in order with 25 µM iodoacetate (to alkylate free sulfhydryl groups) and 20 µl of ice-cold lysis buffer (1% Triton X-100 in 200 mM Tris-HCl, 1.5 mM NaCl, 25 mM EDTA, pH 7.5). After vortexing the mix for 10 s, the insoluble material was pelleted at 16,000 × g for 10 min.
The ER is an organelle that specializes in the folding and oligomerization of proteins for export. We previously described a cell-free assay based on the ability of radiolabeled ([35S]) oligomerization of proteins for export. We previously described this assay, we demonstrated that H2L2 assembly is efficient and that the presence of ATP and cytosol leads to luminal continuity which as shown previously (12) can occur if the membranes are lysed by addition of detergent at the beginning of the incubation. Using intact membranes, there is a rapid onset of H2L2 assembly that proceeds at a linear rate following a brief lag (<1–2 min) and reaches a plateau after 15 min of incubation (Fig. 1). In contrast, in the presence of 0.1% Triton X-100, the kinetics of H2L2 assembly has a prolonged lag period (10 min) and a reduced rate. The reduced kinetics of H2L2 assembly in the presence of detergent undoubtedly reflects the loss of the highly specialized folding/oligomerization environment of the ER (12). Given the kinetic differences between assembly observed in the absence or the presence of detergent, incubations are limited to the 15-min time period where nonluminal H2L2 assembly in response to any potential membrane lysis would contribute only minimally to the signal derived from fusion-related assembly. A detergent-treated sample is always included as an internal control in each experiment, and this value, which measures the maximal contribution of luminal independent assembly, is subtracted from all reported values. All of the basic properties of the ELISA based assay were found to be identical to those reported for ER fusion detected by appearance of radiolabeled H2L2 (12) (not shown).

Previous studies by our group and others using GTP or nonhydrolyzable GTP analogs such as GTPγS have suggested a potential role for GTPases in the fusion of mammalian ER membranes (12, 15, 16). To define the GTP requirement for ER assembly, we first examined whether the GTP-dependent step required a membrane-associated or cytosolic component. Pre-treatment of cytosol with 50 μM GTPγS for 15 min at 32 °C in the presence of ATP and an ATP-regenerating system, followed by the addition of 10 mM GTP to neutralize the inhibitory effect of GTPγS, had no effect on the subsequent ability of cytosol stimulate H2L2 assembly compared with untreated cytosol (not shown). In contrast, the addition of GTPγS to the assay potently inhibited ER fusion (Fig. 2A, lane g) (12). Identical results were observed with GDPβS (Fig. 2A, lane h), suggesting that ER fusion requires a complete GTPase cycle. No effect of either analog was observed on the assembly of H2L2 in the presence of detergent (not shown).

The ability of both GTPγS and GDPβS to inhibit fusion is diagnostic of the activity of small GTPases belonging to the Ras superfamily. Two guanine nucleotide binding proteins associated with the ER and compartments of the early secretory pathway are the ARF1 and Sar1 GTPases. Mutants that restrict these GTPases to the GDP- or GTP-bound forms have potent trans-dominant effects on ER to Golgi transport in vivo (25) and in vitro (22–24) by inhibiting the assembly/disassembly of COPII and COPI coat components, respectively (29–32). To determine if either of these two GTPases affect ER assembly, we incubated microsomes with the GDP- (inactive) or GTP-bound forms. Previous studies by our group and others using GTP or nonhydrolyzable GTP analogs such as GTPγS have suggested a potential role for GTPases in the fusion of mammalian ER membranes (12, 15, 16). To define the GTP requirement for ER assembly, we first examined whether the GTP-dependent step required a membrane-associated or cytosolic component. Pre-treatment of cytosol with 50 μM GTPγS for 15 min at 32 °C in the presence of ATP and an ATP-regenerating system, followed by the addition of 10 mM GTP to neutralize the inhibitory effect of GTPγS, had no effect on the subsequent ability of cytosol stimulate H2L2 assembly compared with untreated cytosol (not shown). In contrast, the addition of GTPγS to the assay potently inhibited ER fusion (Fig. 2A, lane g) (12). Identical results were observed with GDPβS (Fig. 2A, lane h), suggesting that ER fusion requires a complete GTPase cycle. No effect of either analog was observed on the assembly of H2L2 in the presence of detergent (not shown).

