Characterization of *N*-ethylmaleimide-sensitive thiol groups required for the GTP-dependent fusion of endoplasmic reticulum membranes

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The GTP-dependent fusion activity of endoplasmic reticulum membranes is thought to be required for the structural maintenance and post-mitotic regeneration of the endoplasmic reticulum. This fusion is sensitive to the thiol-alkylating agent *N*-ethylmaleimide. In many intracellular fusion events *N*-ethylmaleimide-sensitivity is associated with a homotrimeric ATPase called *N*-ethylmaleimide-sensitive fusion protein or NSF. The addition of cytosol containing NSF is known to restore fusion activity to *N*-ethylmaleimide-treated membranes. We found that the inhibition of fusion of rat liver endoplasmic reticulum membranes (microsomes) by *N*-ethylmaleimide was not reversed by the addition of untreated cytosol. Fusion was also unaffected by treatment with a buffer known to remove NSF from membranes. Accordingly, no membrane-associated NSF was detected by immunoblot analysis. These data suggest that microsome fusion requires an *N*-ethylmaleimide-sensitive component

distinct from NSF. This component was tightly associated with the membranes, so we used a number of chemical probes to characterize it *in situ*. Its thiol groups did not appear to be part of a GTP-binding site. They showed relatively low reactivity with sodium periodate, which induces the formation of disulphide bonds between proximate thiol groups. The thiols were not protected against *N*-ethylmaleimide by Zn^{2+} , a potent inhibitor of fusion which is known to efficiently co-ordinate thiol groups. To characterize the topology of the fusion-related thiol groups we used bulky thiol-specific reagents prepared by conjugating BSA or 10 kDa aminodextran to the bifunctional reagent *N*-succinimidyl 3-(2-pyridyldithio)propionate. The inhibition of fusion by these reagents indicated that these thiols are highly exposed on the membranes. This exposure might be important for the function of these groups during GTP-triggered fusion.

INTRODUCTION

Membrane fusion is crucial for the secretory and endocytotic transport pathways and the formation of cellular organelles. Despite significant progress in the identification of proteins required for intracellular transport, the proteins mediating membrane fusion itself remain unknown. The experimental systems employed to identify the intracellular fusion machinery have generally been *in vitro* reconstitutions of inter-organelle multi-step transport processes culminating in membrane fusion. Typical examples include transport from the endoplasmic reticulum to the Golgi [1] and through the Golgi [2], the formation of 'late' endosomes [3] and transport from endosomes to the trans-Golgi network [4]. Membrane fusion in these experimental systems is measured indirectly by following the modification of content protein markers as they come into contact with modifying agents present in counterpart membrane compartments [1,3,5]. These processes require cytosol and ATP [1,3–5].

Before reaching the point of fusion, membranes must acquire a fusion-competent state and become docked [2,3,6]. In general, the acquisition of a fusion-competent state involves the binding of cytosolic proteins to membranes [7]. The docking stage involves the formation of an intermembrane complex which includes receptor proteins termed SNAREs (SNAP receptors), which provide specificity of transport, and 'general fusion proteins', which stabilize the complex [8,9]. General fusion proteins include

N-ethylmaleimide (NEM)-sensitive fusion protein (NSF) and soluble NSF-attachment proteins (SNAPs) [10]. Both NSF and SNAPs are considered to be universal components of the intracellular transport/fusion machinery [9]. The docking complex dissociates upon ATP hydrolysis by NSF [8]. The mechanism by which fusion then proceeds is completely unknown. Fusion has been broadly defined as 'the set of events that follows docking, up to and including the mixing of lipid bilayers' [11].

The exchange of general fusion proteins between cytosol and membranes is a key feature of these experimental systems and has permitted the biochemical manipulation of the transport machinery. The proteins on the membrane can be inactivated and replaced by active components through the addition of non-treated cytosol [1,3,5]. Cytosolic components that restore function can be further isolated by fractionation. This approach led to the identification of NSF, a cytosolic protein which restored transport between Golgi membranes treated with the thiol-reactive agent NEM [12]. NEM-sensitivity has been used in a similar way for the identification of other cytosolic transport/fusion components unrelated to NSF [4,13,14].

In view of the difficulty inherent in studying complex multi-step transport processes, the employment of simpler experimental systems to study intracellular membrane fusion seems promising. One such system is the homotypic fusion *in vitro* of membrane vesicles, or microsomes, formed by disrupting the endoplasmic reticulum by homogenization [15–18]. The function of micro-

Abbreviations used: DNP, dinitrophenyl; DTT, dithiothreitol; FBA, Fusion Buffer A (10 mM Hepes/KOH, 150 mM sucrose, 50 mM KCl, 0.2 mM EGTA, pH 7.0); GTP[S], guanosine 5'-[γ-thio]triphosphate; NEM, *N*-ethylmaleimide; NSF, *N*-ethylmaleimide-sensitive fusion protein; PDP, 3-(2-pyridyldithio)propionimidate; PEG, poly(ethylene glycol); R18, octadecyl rhodamine B; SNAP, soluble NSF-attachment protein; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate.

