# Identification of a lysosomal protein causing lipid transfer, using a fluorescence assay designed to monitor membrane fusion between rat liver endosomes and lysosomes

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In the present and previous studies [Mullock, Perez, Kuwana, Gray and Luzio (1994) J. Cell Biol. **126**, 1173–1182], we have attempted to investigate endosome–lysosome fusion using an assay based on the dilution of the self-quenching fluorescent lipid probe octadecylrhodamine. Although some characteristics of fluorescence dequenching were consistent with those observed in other cell-free assays, we have now demonstrated that increased fluorescence was due to leakage of an intralysosomal lipid-transfer protein. This protein was purified and found to be a 22 kDa molecule with sequence, immunological and functional characteristics strongly suggesting that it is the rat homologue of human $G_{M2}$-activator protein. Both the 22 kDa protein and recombinant human $G_{M2}$-activator protein caused fluorescence dequenching either when mixed with octadecylrhodamine-loaded endosomes and lysosomal membranes or in a liposome system. The data were consistent with $G_{M2}$-activator protein acting as an octadecylrhodamine-transfer protein. Antibodies to the 22 kDa protein added to cell-free endosome–lysosome content-mixing assays had no effect, although they could inhibit fluorescence dequenching caused by the protein. Thus this protein is not required in any fusion event involved in delivery of ligands from endosomes to lysosomes. The existence within an intracellular organelle of a protein capable of acting as an octadecylrhodamine-transfer protein suggests the need for caution in the interpretation of fluorescence-dequenching assays using mammalian subcellular fractions.

## INTRODUCTION

The final membrane traffic event of the major constitutive endocytic pathway in mammalian cells allows the transfer of endocytosed ligands to lysosomes. Two hypotheses have been proposed to explain the sequential appearance of ligand in endocytic compartments observed *in vivo*. The maturation theory [1,2] suggests that an early endosome gradually turns into a late endosome and then a lysosome by sequential addition of material to and subtraction of material from the organelle containing the ligand. In contrast, the vesicular transfer theory [3] proposes the permanent existence of early endosomes, late endosomes and lysosomes as discrete compartments, through which incoming ligand is sequentially transferred by vesicular traffic. Recent experiments using rat hepatocyte membrane fractions have shown that ligands can be delivered from late endosomes to pre-existing lysosomes using the criterion of ligand transfer to lysosomal positions on density gradients [4,5]. It has been demonstrated that a fusion event occurs by the criterion of content mixing [5].

A widely used membrane-fusion assay based on the dilution of the self-quenching fluorescent lipid probe octadecylrhodamine B chloride (R18) [6] has also been applied to the study of endosome–lysosome interactions [5,7]. In experiments using endosomal membrane fractions loaded with R18 at self-quenching concentrations as donors and lysosomes as acceptors, an increase in fluorescence was observed on incubation at 37 °C. Several characteristics of the increase in fluorescence were consistent with the characteristics of endosome–lysosome interaction determined by density-gradient centrifugation and content-mixing assays, suggesting that the fluorescence assay was measuring true membrane fusion. The time course, temperature-dependence, requirement for lysosomes as acceptors and inhibition by addition of the non-hydrolysable ATP analogue adenosine 5′-[$\beta,\gamma$-imido]triphosphate (p[NH]ppA) were all consistent with data from the density-gradient and content-mixing assays [5,7]. The increased fluorescence activity was sedimentable showing that it remained associated with membranes as would be expected if a membrane-fusion event had occurred. Although the R18 assay could not be used to assess membrane fusion in the presence of cytosol, because of the presence of lipid-transfer proteins [8–11], this was not regarded as a problem, as some-cell-free endosome–lysosome interaction can still be observed, particularly with the centrifugation assay, in the absence of added cytosol, presumably as a result of the presence of primed membranes in isolated fractions [5].

The simplicity of the R18 assay suggested its use to identify endosome and lysosome proteins involved in fusion events in the endocytic pathway. We therefore further fractionated lysosomes in an attempt to identify such proteins. The outcome of these experiments was the demonstration that rat lysosomes contain a protein, identified as the rat homologue of human $G_{M2}$-activator protein, which was responsible for the increase in fluorescence observed after incubation of R18-labelled endosomes with lysosomes. The increase in fluorescence stimulated by this protein was inhibited by p[NH]ppA, and could best be explained by the protein intrinsically possessing R18-transfer activity.

## EXPERIMENTAL

### Materials

R18 was from Molecular Probes (Eugene, OR, U.S.A.). Egg phosphatidylcholine (PC) in chloroform was obtained from

Abbreviations used: p[NH]ppA, adenosine 5′-[$\beta,\gamma$-imido]triphosphate; ASF, asialofetuin; bpIgA, biotinylated polymeric IgA; NHE, 0.15 M NaCl, 0.1 mM EDTA, 10 mM Hepes, pH 7.0; PC, phosphatidylcholine; PC-LUV, phosphatidylcholine-containing large unilamellar vesicles; R18, octadecylrhodamine B chloride; STM, 0.25 M sucrose, 10 mM Tes, 1 mM $MgCl_2$, pH 7.4.

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Avanti Polar Lipids (Alabaster, AL, U.S.A.). Unless otherwise stated all other reagents were from Sigma Chemical Co. Recombinant human $G_{M2}$-activator protein [12] was a gift from Professor K. Sandhoff (Institut für Organische Chemie und Biochemie der Universität Bonn, Bonn, Germany).

### Subcellular fractions

Dense endosomes and lysosomes were isolated, and centrifugation analyses of their interaction performed as previously described [7]. Briefly, for the isolation of dense endosomes, postmitochondrial supernatants were prepared by homogenizing liver (3 ml/g) in STM (0.25 M sucrose, 10 mM Tes, 1 mM $MgCl_2$, pH 7.4) and centrifuging at 1500 *g* for 10 min. The postmitochondrial supernatant was fractionated on 1–22% Ficoll gradients and the dense endosome peak was further purified on Metrizamide step gradients in a vertical rotor. Lysosomes were prepared by differential centrifugation, followed by a wash with 0.25 M KCl to precipitate mitochondria and further purification on a Percoll gradient [13]. A 10 mM Tes extract of lysosomes and one of lysosomal membranes were prepared by resuspending lysosomes in 10 mM Tes/1 mM $MgCl_2$, pH 7.4 (0.5 ml for lysosomes from 1 g of liver) and centrifuging at 288000 *g* for 1 h. The resulting supernatant (extract) was collected, as was the lysosomal membrane pellet which was resuspended in 0.3–0.4 ml of STM (for lysosomes from 1 g of liver). Cytosol was prepared by centrifuging postmitochondrial supernatant at 288000 *g* for 45 min and, unless otherwise stated, was gel-filtered through Bio-Gel P6 columns. The protein concentration was approx. 20 mg/ml.

### Fluorescence-dequenching assays using R18-labelled endosomes

Fluorescence-dequenching assays were performed essentially as previously described [7]. Isolated dense endosomes suspended in STM were loaded with self-quenching concentrations of R18 by addition of an ethanolic solution of R18 at room temperature [7]. Free R18 was removed on small columns of Sepharose CL-4B. The loaded endosomes were incubated under appropriate conditions and increases in fluorescent emission at 590 nm measured (excitation wavelength 560 nm).