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Rab family GTPases are believed to play an unknown but critical role in vesicle targeting and fusion (reviewed in Ref. 21). To test if members of the Rab GTPase family are involved in ER assembly, we treated membranes with Rab GDP dissociation inhibitor (GDI), a protein essential for the cycling of Rab between GDP- and GTP-bound forms. Previous studies have demonstrated that GDI binds exclusively to the GDP-bound form of Rab proteins and that the addition of GDI to a variety of Rab-dependent in vitro fusion assays leads to potent inhibition (33–36), presumably due to the ability of GDI to efficiently extract the GDP-bound form of Rab proteins from the mem-
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inhibition of ER assembly (Fig. 2A) had at most a modest effect on ER fusion (Fig. 2B). In addition, Rho-GDI, which extracts Rho GTPases and inactivates Rho/Rac-dependent events (reviewed in Ref. 40), had no effect on ER assembly at concentrations up to 50 μM (not shown). Assembly does not require the Rab1 isoform involved in ER to Golgi and intra-Golgi transport because addition of a trans-dominant mutant (Rab1A[N124I]), which fails to bind guanine nucleotide and which potently inhibits the fusion of ER-derived vesicles to Golgi compartments (22, 24, 25, 41), had no effect on homotypic fusion (Fig. 2A, lane f). These results demonstrate that a novel Rab protein is required for the homotypic fusion of ER membranes.

To assess whether the requirement for Rab in ER assembly occurs in conjunction with the activity of a NEM-sensitive factor(s), we first examined whether our assay is sensitive to sulphydryl alkylating reagents. Although NEM has been widely used in the past to inactivate AAA ATPase family members and found to inhibit the GTP-dependent assembly of liver microsomes (14), it is membrane permeant and would be expected to inactivate sulphydryl groups required for the assembly of H and L chains in the lumen of the ER. We therefore examined the effects of a number of membrane-impermeant analogs of NEM including sulfo-MBS, sulfo-SMPB, and sulfo-SMCC on the ability of membranes or cytosol to promote fusion (Fig. 3A). Following treatment for 15 min on ice, the reagents were inactivated by the addition of excess glutathione, and the treated membranes or cytosol were subsequently incubated in the presence of ATP for 15 min at 32 °C. Whereas treatment of cytosol had little effect on ER assembly (Fig. 3A, lanes b and c), pre-treatment of microsomes with each of the reagents completely inhibited the appearance of assembled H2L2 (Fig. 3A, lanes d–g). As expected, a similar effect was observed in the presence of detergent (not shown) due to alkylation of the sensitive sulphydryl groups required for H and L chain oligimerization.

To assess the temporal sensitivity of the assay to Rab activation or sulphydryl-blocking reagents, membranes were incubated for 15 min at 32 °C. Treatment of either the H or L chain containing membranes alone was sufficient to inactivate fusion of ER membranes when mixed with the untreated partner (Fig. 3A, lanes h–j). No effect was observed with unmodified BSA (not shown). Attempts to reactivate the fusion of ER membranes pretreated with either the membrane impermeant NEM analogs or BSA-PDP using purified NSF or p97, two AAA ATPases previously implicated in yeast ER and mammalian Golgi reassembly (6, 7, 17), were unsuccessful (not shown).

To assess the temporal sensitivity of the assay to Rab activation or sulphydryl-blocking reagents, membranes were incubated for increasing time at 32 °C. At the indicated time (Fig. 3B, Δt), membranes were transferred to ice and either retained on ice (Fig. 3B, closed squares) or treated with GDI (Fig. 3B, open squares), GTPγS (Fig. 3B, open circles), or BSA-PDP (Fig. 3B, closed circles) and reincubated at 32 °C for a total time of 15 min. The addition of BSA-PDP (Fig. 3B, closed circles) inhibited the assembly of H2L2 only when added within the first 2–5 min of incubation at 32 °C, confirming that H2L2 assembly is inaccessible to the bulky thiol-containing reagent. Similar results were observed with membrane impermeant NEM analogs (not...
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Fig. 3. A, ER fusion is inhibited by pretreatment with sulphydryl-blocking reagents. Cytosol (b and c) or microsomes (d–j) were preincubated separately (b, c, h, and i) or together (a, d–g, and j) on ice for 15 min with the indicated reagents (final concentrations: 0.1 mM for NEM and sulfo-analogs, 1 mM [PDP] for BSA-PDP (14)). Following the addition of 2 mM glutathione to neutralize excess reagent, treated and untreated membranes/cytosol were combined and incubated for 15 min at 32 °C in the presence of ATP, and H2L2 assembly was quantitated by ELISA. B, microsomes incubated in the presence of ATP and cytosol at 32 °C for the indicated time (Δt) were transferred to ice and either held on ice (■) or supplemented with 2 μM GDI (□), 50 μM GTPγS (○), or BSA-PDP (1 mM PDP) (△) (final concentrations). After 30 min on ice, GDI treated samples were transferred to 32 °C and incubated for a total time of 15 min. After 15 min on ice, BSA-PDP of GTPγS treated samples were supplemented with either 2 mM glutathione (BSA-PDP-treated samples) or 10 mM GTP (GTPγS-treated samples) to neutralize excess reagent, transferred to 32 °C, and incubated for a total time of 15 min

shown). Although the temporal sensitivity to GTPγS yielded a similar result to that of sulphydryl blocking reagents (Fig. 3B, open circles), H2L2 assembly remained sensitive to GDI throughout the entire time course (Fig. 3B, open squares). The addition of GDI at any time point abruptly blocked ER fusion, similar to the effect of transferring cells to ice (Fig. 3B, closed squares). Thus, the requirement for Rab is a transient event, occurring immediately prior to membrane fusion.