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somal fusion activity *in vivo* has been suggested to be related to the structural maintenance and post-mitotic morphogenesis of the endoplasmic reticulum [19–21]. Microsomes fuse *in vitro* with each other or with homologous nuclear membranes, but not with the membranes of other intracellular organelles [15,17,20]. Like many other intracellular fusion reactions, microsome fusion is inhibited by NEM [22]. While the homotypic fusion of microsomes studied using different experimental systems is, to a variable extent, cytosol-independent [15,17,18], the nucleotide requirement of fusion depends on the source of microsomes. Fusion of yeast microsomes requires ATP and is insensitive to guanosine 5'-[γ -thio]triphosphate (GTP[S]) [18], fusion of microsomes from cultured myeloma cells is ATP-dependent and is inhibited by GTP[S] [17], and fusion of rat liver microsomes is ATP-independent, requires GTP and is inhibited by GTP[S] [15]. The inhibition of fusion by GTP[S] and non-hydrolysable GTP analogues indicates that hydrolysis of GTP is required for fusion to occur [15,17].

The minimum requirements for the fusion of rat liver microsomes include contact between membranes and the presence of GTP and Mg^{2+} or Mn^{2+} ions [15]. Once microsomes are in contact, fusion takes place upon the addition of GTP and Mg^{2+} with a very short delay and can be measured directly [16,22,23]. Contact between microsomes can be established spontaneously, in the presence of physiological concentrations of Mg^{2+} [15], or induced much more quickly by including high-molecular-mass poly(ethylene glycol) (PEG) [16,24] in the incubation mixture. In the presence of MgGTP, the aggregation and fusion of microsomes at low PEG concentrations (1–5%) are solely a function of the PEG fractional volume occupancy, i.e. the volume occupied by PEG molecules relative to the total volume of the incubation mixture (S. P. Radko and A. V. Sokoloff, unpublished work).

In seeking to characterize microsome fusion biochemically, we explored first the possibility that the microsome fusion component sensitive to NEM would share the properties of NSF, in that it would be present in an exchangeable form in the cytosol and its association with membranes would be controlled by the availability of ATP. Having found that the NEM-sensitive component was distinct from NSF and was strongly associated with the membranes, we used a variety of probes to reveal distinct biochemical and structural features of this component *in situ*.

MATERIALS AND METHODS

GTP and ATP (sodium salts), sodium periodate ($NaIO_4$), PEG 8000, aprotinin, leupeptin, pepstatin and BSA (fraction V, crystalline) were from Sigma (St. Louis, MO, U.S.A.); octadecyl rhodamine B (R18) and aminodextran (10000 Da) were obtained from Molecular Probes (Eugene, OR, U.S.A.); *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) and dimethylformamide were from Pierce (Rockford, IL, U.S.A.); 2,4-dinitrofluorobenzene, dithiothreitol (DTT) and NEM were from Fluka (New York, NY, U.S.A.). [3H]NEM (40–60 Ci/mmol) was purchased from Du Pont-NEN Research Products (Boston, MA, U.S.A.). All other chemicals were of analytical grade. Bio-Gel A-0.5 m (mesh 50–100) was obtained from Bio-Rad (Melville, NY, U.S.A.). Protein was measured using a Pierce BCA kit. Monoclonal antibodies against NSF were generously donated by Dr. J. E. Rothman (Sloan-Kettering Memorial Cancer Center; New York, NY, U.S.A.).

Isolation of microsomes

Microsomes were isolated from frozen rat livers (Pel-Freez, Rogers, AR, U.S.A.) using a protocol modified from that of

Dawson and Irvine [25]. All steps were performed at 0–4 °C. All centrifugation steps were performed using a Beckman SW-28 rotor. A total of nine livers were thawed, minced, washed five times with 200 ml portions of 5 mM Hepes/KOH, 50 mM sucrose, 0.5 mM EGTA and 1 mM DTT (pH 7.0), and resuspended in the same buffer containing 10 μ g/ml aprotinin, 5 μ g/ml leupeptin and 5 μ g/ml pepstatin to give a final volume of 210 ml. The tissue was homogenized in an Elvehjem–Potter homogenizer and the homogenate was centrifuged at 20000 *g* for 20 min. The supernatant was collected and the centrifugation repeated. The resulting supernatant was centrifuged at 38000 *g* for 25 min and the microsome pellet was washed by resuspension in 5 mM Hepes/KOH, 250 mM sucrose, 10 mM KCl and 1 mM DTT (pH 7.0) and pelleted using the same conditions. The microsomes were resuspended in 5 mM Hepes/KOH, 250 mM sucrose, 10 mM KCl and 1 mM DTT (pH 7.0) to give a protein concentration of 25 mg/ml and stored in 100 μ l aliquots at –20 °C. Prior to use, microsomes were thawed, diluted with 3 vol. of Fusion Buffer A (FBA; 10 mM Hepes/KOH, pH 7.0, 150 mM sucrose, 50 mM KCl, 0.2 mM EGTA) and incubated in the presence of 5 mM DTT at 0 °C for 1 h. Unless otherwise indicated, all subsequent steps were performed at 0–4 °C.