During purification of the lysosomal protein that causes fluorescent dequenching of the R18-loaded endosomes, assays consisted of R18-labelled endosomes (0.5 mg), lysosomal membranes (0.1–0.6 mg) and samples from each purification step. The volume of the assay mixture was adjusted to 1.0 ml with STM and incubations were carried out for up to 1 h at 37 °C. At the end of the time course, 60 $\mu$l of 20% (v/v) Triton X-100 was added to the assay mixture to obtain the fluorescence value at infinite dilution of the probe (total fluorescence). Fluorescence dequenching at any point was expressed as a percentage of the fluorescence at infinite dilution and fluorescence-dequenching units were calculated as the percentage of the total fluorescence in the sample after subtraction of the baseline fluorescence obtained from the control sample containing labelled dense endosomes and lysosomal membranes only. To calculate recoveries of the protein with dequenching activity throughout purification procedures, fluorescence dequenching at 30 min incubation was measured.

### Protein purification

#### Step 1: preparation of 10 mM Tes extract of postnuclear supernatant

The livers of eight rats that had been deprived of food overnight were homogenized in 7 ml of ice-cold 0.45 M sucrose/0.5 mM EDTA, pH 7.4, per g of liver using four strokes of a Potter–Elvehjem homogenizer at 2400 rev./min. The homogenate was centrifuged at 500 *g* for 10 min and the resulting supernatant further centrifuged at 27000 *g* for 10 min. The sedimented material was resuspended in 520 ml of 0.25 M sucrose/10 mM Tes/Tris, pH 7.4, and centrifuged at 27000 *g* for 10 min. The pellet obtained was resuspended in 1.75 ml of 10 mM Tes/1 mM $MgCl_2$, pH 7.4, per g of liver and centrifuged at 288000 *g* for 1 h. The resulting supernatant was the 10 mM Tes extract.

#### Step 2: heat treatment

The 10 mM Tes extract was incubated for 2 min in a metal beaker placed in a boiling-water bath, then centrifuged at 12000 *g* for 10 min to precipitate denatured protein. The supernatant (boiled extract) was collected.

#### Step 3: $(NH_4)_2SO_4$ precipitation

The boiled extract was cooled to 4 °C and 22.9 g of solid $(NH_4)_2SO_4$ per 100 ml was slowly added to achieve 40% saturation. The mixture was stirred at 4 °C for 1 h and centrifuged at 12000 *g* for 15 min. The resulting supernatant was collected and another 30 g of $(NH_4)_2SO_4$/100 ml added to give 85% saturation. After further centrifugation under the same conditions, the precipitate was resuspended in 20 ml of 10 mM Tes/1 mM $MgCl_2$, pH 7.4, and dialysed against the same buffer overnight to remove the remaining $(NH_4)_2SO_4$ and give the solubilized 85% precipitate.

#### Step 4: ion-exchange chromatography

The dialysed fraction was added to 10 ml (wet volume) of DE52 (Whatman) equilibrated with 10 mM Tes/1 mM $MgCl_2$, pH 7.4. The mixture was incubated at room temperature for 15 min with occasional mixing and allowed to settle. After the supernatant was removed by pipette, two consecutive aliquots of 20 ml of buffer containing 0.2 M NaCl, 10 mM Tes and 1 mM $MgCl_2$, pH 7.4, were added to elute the protein. The eluate was collected and concentrated to 1–2 ml by ultrafiltration at 138 kPa using an Amicon YM10 membrane (25 mm diameter) to give the DE52 fraction.

#### Step 5: gel filtration with AcA44

A 1–2 ml volume of the concentration was loaded on to an Ultrogel AcA44 column (Sepracor, Villeneuve la Garenne Cedex, France; 1.6 cm × 100 cm; bed volume 190 ml) equilibrated in PBS at 4 °C. The column was eluted at a flow rate of 4.5 ml/h with PBS. Fractions in the peak region of eluted fluorescence-dequenching activity were pooled to give a final volume of 10–20 ml. The pooled fraction was again concentrated using an Amicon cell fitted with a YM10 membrane to give the AcA fraction.

#### Step 6: gel filtration in FPLC system

The AcA fraction (100 $\mu$l; approx. 100 $\mu$g of protein) was loaded on to a TSK G2000SW column (Pharmacia–LKB; 7.5 mm × 600 mm; bed volume 26 ml) equilibrated with 0.1 M sodium phosphate buffer, pH 6.3. The column was eluted at a flow rate of 12 ml/h with 0.1 M sodium phosphate buffer, pH 6.3. Fractions (0.5 ml) in the peak region of eluted fluorescence-dequenching activity were pooled and concentrated by centrifugation at 4000 *g* for 30 min using a Centricon 10 (Amicon) fitted in an SS-34 rotor (Sorvall) to give the FPLC-purified protein.

### Content-mixing assay

The content-mixing assay was based on the formation of a tightly bound complex between avidin–asialofetuin (avidin–ASF) preloaded into late endosomes and biotinylated polymeric IgA (bpIgA) preloaded into lysosomes and was carried out as previously described [5]. Dense endosomes were prepared from a rat which had received an intravenous injection of approx. 1–3 nmol of avidin–ASF 6 min before killing and were resuspended in cytosol (~ 20 mg of protein/ml). Lysosomes were prepared from a rat which had received an intravenous injection of approx. 50 pmol ($3 \times 10^8$–$4 \times 10^8$ d.p.m.) of $^{125}$I-labelled bpIgA 35–45 min before killing and were resuspended in STM. The content-mixing assay contained 100 $\mu$l of the avidin–ASF-loaded dense endosome suspension, 100 $\mu$l of the $^{125}$I-labelled bpIgA lysosome suspension and 25 $\mu$l of a 1 mg/ml solution of biotinylated insulin or 0.3 mg/ml biocytin. An ATP-regenerating system composed of 10 $\mu$l of an equal mix of 0.8 M phosphocreatine and 4 mg/ml creatine kinase was added and the mixture incubated for 10 min at 37 °C. Subsequent immunoprecipitation of the avidin–biotin complex, counting of radioactivity and calculation of fusion were as previously described [5].

### Phospholipid vesicles (liposomes)

Phosphatidylcholine-containing large unilamellar vesicles (PC-LUV) were prepared as described by MacDonald et al. [14]. Egg PC in chloroform was dried under nitrogen, dehydrated under high vacuum for 3 h and kept at −20 °C until use. The lipid was resuspended at 25 mg/ml in NHE buffer (0.15 M NaCl, 0.1 mM EDTA, 10 mM Hepes, pH 7.0) and freeze–thawed ten times to ensure entrapment of the buffer in the inner space of vesicles formed. The vesicles were extruded through two polycarbonate filters (pore diameter 100 nm) fitted in a mini-extruder (Avestin, Ottawa, Ont., Canada) with 19 passes. The PC-LUV were made fresh each day.