DISCUSSION

We have developed a convenient ELISA assay to measure homotypic ER fusion based on the unique protein folding environment of the ER (12). Fusion of H and L chain-containing microsomes requires a factor sensitive to sulphydryl blocking reagents, as has been observed previously in other assays that measure ER assembly using fluorescent lipid probes (11, 13, 14). Consistent with the observation that NSF cannot reverse the GTP-dependent fusion of microsomes prepared from rat liver (14), we have been unable to reverse NEM-inhibited ER fusion by either purified NSF or p97, proteins that are required for the reassembly of NEM-treated Golgi membranes (6, 7). The latter reagent (p97/yeast Cdc48p) is involved in the assembly of yeast ER fragments (17). It would appear that the fusion of mammalian ER microsomes may be mediated by a novel member of the AAA ATPase family. Alternatively, the inactivated factor(s) may remain associated with a docking/fusion complex(es), functioning as a dominant inhibitor.

The principle focus of our study was to examine the hypothesis that a Rab GTPase may mediate ER fusion. Previous observations using GTP and/or GTP analogs have implicated the involvement of a GTPase(s) in the homotypic assembly of the mammalian ER membranes (12, 15, 16). We eliminated the possibility that Sar1, ARF1, and Rab1 are involved in ER fusion as trans-dominant inhibitory forms of these proteins, which inhibit ER to Golgi transport, had no effect on ER fusion in vitro. The inability of the GTP-restricted forms of either the Sar1 or ARF1 GTPases to inhibit fusion eliminates the possibility that the inhibition observed with GTPγS is somehow related to the activation of endogenous Sar1 or ARF1 leading to the stable coating of ER membranes with either COPII or COP1 coats, respectively (30, 32, 43). However, we did find that Rab-GDI, but not Rho-GDI, had potent effects on homotypic fusion and that this inhibition was specific, because GDI mutants defective in Rab binding were not inhibitory. These results demonstrate for the first time the involvement of a member of the rab gene family in ER assembly. A requirement for Rab in ER fusion is paralleled by the need for Ypt7p in the homotypic fusion of vacuolar membranes in yeast (8) and Rab5 in the homotypic fusion of early endosomes in vitro (26, 44). Curiously, the homotypic assembly of Golgi cisternae has been reported to be insensitive to both Rab-GDI and GTPγS (6, 7). Likewise, the homotypic fusion of yeast ER membranes is GTPγS-insensitive (11). These results are at odds with a large body of data that suggests that most if not all cellular fusion events involve Rab (reviewed in Ref. 21). We suggest that the Rab GTPases likely to be involved in each case remain to be detected.

We found that the timing of Rab activation is coupled to the formation of a pore that provides luminal continuity between H and L chain-containing membranes. This is because H2L2 assembly occurs immediately upon fusion (12), and GDI, which only recognizes the GDP-bound form of Rab (reviewed in Refs. 34 and 35), was able to inactivate fusion at early and late time points. Our results are similar to the late requirement for Ypt7p in the homotypic fusion of vacuolar membranes (8). Although GDI blocked a late step coincident with fusion, the temporal effects of GTPγS mimicked that of sulhydryl reagents, which only blocked an early membrane priming step. If the endogenous Rab responsible for ER fusion is also a target for GTPγS, these results suggest that although Rab can become activated during priming, premature stable activation by the nonhydrolyzable analog is inhibitory. Consistent with this conclusion, the sensitivity to GDI throughout the time course of incubation suggests that functional activation from the GDP- to the GTP-bound form occurs immediately prior to or coincident with fusion. These results are, in part, similar to the observation that Rab5 continuously cycles between GDP- and GTP-bound forms prior to membrane interaction (45). This cycling has been proposed to serve as a timer to kinetically proofread membrane fusion events (45, 46). In ER assembly, a similar timer function may be in effect. Therefore, an ordered reaction involving a protein belonging to the AAA ATPase gene family during the priming of membranes for fusion followed by a transient activation of Rab at the fusion site may be a common feature of the homotypic fusion of both endocytic and exocytic compartments.
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