'Stripped' microsomes (microsomes from which ribosomes and peripheral membrane proteins were removed) were prepared from rough endoplasmic reticulum [15] isolated according to Morré [26] with some modifications. Livers were homogenized as above, except that 5 mM $MgCl_2$ was included in the homogenization buffer. The homogenate was centrifuged at 10200 *g* for 10 min and the supernatant was collected and re-centrifuged using the same conditions. Microsomes were pelleted on to a 5 ml cushion of 2 M sucrose/5 mM Hepes/KOH (pH 7.0) by centrifugation at 38000 *g* for 35 min and resuspended in 150 ml of 1.5 M sucrose/5 mM Hepes/KOH (pH 7.0). Portions of 25 ml of the suspension were layered on to 5 ml cushions of 2 M sucrose/5 mM Hepes/KOH (pH 7.0), overlaid with 1.3 M sucrose/5 mM Hepes/KOH and centrifuged at 94000 *g* for 16 h. Microsomes from the 1.5/2.0 M sucrose interphase were collected (30 ml), diluted with 5 mM Hepes/KOH (pH 7.0) to 112.5 ml and extracted for 60 min with 1 M NaCl. NaCl (37.5 ml) was added dropwise from a 4 M stock solution to the microsome suspension with stirring in the presence of 1 mM DTT, 1 mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin and 10 μ g/ml pepstatin A. The extract (25 ml) was overlaid on to a gradient of 1.5 M (2 ml), 1.3 M (5 ml) and 1 M (5 ml) sucrose buffered with 5 mM Hepes/KOH (pH 7.0) and centrifuged at 104000 *g* for 2 h. Stripped microsomes were collected from the 1.0/1.3 M sucrose interphase, diluted with 10 vol. of 5 mM Hepes/KOH/10 mM KCl, pelleted by centrifugation at 38000 *g* for 35 min and stored at –20 °C as described above.

Preparation of cytosol

Rat liver cytosol was prepared in 25 mM Hepes, 5 mM $MgCl_2$, 2 mM ATP, 2 mM DTT, 100 mM KCl and 1 mM PMSF (pH 7.2) as described by Tagaya et al. [27], diluted to a protein concentration of 10 mg/ml and kept in aliquots at –70 °C.

Preparation of NSF-containing brain homogenate

Rat brains were homogenized in an equal volume of a buffer consisting of 250 mM sucrose, 5 mM Hepes, pH 7.0, 0.01% NaN_3 , 2.5 mM $MgCl_2$ and 2 mM adenosine 5'-[γ -thio]triphosphate, and 200 μ l aliquots were immediately frozen in solid CO_2 . An aliquot was mixed with 2 vol. of sample buffer consisting of 112 mM Tris/HCl, pH 8.8, 3% SDS, 20 mM DTT, 1.5 mM

EDTA, 11 % sucrose and 0.01 % Bromophenol Blue. It was then sonicated and boiled for 2 min. The sample was extracted with methanol/chloroform [28] and dissolved in 1 ml of sample buffer.

Synthesis of dextran 3-(2-pyridyldithio)propionimide (dextran-PDP) and BSA-PDP

Aminodextran was passed over a Sephadex G-25 (fine) column in 0.1 M potassium phosphate, 0.1 M NaCl and 0.01 % NaN_3 (pH 7.5), and the void volume material was collected. BSA was prepared in the same way, except that a Sephadex G-50 (fine) column was used, and prior to gel filtration the protein was treated for 60 min with 10 mM NEM. PDP groups were introduced into the carriers by reacting gel-filtered aminodextran (25 mg/ml) and BSA (50 mg/ml) with SPDP (10:1, w/w) or with SPDP and 2,4-dinitrofluorobenzene (20:1:1, by wt.) respectively in 0.1 M potassium phosphate, 0.1 M NaCl, 0.1 % NaN_3 and 20 % dimethylformamide (pH 7.5) for 30 min at room temperature [29]. Dinitrophenyl (DNP) groups were introduced into activated BSA (BSA-PDP) as a visual label to localize the reagent during its preparation and application to membranes. The modified aminodextran (dextran-PDP) was purified by gel filtration over a Sephadex G-25 column equilibrated with 0.1 M potassium phosphate, 0.1 M NaCl and 0.01 % NaN_3 (pH 7.5), concentrated with Centricon 3 microconcentrators (Amicon, Beverly, MA, U.S.A.) and stored before use at -20°C . BSA-PDP was isolated in the same way, except that Sephadex G-50 and Centricon 30 microconcentrators were used.

The concentration of PDP groups in the final preparations was determined by measuring the absorption at 343 nm of pyridine-2-thione formed in the reaction of PDP groups with DTT [29]. The concentration of dextran was measured using glucose as a standard [30]. The molar ratio of PDP groups to hexose monomers was found to be 12–14. The incorporation of DNP groups into BSA-PDP was qualitatively estimated by measuring the absorption at 343 nm of the preparation before the addition of DTT. The ratio of the absorption of pyridine-2-thione released in the presence of DTT to the absorption of DNP groups was in the range 2.2–2.7. The concentration of PDP groups in the BSA-PDP preparation was in the range 20–30 mM.