### Fluorescence-dequenching assays using R18-labelled liposomes

Fluorescence-dequenching assays using R18-labelled liposomes were essentially the same as those using labelled endosomes except that the buffer used for R18 loading and subsequent incubation was different and gel-filtration conditions to remove free R18 were altered. The PC-LUV (0.44 $\mu$mol) were mixed at room temperature for 15 min with 0.04 $\mu$mol of R18 (kept at 20 mM in 100% ethanol) in a total volume of 0.5 ml of NHE to achieve 9 mol% concentration of R18 in the total lipid. Free R18 was removed by Sephadex G-50 gel filtration on two consecutive columns (each 0.5 cm × 7 cm); elution was with NHE. This preparation of R18-labelled PC-LUV was sufficient for ten assays. In each assay, 630 nmol of unlabelled PC-LUV were mixed with appropriate samples of lysosomal protein or recombinant human $G_{M2}$ activator protein and the assay was initiated by the addition of 44 nmol of R18-labelled PC-LUV. The assay mixture was made up to 1 ml with STM and fluorescence dequenching measured as described above.

### Antibodies

Polyclonal antibodies were produced in a rabbit by immunization using multiple subcutaneous injection. Protein (100 $\mu$g) in 0.5 ml of PBS was mixed with an equal volume of Freund's complete adjuvant and injected at two to three sites. After 1 month and 2 months, the rabbit was boosted by again injecting the same amount of protein mixed with an equal volume of Freund's incomplete adjuvant. Blood was taken 10 days after the booster injections and the presence of antibodies evaluated by immunoblotting. When required, IgG fractions were purified from rabbit antisera using AffiGel Protein A (Bio-Rad) according to the manufacturer's instructions. Antibodies were affinity-purified on immunoblots as previously described [15].

### Assays of enzyme and protein

$\beta$-Hexosaminidase was assayed as described by Maguire et al. [16]. Protein was determined by measuring the $A_{280}$ or using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, U.S.A.) which produced a coloured product detected at 620 nm.

SDS/PAGE, immunoblotting and densitometry of blots were essentially as described in previous work from this laboratory [17]. N-Terminal amino acid analysis of purified protein was performed after SDS/PAGE using a gel constructed with half the normal volumes of ammonium persulphate and *NNN'N'*-tetramethylethylenediamine to avoid N-terminus blockage. The gel was pre-run for 30 min at 10 mA with the running buffer containing 2 mM mercaptoacetic acid in the cathode reservoir to prevent the N-terminus being blocked. The sample was run on the gel at 9 mA for 15 h, then at 25 mA for 3 h and subsequently electroblotted on to a poly(vinylidene difluoride) membrane (ProBlott; Applied Biosystems). Protein bands were stained with Coomassie Brilliant Blue and destained with 50% methanol/10% acetic acid. The appropriate band was excised and subjected to N-terminal microsequencing by the Microchemical Division at Babraham AFRC Research Institute using an automated Applied Biosystems protein sequencer.

Where appropriate, data are presented as means ± S.E.M. with the number of experiments given in parentheses.

## RESULTS

Incubation of R18-labelled dense endosomes with lysosomes at 47 °C in STM in the presence of ATP resulted in fluorescence dequenching observed as an increase in fluorescence over a time course of 1 h (Figure 1 [5,7]). No increase in fluorescence was

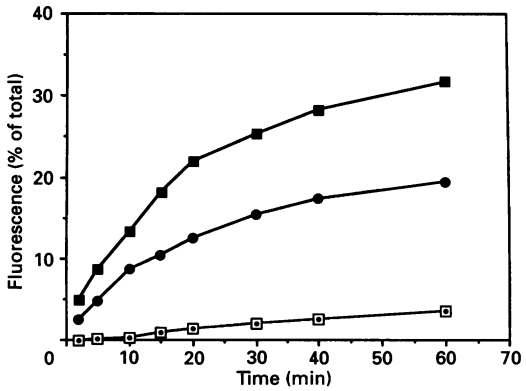


**Figure 1 Fluorescence dequenching of R18-labelled endosomes incubated with lysosomes**

R18-labelled dense endosomes (0.05 mg of protein) were incubated with lysosomes (1 mg of protein) (●) or lysosomal membranes (1 mg of protein) with (■) or without (⊡) the addition of Tes extract of lysosomes (0.3 mg of protein). All assays were performed in STM at 37 °C in the presence of 2 mM ATP. A representative experiment is shown. It should be noted that fluorescence dequenching is proportional to the amount of lysosomes added [7] and that 0.15 mg of Tes extract protein and 0.85 mg of lysosome membrane protein are obtained from 1 mg of lysosome protein.

**Table 1 Summary of the properties of fluorescence-dequenching activity in the 10 mM Tes extract of lysosomes and in cytosol**

| | 10 mM Tes extract of lysosomes | Cytosol |
|---|---|---|
| Acceptor membrane requirement for maximal activity | Yes | No |
| p[NH]ppA (2 mM) inhibition (%) | 65±17 (5) | 9±6 (3) |
| Activity at 20 °C (% of that at 37 °C) | 10–20% | 40–50% |
| Ratio of activity at pH 6.5 to that at pH 7.4 | 2.3 | 1.3 |

observed when R18-labelled endosomes were incubated on their own (results not shown) or when incubated with lysosomal membranes prepared by centrifugation after incubating lysosomes with 10 mM Tes/1 mM $MgCl_2$, pH 7.4 (Figure 1). When the 10 mM Tes extract was added back in the presence of lysosomal membranes, a considerable increase in fluorescence was observed (Figure 1). A study of the properties of the fluorescence-dequenching activity in the 10 mM Tes extract showed that it differed considerably from lipid-transfer proteins present in rat liver cytosol (Table 1). Maximal activity of the Tes extract required the presence of acceptor membranes whereas incubation of R18-labelled endosomes with cytosol resulted in large increases in fluorescence with no requirement for acceptor membranes. The temperature-dependence of fluorescence dequenching by the Tes extract and by cytosol was different, with a reduction in incubation temperature to 20 °C inhibiting the Tes extract much more than the cytosol activity. The pH-dependence was also different, with the Tes extract showing increased activity at acid pH. In contrast with inhibition of the fluorescence-dequenching activity seen on addition of p[NH]ppA to the Tes extract, this non-hydrolysable analogue of ATP had no effect on fluorescence dequenching by cytosol. This result was expected since no lipid-transfer proteins have been reported to show any requirement for a supply of energy.

Since the fluorescence assay for endosome–lysosome interaction was designed to measure membrane fusion and, as described in the Introduction, had many characteristics expected of endosome–lysosome fusion, it was possible that 10 mM Tes treatment of lysosomes was releasing a fusogenic protein(s) detected by its ability to cause fluorescence dequenching of R18-labelled endosomes in the presence of lysosomal membranes. The fluorescence assay was known to be quantitatively dependent on the amount of lysosomes present when whole lysosome preparations were used [7] and in the present study was shown to be quantitatively dependent on the amount of 10 mM Tes extract added when lysosomal membranes were used as acceptors (results not shown). Thus it was possible to use the assay to monitor purification of the lysosomal protein(s) causing fluorescence dequenching.