R18 labelling of microsomes

Microsomes were labelled with R18 by injecting 3.5 μl of 1.7 mM R18 in ethanol into 250 μl of FBA/1 mM DTT containing 0.4 mg of microsomes, with vigorous mixing [23]. Non-incorporated R18 was removed by passing the microsomes through a 2 ml Bio-Gel A-0.5 m column.

Treatment of microsomes with thiol-specific reagents

Portions of 150 μl of non-labelled microsomes in FBA/5 mM DTT (see above) were mixed with 50 μl of R18-labelled microsomes and passed through a 3 ml Bio-Gel A-0.5 m column in FBA. Microsomes collected in the void volume were treated with BSA-PDP, dextran-PDP, sodium periodate or NEM at $0-4^\circ\text{C}$ (unless specified otherwise in the Figure legends), gel-filtered as above and tested for fusion activity as described below. The oxidation of thiol groups by sodium periodate was estimated by measuring the decrease in the incorporation of [^3H]NEM into the gel-filtered microsomes. Microsomes were incubated with 0.1 mM [^3H]NEM for 15 min, then pelleted by centrifugation through a cushion of 0.5 M sucrose in FBA at 36000 g for 25 min; ^3H radioactivity in the pellet was measured in a liquid scintillation counter. The background incorporation of [^3H]NEM was determined with microsomes pretreated with 10 mM NEM.

Fusion assay

The fusion assay was based on R18 fluorescence dequenching upon merging of labelled and non-labelled lipid bilayers [22,23,31]. The fusion conditions were a modification of those used by Comerford and Dawson [24]. Portions of 100 μl of the mixture of R18-labelled and non-labelled microsomes (protein concentration 0.3–0.5 mg/ml; the ratio of labelled to non-labelled microsomes was 1:15) and 104 μl of 10 mM Hepes/KOH, 150 mM sucrose, 50 mM KCl, 10 % (w/v) PEG and 1.92 mM MgCl_2 (pH 7.0) were incubated for 2.5 min at 37°C , mixed (at $t = 0$), and transferred to a fluorescence microtitre plate. R18 fluorescence was measured at 590 nm (bandwidth 14 ± 2 nm) with excitation at 544 nm (bandwidth 15 ± 2 nm) at 37°C using a Fluoroscanner II plate reader (Labsystems, Helsinki, Finland) before and after the addition of GTP to a final concentration of 200 μM . Results were normalized by presenting the increase of R18 fluorescence during the incubation as a percentage of the maximum increase of fluorescence observed after dissolving the microsomes with 4 % Triton X-100. Unless specified otherwise, the results shown are the means $\pm$ SEM from three independent experiments.

Stripped microsomes were labelled with R18 as described above and pelleted on to a 50 μl cushion of 2 M sucrose and 5 mM Hepes/KOH (pH 7.0) by centrifugation through a 1.5 ml layer of FBA containing 0.5 M sucrose at 100000 g for 10 min in a Beckman Optima-TL centrifuge (rotor TLS 55). Microsomes were collected in a minimum volume and resuspended in 200 μl of FBA. Non-labelled microsomes (340 μl) were pelleted in the same way and resuspended in 340 μl of FBA. R18-labelled and non-labelled microsomes were mixed (at a final ratio of 1:15), diluted 3-fold with FBA and tested for fusion activity as above.

Electron microscopy

Microsomes were incubated under fusion conditions for 7 min and EDTA was added to give a final concentration of 2 mM. The microsomes were pelleted at 10000 g in a bench Microfuge. The pellets were fixed in a series of 0.3–3.0 % glutaraldehyde in 0.2 M sodium phosphate (pH 7.0) followed by 1 % osmium tetroxide, stained *en bloc* with saturated uranyl acetate, dehydrated and embedded in Epon. The sections were stained with saturated uranyl acetate followed by Reynolds' lead citrate and analysed in a Philips CM-10 microscope operated at 80 kV.

Electrophoresis and Western blotting

Proteins for electrophoresis were dissolved in sample buffer and subjected to electrophoresis in 12 % polyacrylamide gels as described previously [32], and then transferred to poly(vinylidene difluoride) membranes (Millipore, Bedford, MA, U.S.A.). Blots were blocked for 30 min at room temperature in a blocking solution which comprised 140 mM NaCl, 10 mM NaPO_4 , 0.5 % Triton X-100 and 20 mg/ml BSA, pH 7.4. Blots were incubated overnight at 4°C with the anti-NSF antibody 6E6 diluted in blocking solution at a concentration of 1 $\mu\text{g}/\text{ml}$. Proteins were visualized using rabbit anti-[mouse (H + L)] antibody (Pierce) at a concentration of 1 $\mu\text{g}/\text{ml}$ followed by ^{125}I -labelled Protein A (Amersham Corp., Arlington Heights, IL, U.S.A.) used at a concentration of 10 ng/ml.