### Purification of a soluble lysosomal protein causing fluorescence dequenching of R18-labelled endosomes in the presence of lysosome membranes

A 10 mM Tes extract of a postnuclear supernatant showed the same dequenching activity as an extract of purified lysosomes, therefore this preparation was used as the starting material for protein purification rather than lysosomes. Preliminary experiments (described in greater detail in ref. [18]) showed that the protein(s) with fluorescence-dequenching activity was heat-resistant (< 8% inactivation after boiling for 2 min and < 70% inactivation after boiling for 1 h), was more than 70% precipitated by 40–85%-satd. $(NH_4)_2SO_4$ and could bind to DE52 ion-exchange resin and be eluted with a gradient of NaCl (Figure 2a). Gel filtration suggested that the protein had an apparent molecular mass of ~ 25 kDa (Figure 2b). These properties were used to devise a purification protocol which resulted in a 40% yield and ~ 50-fold purification after five steps starting from the 10 mM Tes extract of a postnuclear supernatant (Table 2). A further increase in purification to more than 100-fold was achieved by a final FPLC gel-filtration step (Table 2). SDS/PAGE of fractions from each purification step showed that purification of the fluorescence-dequenching activity coincided with purification of a protein of apparent molecular mass 22 kDa (Figure 3a). Proof that this protein was responsible for the fluorescence-dequenching activity was obtained by demonstrating linearity between specific activity and the percentage of this protein determined by SDS/PAGE in samples from the DE52 and AcA44 purification steps of three independent purifications (Figure 3b). On subsequent SDS/PAGE analysis of the purest samples of the protein, it appeared as a triplet of closely

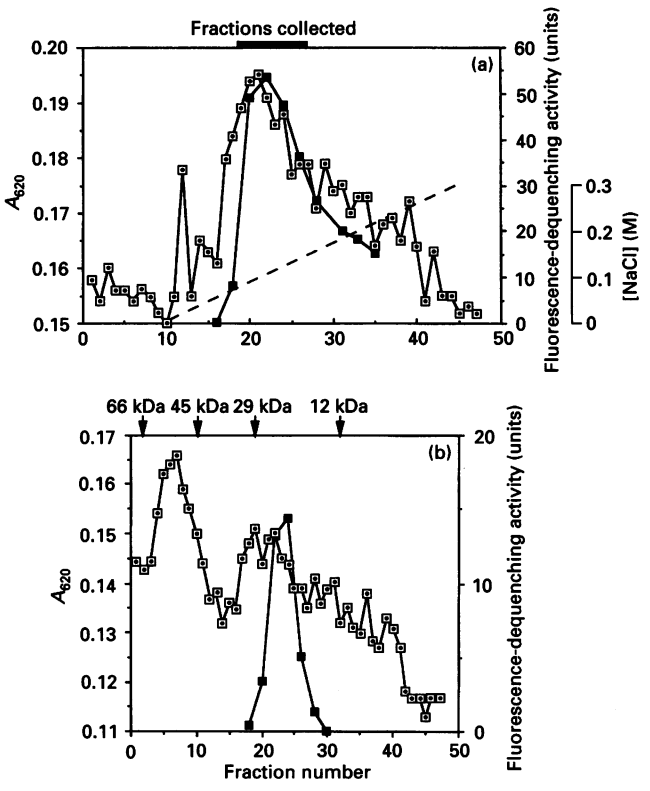


**Figure 2 Column chromatography of fluorescence-dequenching activity in the Tes extract of lysosomes**

(**a**) Elution profile from DE52: 20 ml of an 85%-satd. $(NH_4)_2SO_4$ precipitate of the Tes extract of lysosomes was loaded on to a column (1.6 cm × 5 cm) packed with DE52 equilibrated in 10 mM Tes/1 mM $MgCl_2$, pH 7.4. The first 20 ml was discarded and the column was eluted with a linear gradient of 0–0.3 M NaCl in 10 mM Tes/1 mM $MgCl_2$, pH 7.4, at a flow rate of 11 ml/h. Fractions (1.1 ml) were collected and concentrated using an Amicon ultrafiltration membrane. $A_{620}$ represents protein concentration using a colorimetric assay. ⊡, $A_{620}$; ■, fluorescence-dequenching activity; ——, [NaCl]. (**b**) Gel-filtration profile using Ultrogel AcA44. The concentrated sample produced after eluting the DE52 column was loaded on to a column (1.6 cm × 100 cm) packed with Ultrogel AcA44 equilibrated in PBS. The column was run at a flow rate of 4.5 ml/h with PBS. Fractions (1.85 ml) were collected. The AcA44 column was calibrated with markers of known molecular mass as shown. $A_{620}$ (⊡) represents protein concentration using a colorimetric assay. ■, Fluorescence-dequenching activity.

**Table 2 Purification of a protein with fluorescence-dequenching activity from the 10 mM Tes extract of postnuclear supernatant from eight rat livers**

The activity was determined using the fluorescence-dequenching assay as described in the Experimental procedures section.

| Step | Volume (ml) | Total protein (mg) | Total activity (units) | Specific activity (units/mg of protein) | Purity (fold) | Yield (%) |
|---|---|---|---|---|---|---|
| 1. 10 mM Tes extract | 124 | 131 | 12648 | 96 | 1 | 100 |
| 2. Heat treatment (boiled extract) | 118 | 91 | 12036 | 132 | 1.4 | 95 |
| 3. $(NH_4)_2SO_4$ (85%-satd. precipitate) | 23.5 | 19.5 | 9870 | 506 | 5.3 | 78 |
| 4. Ion exchange (DE52) | 1.1 | 7.3 | 7842 | 1071 | 11 | 62 |
| 5. AcA44 gel filtration | 0.95 | 1.1 | 5059 | 4611 | 48 | 40 |
| 6. FPLC gel filtration | 0.30 | 0.093 | 1033 | 11051 | 115 | 8.2* |

* Only the central peak fractions recovered from the AcA44 gel-filtration step were subjected to FPLC gel filtration.

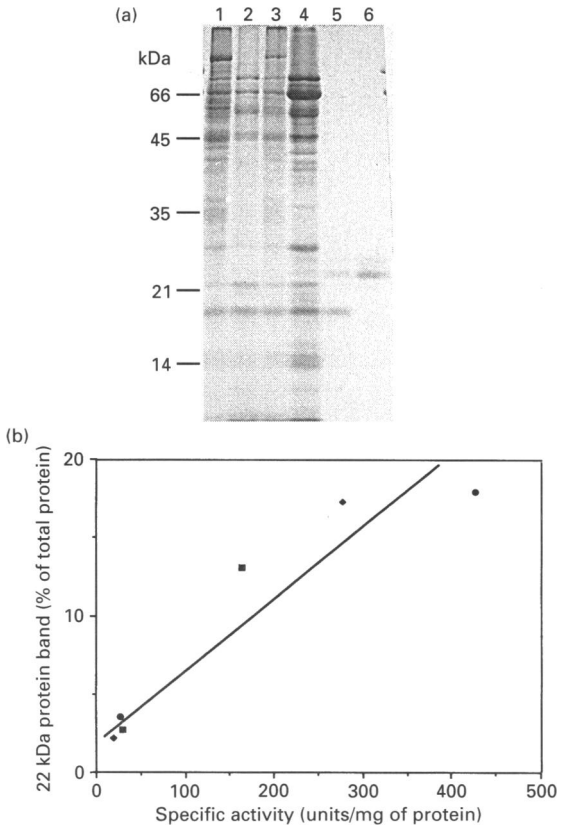


**Figure 3 SDS/PAGE of samples from various steps of the purification of the fluorescence-dequenching activity in the Tes extract of lysosomes**