RESULTS

Fusion assay

Incubation of unlabelled and R18-labelled microsomes with PEG, GTP and Mg^{2+} led to a significant increase in fluorescence

Figure 1 Fusion of microsomes washed with NSF extraction buffer

Mixed R18-labelled and non-labelled gel-filtered microsomes (500 μ l) were incubated with an equal volume of NSF extraction buffer (see the Materials and methods section) in the presence (●, ▼) of 2 mM ATP for 30 min and overlaid on to 1.5 ml of the same buffer containing 0.25 M sucrose and 2 mM ATP placed on a 2 ml cushion of FBA/0.5 M sucrose. In controls (○, ▽), microsomes were treated in the same way except that ATP was omitted from all solutions. Microsomes were pelleted at 40000 g at 4 °C in an SW 50.1 rotor for 25 min. The supernatant was discarded, and microsomes were resuspended in 200 μ l of FBA/1 mM DTT, gel-filtered and tested for fusion activity (●, ○) as described in the Materials and methods section. Background fluorescence (▼, ▽) was measured in the presence of ATP instead of GTP.

(Figure 1). This is an expected result of the fusion between unlabelled and labelled membranes, which leads to a decrease in the membrane concentration of R18 [22,31]. To confirm that R18 fluorescence dequenching was associated with membrane fusion, we examined the products of the reaction using electron microscopy. Dequenching of R18 fluorescence in the presence of GTP correlated with the appearance of fusion products (Figure 2a), represented by large membrane compartments [15,16]. Very few of these structures were observed when GTP was replaced by ATP (Figure 2b), and ATP did not support a dequenching of R18 fluorescence (Figure 1). While the results from R18 fluorescence dequenching experiments and electron microscopy are qualitatively in good agreement, a quantitative correlation between the appearance of fusion products and an increase in R18 fluorescence is difficult to establish. In particular, a contribution of morphologically undetectable hemifusion (fusion of outer bilayer leaflets only) to the R18 fluorescence increase cannot be ruled out. The reason for the slight decrease in R18 fluorescence induced by ATP (used as a control for GTP) is unknown. This effect was not unique to ATP and was also observed with GTP when it was added to microsomes treated with inhibitors of fusion (see below). ATP controls showing background fusion were routinely performed for all experiments; they were very similar and generally are not presented for the sake of clarity.

The NEM-sensitive component has properties distinct from those of NSF

It has previously been established that microsome fusion is inhibited by NEM [22]. To examine whether the NEM-sensitive fusion component of microsomes is related to NSF in terms of the mechanism of its interaction with membranes, microsomes were tested for fusion activity after being washed with a MgATP-

Figure 2 Electron microscopy of microsome fusion products

Microsomes were incubated under fusion conditions (a) and processed for electron microscopy as described in the Materials and methods section. In controls (b), microsomes were treated in the same way except that GTP was replaced with ATP. Both micrographs are at the same magnification. The scale bar represents 1 μ m.

containing buffer known to efficiently extract membrane-bound NSF [27]. The washing procedure had no effect on background fluorescence (Figure 1). It was found that washing did not affect fusion, indicating that the NEM-sensitive component did not dissociate from the membrane (Figure 1). This suggested that the NEM-sensitive component, if it were present in cytosol, would not exchange with homologous membrane-bound molecules in the presence of MgATP, as NSF does [27]. In keeping with this suggestion, we found that cytosol prepared in the same buffer and applied to NEM-treated microsomes at concentrations as high as 5 mg/ml did not restore fusion (Figure 3). Fusion of untreated microsomes was not affected by the provision of cytosol (Figure 3).

The NEM-sensitivity of microsome fusion was associated with component(s) tightly bound to the membrane. Fusion remained highly sensitive to NEM after treating microsomes with 1 M NaCl, a procedure known to remove, in addition to ribosomes, a significant proportion of many peripheral membrane proteins [15] including SNAPs, proteins which mediate the binding of NSF to membranes [33] (Figure 4). Using immunoblot analysis we found that a crude microsome fraction contained low levels of NSF together with lower-molecular-mass bands which might be proteolysis products. However, in stripped microsomes, which have a higher fusion activity, we were unable to detect any NSF or the lower-molecular-mass species (Figure 5). These data are in good agreement with the functional data indicating that the microsomal NEM-sensitive component has membrane-binding properties different from those of NSF. Furthermore, incubation of microsomes with high concentrations of functionally inhibitory anti-NSF antibodies [11] had no effect on GTP-triggered

Figure 3 Fusion of NEM-treated microsomes after incubation with cytosol

Mixed R18-labelled and non-labelled gel-filtered microsomes (500 μ l) were treated with 75 μ M NEM (∇ , $\blacktriangledown$) added from stock solution in methanol or, in controls ($\circ$, $\bullet$), with a corresponding amount of methanol (5 μ l) for 10 min at 0–4 $^{\circ}$ C, and the reaction was stopped with 5 μ l of 0.1 mM DTT. After 5 min, microsomes were mixed with an equal volume of cytosol ($\blacktriangledown$, $\bullet$) or NSF extraction buffer (∇ , $\circ$) and 30 min later they were pelleted by centrifugation through a 3.5 ml cushion of FBA/0.5 M sucrose/1 mM DTT at 40 000 g at 4 $^{\circ}$ C for 25 min in an SW 50.1 rotor. The supernatant was discarded, and microsomes were resuspended in 200 μ l of FBA/1 mM DTT, gel-filtered and tested for fusion activity.