(**a**) Coomassie Blue-stained SDS/15% polyacrylamide gel. Lane 1, 10 mM Tes extract (30 $\mu$g of protein); lane 2, boiled extract (30 $\mu$g); lane 3, 85%-satd. $(NH_4)_2SO_4$ precipitate (30 $\mu$g); lane 4, DE52 (60 $\mu$g); lane 5, AcA (8 $\mu$g); lane 6, FPLC (6 $\mu$g). The positions of molecular-mass markers are shown. (**b**) Relationship between the amount of 22 kDa protein band and specific activity in the fluorescence-dequenching assay. Data from samples taken after the DE52 and AcA44 steps from three different purifications are shown, each purification being represented by a different symbol.

packed bands of mean molecular mass 22 kDa. In some preparations the upper band of the triplet was most prominently stained and in others the middle band.

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22 kDa protein FSFDNDDEGKDPAVIKSLTL
                ** ** ********* ****
                ***** **************
Human GM2-activator protein FSWDNCDEGKDPAVIRSLTLEPDPIVVPGNVT
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**Figure 4 N-Terminal amino acid sequence of the 22 kDa protein**

Comparison with the N-terminal sequence of human $G_{M2}$-activator protein. Amino acid residues that are identical in the two proteins are denoted by two asterisks, and those residues with conventional substitution are denoted by one asterisk.

## The lysosomal protein with fluorescence-dequenching activity is the rat homologue of human $G_{M2}$-activator protein

The purified protein was subjected to SDS/PAGE, transferred to a poly(vinylidene difluoride) membrane and the uppermost band of the triplet subjected to N-terminal amino acid analysis. Unambiguous assignments were obtained from the first 20 amino acids, and comparison with sequences in the EMBL database showed 85% identity with the N-terminal sequence of the mature form of human $G_{M2}$-activator protein (Figure 4) This is an intralysosomal 22 kDa protein which is one of the glycoprotein cofactors that assist acid hydrolases to degrade the sugar moieties of glycolipids in a stepwise manner in lysosomes and preferentially binds the ganglioside $G_{M2}$ [19–23].

Evidence that all three SDS/PAGE bands of the purified rat lysosomal 22 kDa protein were related to each other and to human $G_{M2}$-activator protein was provided by immunoblotting using an antiserum raised against the purified protein. This antiserum reacted with the Tes extract of lysosomes and the purified rat protein but not with lysosomal membranes or endosomes (Figure 5a). It also reacted with recombinant human $G_{M2}$-activator protein (Figure 5b), providing further evidence that the rat homologue of the human protein had been purified. Antibodies immunoaffinity-purified on the middle SDS/PAGE band of the purified rat protein reacted with all three bands present in the purified protein (Figure 5c). Coomassie Blue staining of the gels showed that the upper band was most similar in molecular mass to the recombinant human $G_{M2}$-activator protein (Figure 5d).

The availability of the human recombinant $G_{M2}$-activator protein allowed us to demonstrate that, when added to R18-labelled endosomes and lysosomal membranes, it was able to cause fluorescence dequenching (Figure 6) in an analogous manner to the purified rat lysosomal protein. The presence of p[NH]ppA caused some inhibition of the increase in fluorescence.

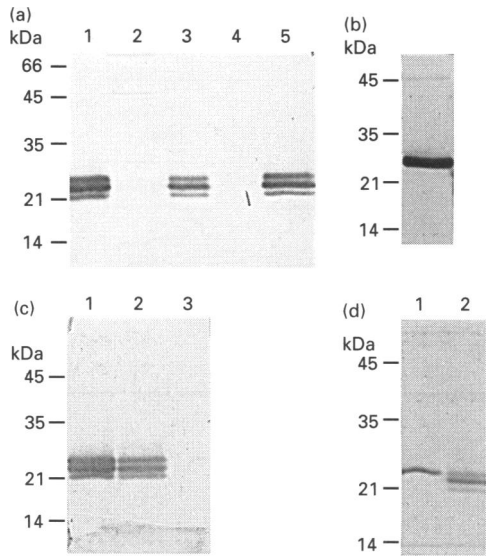


**Figure 5 SDS/PAGE and immunoblotting of $G_{M2}$-activator proteins**

(**a**) Immunoblots with antiserum raised against the purified 22 kDa protein. Lane 1, FPLC-purified 22 kDa protein (0.4 $\mu$g); lane 2, dense endosomes (12 $\mu$g); lane 3, lysosomes (12 $\mu$g); lane 4, lysosomal membranes (12 $\mu$g); lane 5, Tes extract of lysosomes (12 $\mu$g). (**b**) Immunoblot of recombinant human $G_{M2}$-activator protein with antiserum raised against the purified 22 kDa protein (0.6 $\mu$g of protein was loaded on the gel). (**c**) Immunoblots with antibodies affinity-purified on the middle band of the 22 kDa protein. Lane 1, FPLC-purified 22 kDa protein (0.4 $\mu$g); lane 2, Tes extract of lysosomes (12 $\mu$g); lane 3, lysosomal membranes (12 $\mu$g). (**d**) Coomassie Blue stain of SDS/polyacrylamide gel. Lane 1, recombinant human $G_{M2}$-activator protein (0.9 $\mu$g); lane 2, FPLC-purified 22 kDa protein (1.2 $\mu$g). The positions of molecular-mass markers are shown. The antiserum and affinity-purified antibodies were used at 1:1000 dilution.

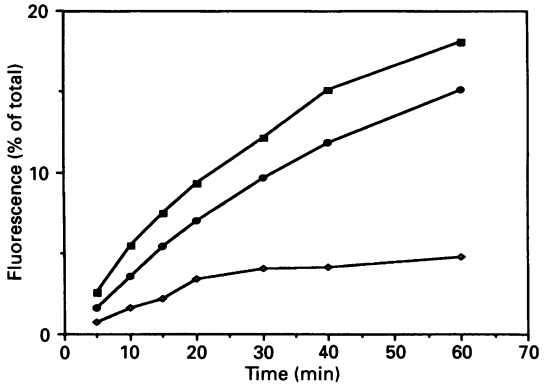


**Figure 6 Fluorescence dequenching of R18-labelled endosomes incubated with lysosomal membranes and recombinant human $G_{M2}$-activator protein**

R18-labelled dense endosomes (0.05 mg of protein) were incubated with lysosomal membranes (0.5 mg of protein) and 2.25 mg of recombinant human $G_{M2}$-activator protein with (●) or without (■) the addition of 2 mM p[NH]ppA. Incubation of R18-labelled dense endosomes with lysosomal membranes alone is also shown (◇). All assays were performed in STM at 37 °C. A representative experiment is shown.

## The rat homologue of human $G_{M2}$-activator protein is not involved in endosome–lysosome membrane fusion

Human $G_{M2}$-activator protein has been shown to be capable of acting as a lipid-transfer protein [24,25], but for this reason alone one could not rule out the possibility that the rat

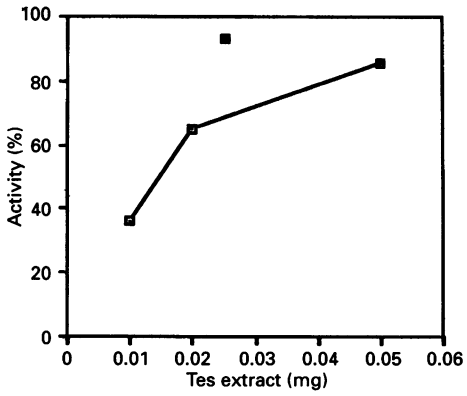


**Figure 7 Effect of antibodies to the 22 kDa protein on fluorescence-dequenching and content-mixing assays**

⊡, Inhibition of fluorescence dequenching. R18-labelled dense endosomes (0.05 mg of protein) were incubated in a total volume of 1 ml of STM for 10 min at 37 °C with lysosomal membranes (0.64 mg of protein) plus Tes extract of lysosomes (10–50 $\mu$g of protein) plus 20 $\mu$g of IgG from the antiserum to the 22 kDa protein. ■, Effect on content-mixing assay. Avidin–ASF-loaded dense endosomes (0.67 mg) were incubated in a total volume of 235 $\mu$l for 10 min at 37 °C with $^{125}$I-bpIgA-loaded lysosomes (0.32 mg) in the presence of cytosol plus 20 $\mu$g of IgG from the antiserum to the 22 kDa protein. In both experiments shown, 100% activity values were obtained under the same incubation conditions in the presence of 20 $\mu$g of non-immune IgG. The content-mixing assay mixture contained a similar ratio of IgG to total protein as the fluorescence-dequenching assay, but the IgG concentration was 4-fold higher. It is expected that 0.32 mg of lysosomes would give ~ 70 $\mu$g of Tes extract. Assuming a 40% leak of the 22 kDa protein, the content-mixing assay should have equivalence to ~ 25 $\mu$g of Tes extract, under the conditions of the fluorescence-dequenching assay, as shown. Representative experiments are shown with data points representing the mean of two readings.

homologue was involved in endosome–lysosome fusion events in the cell-free system established for the present experiments. Evidence that it could not be acting from its site of normal residence within the lysosome was provided by manipulation of intralysosomal pH. Using published methods [26,27], we measured the intralysosomal pH of fluorescein isothiocyanate–dextran-loaded lysosomes isolated by the methods used in the present study. Different preparations showed a pH in the range 5.5–5.9, which increased on incubation in STM at 37 °C. Addition of 2 mM ATP drove the pH down by about 0.2 units whereas addition of 2 mM p[NH]ppA raised it by about 0.3 unit, consistent with the presence of a protein-pumping ATPase in the lysosomal membrane. Addition of 0.5 $\mu$M nigericin (which mediates electroneutral exchange of $K^+$ for protons thereby raising intralysosomal pH [28]) in the presence of 50 mM KCl raised intralysosomal pH by about 1 unit yet had no effect on a content-mixing assay for endosome–lysosome fusion [5]. This effectively rules out a role for rat intralysosomal $G_{M2}$-activator in fusion, as the activity of the purified protein in the fluorescence-dequenching assay was 50–100% greater at pH 5.5 than at pH 6.5, consistent with earlier observations on the effect of pH on the fluorescence-dequenching activity in the lysosomal Tes extract (Table 1).

If $G_{M2}$-activator was not acting from inside the lysosome, then it must have been released from lysosomes to cause fluorescence dequenching when R18-labelled endosomes were incubated with lysosomes in this and previous studies [5,7]. At first sight this is surprising, as the lysosomal preparation used in all of these studies shows less than 10% leakage of $\beta$-hexosaminidase within 2 min of resuspension in STM and only up to 20% leakage after incubation in STM at 37 °C for 1 h, in agreement with previous observations [13]. However, assay of the release of fluorescence-

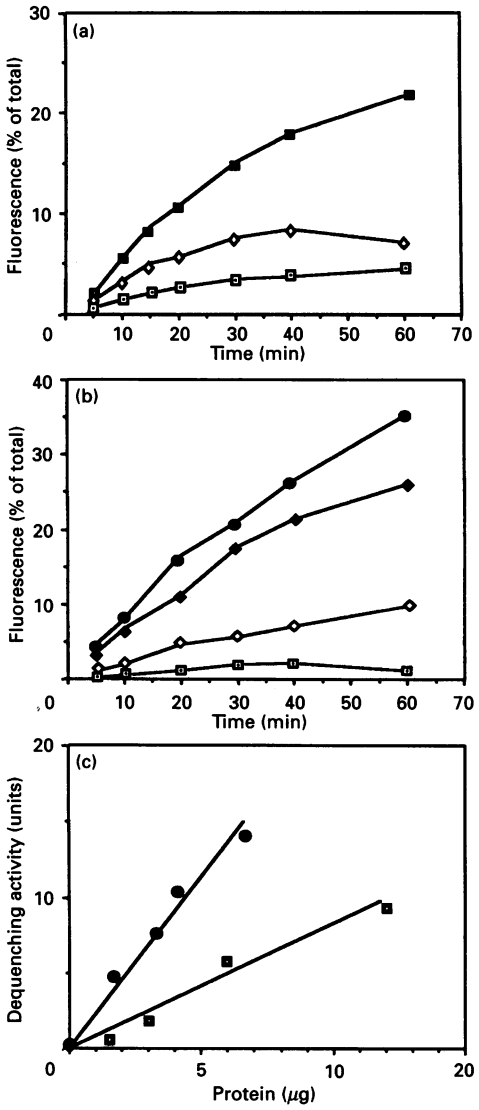


**Figure 8 Fluorescence dequenching of R18-labelled PC-LUV liposomes**

(**a**) Effect of Tes extract of lysosomes. R18-labelled PC-LUV (44 nmol) were incubated in a total volume of 1 ml of STM at 37 °C plus unlabelled PC-LUV (630 nmol) with (■) or without (◇) the addition of Tes extract of lysosomes (70 μg of protein). R18-labelled PC-LUV incubated alone are also shown (⊡). (**b**) Effect of $G_{M2}$-activator protein. Incubation conditions were as in (**a**) but instead of Tes extract, FPLC-purified 22 kDa protein (◆ ; 0.84 μg) or recombinant human $G_{M2}$-activator protein (● ; 3 μg) was added. R18-labelled PC-LUV incubated alone (⊡) and with unlabelled PC-LUV (◇) are also shown. (**c**) Comparison of fluorescence-dequenching activity obtained with FPLC-purified 22 kDa protein (●) or recombinant human $G_{M2}$-activator protein (■) in the liposome assay.

dequenching activity from the same lysosomes showed that more than 36% was released within 2 min of resuspension in STM. This presumably reflects the low molecular mass of the $G_{M2}$-activator protein compared with that of β-hexosaminidase.