Figure 4 Inhibition of fusion of NaCl-extracted microsomes by NEM

Stripped microsomes were routinely incubated with ($\bullet$) or without ($\circ$) 75 μ M NEM in the presence of 0.2 mM GTP and 1 mM $MgCl_2$ for 10 min at 20 $^{\circ}$ C. They were washed by centrifugation through a sucrose cushion and tested for fusion activity as described in the Materials and methods section. In a parallel experiment, microsomes were directly tested for fusion activity; NEM (75 μ M) was added either prior to preincubation of microsomes at 37 $^{\circ}$ C (∇) or 4 min 10 s (indicated by the arrow) after the commencement of the preincubation step ($\blacksquare$). The results shown are typical of three independent experiments. The same results were obtained when microsomes were incubated at 0–4 $^{\circ}$ C.

fusion (results not shown). In addition to high salt, a number of other extraction protocols were used prior to measuring fusion. We found that more drastic extraction of microsomes using alkaline buffers irreversibly inhibited fusion activity. Adding back the extracted proteins followed by gradual neutralization of

Figure 5 Western blot analysis of NSF in microsomal fractions

Crude microsomes, stripped microsomes and rat brain homogenate were analysed for the presence of NSF. Proteins were run on a 10% polyacrylamide gel and transferred to poly(vinylidene difluoride) membranes. NSF was visualized using an anti-NSF monoclonal antibody (6E6), rabbit anti-mouse IgG and ^{125}I -labelled Protein A. Lane 1, 50 μ g of crude microsomes; lane 2, 50 μ g of stripped microsomes; lane 3, 50 μ g of rat brain membranes incubated with cytosol and 2 mM adenosine 5'-[γ -thio]triphosphate. Molecular masses (kDa) are shown on the right and were determined by reference to ^{14}C -labelled proteins. Similar results were obtained using a different anti-NSF monoclonal antibody (2E5).

Figure 6 Inhibition of microsome fusion by dextran-PDP

Dextran-PDP (D_{10} PDP) was added to mixed R18-labelled and non-labelled gel-filtered microsomes (200 μ l) to give a final concentration of PDP groups of 1 mM. The reaction was allowed to proceed for 10 min. Alternatively, the corresponding buffer was added instead of dextran-PDP ($\circ$, $\square$). Microsomes were gel-filtered and tested for fusion activity either directly ($\circ$, $\bullet$) or after being treated either with 75 μ M NEM followed by 50 mM DTT for 10 and 30 min respectively ($\blacktriangledown$, $\square$), or with DTT alone (∇).

the incubation mixture failed to restore fusion. Irreversible inactivation of fusion was also observed after solubilization and reconstitution of membranes using various detergents (results not shown).

PDP compounds reversibly inhibit fusion and protect fusion activity from inhibition by NEM

To characterize the microsomal NEM-sensitive fusion component in terms of the exposure of its NEM-sensitive site relative to the membrane, we examined the accessibility of NEM-reactive thiol groups required for fusion to bulky reagents. To do this we used PDP compounds, which react reversibly with thiols forming

Figure 7 Inhibition of microsome fusion by BSA-PDP

(a) Mixed R18-labelled and non-labelled gel-filtered microsomes (200 μ l) were treated with BSA-PDP (●, ▽) added to the samples to give a final concentration of PDP groups of 1 mM, or with a corresponding amount of buffer (○, ▴) and the reaction was allowed to proceed for 40 min. After that, microsomes were gel-filtered and tested for fusion activity either directly (●) or after being treated with 75 μ M NEM followed by 50 mM DTT for 10 and 30 min respectively (○, ▽), or with DTT alone (▴). (b) Microsomes were treated with BSA-PDP (○) or with BSA-PDP preincubated for 5 min with an equal volume of 0.1 M DTT as described above (●), gel-filtered in FBA and tested for fusion activity.

disulphide bonds [29]. The modified thiols (and, therefore, fusion of microsomes) were expected to be restored after reducing the disulphide bonds with DTT [29]. To test if these inhibitors reacted with the same thiols that were alkylated by NEM, we blocked thiols with PDP compounds, treated 'blocked' microsomes with NEM, inactivated the NEM and reduced the disulphide bonds with DTT, and then tested fusion. We found that bulky PDP-containing reagents, dextran-PDP and BSA-PDP, completely inhibited fusion (Figures 6 and 7a). This inhibition was significantly reversed by treating microsomes with DTT (Figure 6). In the presence of 50 mM DTT at pH 7.0, the fusion of dextran-PDP-treated microsomes was typically restored to a constant level in 30 min or less, while the maximum level of fusion of BSA-PDP-treated microsomes was reached in 1.5–2 h. To accelerate the restoration of fusion, BSA-PDP-treated micro-

Figure 8 Inhibition of microsome fusion by NaIO₄

Mixed R18-labelled and non-labelled gel-filtered microsomes (200 μ l) were treated with 10 μ M NaIO₄ (added from a 1 mM stock solution in water) for 10 min in the dark. The reaction was stopped by the addition of 4 μ l of 0.5 M Tris/HCl (pH 7.0), and microsomes were gel-filtered, treated with 75 μ M NEM followed by 50 mM DTT (pH 7.0) for 10 and 30 min respectively, and tested for fusion activity. Treatment of microsomes with BSA-PDP (□) was performed after quenching the periodate reaction with Tris and before gel filtration, as described in the legend to Figure 6.

somes were incubated with DTT at pH 8. The pretreatment of microsomes with either reagent considerably protected the fusion activity against irreversible inhibition by NEM (Figures 6 and 7a). When the pretreatment step was omitted, DTT did not restore fusion (Figures 6 and 7a). Although the standard concentration of NEM in these experiments was 75 μ M, very similar protection of fusion activity by both BSA-PDP and dextran-PDP was also observed at concentrations of NEM as high as 2 mM (results not shown). The inhibitory effect of PDP compounds was essentially abolished by their pretreatment with DTT, as shown for BSA-PDP in Figure 7(b).

It should be noted that NEM can react, albeit less efficiently, with protein chemical groups other than thiols [34]. Therefore the protection of fusion against NEM-inhibition conferred by thiol-specific PDP compounds provides direct evidence that the inhibitory effect of NEM on fusion is due to alkylation of thiol groups. The inclusion of 1 mM MgGTP did not protect fusion activity against inhibition by NEM added to microsomes either before fusion was triggered or with fusion in progress (Figure 4). Neither did it interfere with the protection of fusion against NEM-inhibition by PDP compounds (results not shown). This suggests that the inhibitory effect of these compounds was not due to modification of the GTP-binding site in a fusion-related GTPase. The accessibility of functionally important thiols to bulky reagents indicates that they are significantly exposed, being accessible to macromolecules at least as large as BSA.

Inhibition of fusion by periodate and by Zn²⁺

Low concentrations of sodium periodate can oxidize closely spaced thiol groups to produce disulphide bonds [35]. We used this reagent to discern the location of fusion-related thiols relative to each other. Sodium periodate at 10 μ M strongly inhibited the fusion of microsomes (Figure 8). The inhibition was efficiently reversed with DTT (Figure 8). Treatment of microsomes with

Figure 9 Effect of Zn^{2+} on fusion and its sensitivity to NEM

(a) Stripped microsomes were tested for fusion activity as described in the Materials and methods section, with $ZnCl_2$ being added to microsomes either at 0 min or 4 min 10 s (arrow). (b) Microsomes were preincubated with (○) or without (●) 75 μM NEM in the presence of 40 μM Zn^{2+} , 0.2 mM GTP and 1 mM $MgCl_2$ for 10 min at 20 °C, washed by centrifugation through a sucrose cushion and tested for fusion activity. In the control (▽), neither NEM nor Zn^{2+} was present. The results shown are typical of three independent experiments.

sodium periodate resulted in a decrease of about 20% in the incorporation of [3H]NEM into the microsomes, indicating that the inhibition of fusion correlated with the oxidation of a considerable portion of reactive microsomal thiols. Pretreatment of microsomes with sodium periodate protected fusion activity against inhibition by NEM very poorly, if at all (Figure 8). Moderate protection of the fusion activity against NEM inactivation was observed when the concentration of sodium periodate was raised to 50 μM (results not shown). Pretreatment of microsomes with 10 μM periodate followed by BSA-PDP had no effect on the ability of BSA-PDP to protect fusion activity against irreversible inhibition by NEM (Figure 8; compare with Figure 7a).

Microsome fusion was very sensitive to Zn^{2+} ions, being completely inhibited in the presence of 5–10 μM $ZnCl_2$ (Figure 9a). It should be noted that inhibition of fusion by Zn^{2+} was

reversible (Figure 9b). Given that Zn^{2+} can co-ordinate thiol groups, thereby protecting them against NEM [36], we tested the effect of Zn^{2+} on the NEM-sensitivity of fusion. Up to 40 μM Zn^{2+} did not protect fusion activity against inhibition by NEM added either before fusion was triggered (Figure 9b) or with fusion in progress (Figure 9a).