The polyclonal antiserum raised against the rat homologue of the human $G_{M2}$-activator protein was used to test whether the released protein had a fusogenic function when endosomes were incubated with lysosomes *in vitro*. When a fixed amount of immune IgG was added to an assay system containing R18-labelled endosomes, lysosomal membranes and different amounts of lysosomal Tes extract, inhibition of fluorescence dequenching was observed (Figure 7). The extent of inhibition was inversely

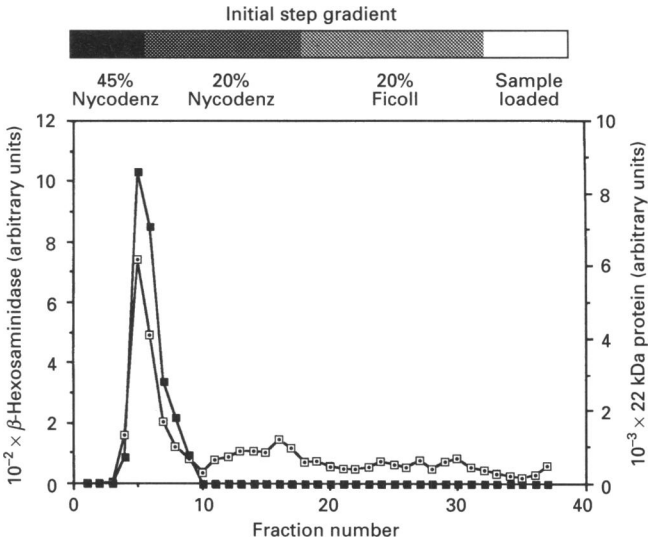


**Figure 9 Distribution of 22 kDa protein and β-hexosaminidase on an isopycnic Nycodenz gradient**

Rat liver postmitochondrial supernatant (7 ml) was loaded on to a step gradient consisting of 4 ml of 45% (w/v) Nycodenz, 14 ml of 20% (w/v) Nycodenz, 14 ml of 20% (w/v) Ficoll [29]. After centrifugation for 206000 *g* for 1 h in a Vti 50 rotor (Beckman), 1 ml fractions were collected. They were assayed for β-hexosaminidase (⊡) and for the rat homologue of human $G_{M2}$-activator protein (■) by SDS/PAGE, immunoblotting and densitometry. A representative experiment is shown.

proportional to the amount of Tes extract present. In contrast, addition of a similar amount of immune IgG to an endosome–lysosome content-mixing assay had no effect despite there being more than enough antibody present to cause inhibition if leaked $G_{M2}$-activator protein had a function in the fusion process (Figure 7). There was also no increase in content mixing on addition of exogenous FPLC-purified rat $G_{M2}$-activator protein (results not shown).

### The rat homologue of human $G_{M2}$-activator protein and recombinant human $G_{M2}$-activator protein can transfer R18 between PC liposomes

As the rat homologue of $G_{M2}$-activator protein did not cause membrane fusion but did cause fluorescence dequenching when R18-labelled endosomes were incubated with lysosome membranes, it was presumably acting as a lipid-transfer protein. Human $G_{M2}$-activator protein has been shown to be capable of transferring $G_{M2}$ from donor to acceptor liposomes [24,25], although the specificity for glycolipids in the transfer is not limited to $G_{M2}$ and $G_{M1}$ can also be transferred [23]. It has little or no affinity for phospholipids such as PC [19] and does not transfer this lipid [24]. In the present study, although it was possible that $G_{M2}$-activator protein caused fluorescence dequenching by transferring gangliosides between the endosome and lysosomal membranes, this seemed unlikely because in the endosome-plus-lysosome system such glycolipids would be restricted to the lumenal leaflet of the phospholipid bilayer. An alternative possibility is that the $G_{M2}$-activator protein showed specificity for R18 and therefore transferred and diluted the fluorescent probe. This was tested using a PC-LUV liposome system with either the rat homologue of human $G_{M2}$-activator protein or recombinant human protein added to a mixture of donor PC-LUV liposomes labelled with self-quenching con-

centrations of R18 and acceptor unlabelled PC-LUV liposomes. Both the rat homologue and recombinant human $G_{M2}$-activator protein caused fluorescence dequenching (Figures 8a and 8b) in a dose-dependent manner (Figure 8c), allowing us to calculate that the specific activity of the purified rat homologue assayed in this system was approximately double that of the recombinant human protein. Interestingly, the presence of p[NH]ppA inhibited fluorescence dequenching caused by both proteins in the liposome system.

### The rat homologue of human $G_{M2}$-activator protein is localized in rat liver lysosomes

The antiserum raised against the purified rat homologue of human $G_{M2}$-activator protein was not suitable for immunofluorescence microscopy. However, the ability to immunoblot subcellular fractions with this antiserum allowed comparison of the distribution of the rat homologue of human $G_{M2}$-activator protein with the distribution of $\beta$-hexosaminidase after isopycnic density-gradient centrifugation (Figure 9). The Nycodenz gradient used achieves maximum separation of lysosomes from endosomes [29]. The distribution of the immunoblotted 22 kDa bands clearly demonstrated that the protein was localized in lysosomes. None could be detected at non-lysosomal positions on the gradient.

## DISCUSSION

Fluorescence assays to measure membrane fusion have achieved wide popularity because they are simple, rapid, can cope with relatively high sample numbers and can measure the actual fusion event rather than a downstream occurrence such as content mixing between two vesicles. R18 is often used for labelling membranes because it is self-quenching and can be readily incorporated into lipid bilayers by incubation with membranes or vesicles [6,30]. An increase in fluorescence is observed as a result of dequenching when fusion occurs with an acceptor membrane. R18 has thus been used in situations as diverse as detecting virus–cell fusion [31–33] and investigating exocytic fusion events [34]. The advantages of R18 as a labelling probe include the possibility of investigating the kinetics of biological membrane fusion and the ability to obtain visual images of fusion as a result of the R18 spreading into the acceptor membrane [35]. Some difficulties have been encountered in membrane-fusion assays based on the measurement of dequenching of R18. These include non-random distribution of R18 within the phospholipid bilayer [34], interaction with proteins [31], effects of incorporated R18 on membrane properties [36] and non-specific probe transfer due to random membrane collisions in the absence of fusion [32].

In the present study, we have demonstrated that the increase in fluorescence observed when rat liver lysosomes are mixed with endosomes labelled with self-quenching concentrations of R18 is not due to membrane fusion, but can be accounted for by the leakage of a lysosomal protein that acts as an R18-transfer protein. All of the observations that lend support to the view that the R18 assay measures true fusion [5,7] can now be explained by the actions of the rat homologue of human $G_{M2}$-activator protein. The similarity to other assays of the time course and temperature-dependence was presumably fortuitous. It may be accounted for by intrinsic properties of the phospholipid bilayer required for fusion or lipid-transfer events. The specificity of lysosomes as acceptors and the dependence on the amount of lysosomes added [7] were due to the great majority of intracellular $G_{M2}$-activator protein being located in this organelle. Indeed, experiments using the purified rat homologue of $G_{M2}$-activator protein showed that any subcellular membrane fractions could be used as donor and acceptor membranes in R18 fluorescence-dequenching assays (results not shown). Inhibition by p[NH]ppA was previously interpreted as a requirement for hydrolysable ATP as an energy source [5], but appears to be an intrinsic feature of the function of $G_{M2}$-activator protein when transferring R18 between membranes. The reason for this remains unclear, especially as the published sequence of human $G_{M2}$-activator protein indicates no obvious nucleotide-binding motifs [21] and in its role in ganglioside degradation it functions without an energy requirement [24,25]. The presence of ATP in dequenching assays using R18-labelled endosomes and lysosomes was thought to be necessary for maximal activity [7], but it has been difficult to reproduce this observation which was probably the result of imperfect buffering of ATP solutions, allowing the pH to fall sufficiently to cause increased activity of the $G_{M2}$-activator protein.