DISCUSSION

We have used the GTP-dependent fusion of post-mitochondrial rat liver endoplasmic reticulum membranes (microsomes) as a simple model of intracellular fusion [15,16]. The high local concentration of microsomes achieved in the presence of PEG allows fusion to proceed much more quickly than in its absence [24]. Omitting PEG can be compensated for by increasing the concentration of microsomes in the incubation mixture [24]. The morphogenesis of the endoplasmic reticulum both *in vivo* and *in vitro* is mediated to a large extent by fusion of endoplasmic reticulum membranes which are closely apposed due to their association with cytoskeletal elements, particularly microtubules [37,38]. Bringing microsomes into close proximity *in vitro* by volume-excluded aggregation in the presence of polymers such as PEG may be considered to a certain extent as a substitute for this 'concentrating' of endoplasmic reticulum membranes in the cell through their association with the cytoskeleton.

The property of microsome fusion that we chose to study in order to establish a chemical basis for the identification of fusion components was its sensitivity to NEM [22]. Attempting to restore NEM-inhibited fusion by analogy with the restoration of fusion to NEM-treated transport reconstitutions [1,3,5], we found that the NEM-sensitive component required for fusion was not extracted with a MgATP-containing buffer that is known to remove NSF [27]. Nor was this component present in an exchangeable form in the cytosol (Figure 3). This strongly suggests that the microsomal NEM-sensitive component is unrelated to NSF. These results are consistent with the observation that the rat liver microsomal fusion machinery is insensitive to high salt [15], a treatment which decreases the binding of NSF to membranes [33]. Furthermore, microsome fusion remains active in the absence of ATP but is inactivated in the presence of ATP but the absence of GTP at 37 °C [39]. In contrast, incubation of NSF in the absence of ATP at 37 °C results in its rapid complete inactivation [12]. Detection of the presence of NSF in crude microsomes using antibodies does not necessarily indicate any functional involvement in fusion. Thus traces of the yeast homologue of NSF, Sec18, have been shown to be tightly associated with microsome membranes without being functionally involved in fusion [18]. In contrast to yeast microsomes, rat liver microsomes treated with high concentrations of salt (stripped microsomes), which increased fusion activity, did not have detectable levels of NSF.

Further characterization of the microsomal NEM-sensitive component was performed using bulky reagents which react specifically and reversibly with thiol groups and inhibit fusion. It should be emphasized that unless the modification can be reversed, the inhibition of fusion by bulky reagents provides by itself little information regarding the specificity of the inhibition. There is always the possibility that fusion is inhibited due to the obstruction of the interaction between membranes by the presence of bulky reagents on the membrane surfaces. Taking this consideration into account, we detected the reaction of fusion-related thiol groups with bulky reagents by the appearance of a resistance of fusion activity to inhibition by NEM. Fusion activity was tested upon removal of covalently bound reagents from NEM-treated microsomes by DTT. We found that the

NEM-sensitive groups required for fusion are highly exposed thiols. The exposure of thiols was inferred from their accessibility to two bulky reagents prepared using very different macromolecules. The first reagent was dextran-PDP, which is expected to be a mixture of neutral and positively charged molecules that behave in solution as random coils [40]. The other reagent was the highly negatively charged BSA-PDP, which is a rigid globule stabilized by multiple disulphide bonds [41]. The fact that very different bulky reagents modify fusion-related thiols in a similar way means that the efficiency of modification does not depend upon the type of macromolecule used to prepare the reagent. As judged from the reactivity of fusion-related thiols to sodium periodate, they are not likely to be represented by vicinal thiols [35]. Presumably, periodate inhibited fusion by cross-linking membrane proteins in a non-specific way [42]. The lack of any noticeable protection against NEM in the presence of Zn^{2+} indicates that fusion-related thiols are unlikely to be located in the vicinity of electron donor groups known to be capable, together with cysteine, of forming strong Zn^{2+} -binding sites [43].

The properties of this NEM-sensitive microsome fusion component distinguish it from most NEM-sensitive proteins found to be required for reconstituted cytosol and ATP-dependent inter-organellar transport [4,12–14]. While these latter proteins exchange between membranes and cytosol under specific conditions, the microsomal component appears to be bound to the membrane constitutively. An exception to this is the NEM-sensitive factor required for the ATP- and cytosol-dependent reconstituted transport of transferrin receptors back to the plasma membrane, which also appears to be constitutively bound to the membrane [44]. The resistance of fusion activity to NSF-removing buffer and the failure of cytosol to restore activity to NEM-treated membranes has been described for the Ca^{2+} -triggered exocytosis of sea urchin egg cortical granules [45]. The employment of bulky reagents revealed that, similarly to microsome fusion, cortical granule exocytosis required highly exposed thiol groups whose modification resulted in complete protection of the fusion activity against inhibition by NEM [45]. This similarity gives rise to speculation that the fusion machinery required for these two apparently disparate processes may have structurally conserved features associated with the same function. For instance, exposed fusion-related thiols could be required for the same type of inter-membrane interactions.

The exposure of microsomal fusion-related thiols, combined with the properties of the bulky reagents used in the present study, appears to provide a starting point for the affinity isolation of a membrane protein fraction that is considerably enriched in fusion components. An attempt to raise antibodies against proteins isolated through their modification with BSA-PDP which contains DNP groups that can be recognized by anti-DNP antibodies as affinity tags is now in progress.

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