The size (22 kDa), N-terminal amino acid sequence, intracellular location and the cross-reaction of polyclonal antibodies all indicated that the protein isolated in the present study responsible for fluorescence dequenching was the rat homologue of human $G_{M2}$-activator protein. In the final FPLC-purified material, three bands were visible on SDS/PAGE, the relative intensity of each differing in independent preparations. This is consistent with data on purified human $G_{M2}$-activator protein in which three major glycosylated forms have been identified of molecular masses 21, 22 and 23 kDa [37].

$G_{M2}$-activator protein is required for $\beta$-hexosaminidase hydrolysis of $G_{M2}$ and other gangliosides [38]. It is thought to extract the glycolipids from the membrane bilayer to present to the enzyme [23]. It is also known that $G_{M2}$-activator protein can act as a ganglioside-transfer protein [24], but in the present study it was a surprise to find that it could transfer R18 between membranes including PC-LUV liposomes. R18 has a molecular structure consisting of a fatty acyl chain with a water-soluble head group (rhodamine B chloride). Presumably this structure is sufficiently similar to a glycolipid to allow recognition by $G_{M2}$-activator protein, which is thought to possess a hydrophobic binding pocket for fatty acyl chains made up of three amphipathic $\alpha$-helices [21] and a separate binding site for terminal sugar(s) [23]. $G_{M2}$–$G_{M2}$-activator-protein complexes are easily formed but hard to isolate if the ganglioside is offered in the form of micelles [24,25]. These observations are relevant to the present experiments in which fluorescence dequenching was observed with little fluorescence (R18) being detected in the high-speed supernatant of the assay mixture (results not shown). A possible mode of action of $G_{M2}$-activator protein in causing fluorescence dequenching is extraction of R18 from one membrane and transfer to another through the formation of a water-soluble complex with the probe. An alternative mechanism is the formation of a short-lived complex of $G_{M2}$-activator protein and R18 associated with the donor membrane, the R18 probe being transferred to the acceptor membrane when membrane collision occurs. These mechanisms are analogous to those proposed for phospholipid-transfer proteins. PC-transfer protein and phosphatidylinositol-transfer protein form water-soluble complexes with the respective lipids to mediate their transfer *in vitro* [11]. In contrast, non-specific lipid-transfer protein can form a complex only in the presence of vesicles, and the complex is difficult to isolate [11,39].

In the present experiments the properties of $G_{M2}$-activator proteins were compared with those of cytosolic lipid-transfer proteins by adding cytosol to R18-labelled endosomes and lysosomal membranes. In the latter case, fluorescence de-

quenching occurred in an ATP-independent manner and was relatively insensitive to alterations in temperature and pH, consistent with the known properties of lipid-transfer proteins in the cytosol [8–10,40,41]. It was found that the presence of unlabelled membranes inhibited the dequenching (results not shown). Hence optimal dequenching induced by cytosol did not require acceptor membranes, in contrast with the lipid-transfer action of $G_{M2}$-activator protein. In this system, dequenching may have been caused by the transfer of lipids present on cytosolic phospholipid-transfer proteins [11] to the endosome membranes containing the R18. We cannot rule out an alternative possibility that the increase in fluorescence may be due to the cytosolic transfer proteins solubilizing some of the R18 probe. However, centrifugation studies showed that more than 90% of the R18 remained associated with membranes in the dequenching system with cytosol.

It is clear from the experiments reported above that $G_{M2}$-activator protein is not required for and does not cause fusion between endosomes and lysosomes *in vitro*. However, $G_{M2}$-activator protein does have an important function in the endocytic pathway. This is most clearly illustrated by the accumulation of $G_{M2}$ in lysosomes in the pathological condition called variant AB gangliosidosis in which $G_{M2}$ activator is functionally deficient [19,23,42]. The protein may be particularly important in the first steps of degradation of intraendosomal and intralysosomal vesicles formed by inward budding of the limiting membrane into the organelle lumen [23,43]. It is noticeable that the lysosomes of patients with a defect in one of the enzymes or activators responsible for the degradation of gangliosides are enlarged and enriched with membranous structures [23,44]. Measurement of the endocytosis and lysosomal incorporation of the fluid-phase marker horseradish peroxidase in normal and AB variant fibroblasts has shown no quantitative differences, consistent with $G_{M2}$-activator protein not being required for efficient fusion of lysosomal and endosomal membranes *in vivo* [45].

The results of the present experiments strongly suggest that the fluorescence dequenching observed in an assay system consisting of R18-labelled endosomes and lysosomes was caused by leakage of the rat homologue of human $G_{M2}$-activator protein which acted as an R18-transfer protein. Although R18 fluorescence-dequenching assays may still prove useful in detecting membrane fusion in other systems, it is clear that they cannot be used for intracellular membrane-fusion assays in the presence of $G_{M2}$-activator protein or cytosol. It is worth noting that, despite the major residence of $G_{M2}$-activator protein in lysosomes, it may also be present at various sites throughout the secretory pathway and from early endosomes to lysosomes along the endocytic pathway, as some is secreted into the medium when fibroblasts are cultured [46] and it is thought to be delivered to lysosomes by the mannose-6-phosphate receptor [23]. Even in the case of viral fusion with cell membranes, it is worth considering artifacts that may occur as a result of the presence of $G_{M2}$-activator protein in the endocytic pathway. In recent experiments [31], it was reported that non-specific dequenching not due to membrane fusion was observed after R18-labelled influenza virus or Semliki Forest virus was delivered to endocytic compartments in MDCK cells or BHK-21 cells respectively. Both viruses enter cells by fusion with cell membranes through low-pH-induced conformational change in their respective fusion proteins [47]. Even in the presence of reagents that prevent acidification of endosomes/lysosomes, such as $NH_4Cl$ or chloroquine, a prolonged increase in fluorescence was observed to varying degrees [31]. The involvement of some cellular proteins capable of removing R18 from the virus was suspected and $Ca^{2+}$-activator protein must now be regarded as a candidate.

Cell-free investigation of ligand delivery from endosomes to lysosomes and the determination of proteins and other molecules required for this process may now be taken forward using density-gradient centrifugation assays to detect organelle association and content-mixing assays to detect membrane fusion [5]. The observation that $G_{M2}$-activator protein shows specificity of binding to R18 and is the major lysosomal protein responsible for R18 dequenching in fluorescent endosome–lysosome fusion assays demonstrates the limits of usefulness of these assays.

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