Fluoroaluminate treatment of rat liver microsomes inhibits GTP-dependent vesicle fusion

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1. Inhibition of GTP-dependent membrane fusion of rat liver microsomes requires preincubation of the membranes with GDP (17 μ M) and relatively high Mg^{2+} concentration (0.5 mM) as well as $AlCl_3$ (30 μ M) and KF (5 mM). Preincubation is required for maximal inhibition (75%). 2. Vesicle fusion in rat liver microsomes has been demonstrated in the absence of polyethylene glycol (PEG). Further, inhibition by AlF_4^- of GTP-dependent vesicle fusion in the absence of PEG has been demonstrated. 3. Under similar preincubation conditions AlF_4^- can bring about inhibition (80%) of the high-affinity PEG-stimulated GTPase activity in rat liver microsomes, previously described by Nicchitta, Joseph & Williamson [(1986) FEBS Lett. 209, 243–248]. 4. Preincubation of small- M_r GTP-binding proteins (Gn proteins) on nitrocellulose strips with GDP (20 pM), $AlCl_3$ (30 μ M) and KF (5 mM) results in inhibition of binding of guanosine 5'-[γ - ^{35}S]thiotriphosphate to Gn proteins. The extent of inhibition of this binding differs for different Gn proteins.

INTRODUCTION

Previously we have reported that in rat liver microsomal fractions (microsomes) $Ins(1,4,5)P_3$ -stimulated Ca^{2+} release is greatly increased by micromolar concentrations of GTP, in the presence of poly(ethylene glycol) (PEG) (Dawson, 1985; Dawson *et al.*, 1986). Under these conditions GTP mediates slow but extensive Ca^{2+} mobilization (Dawson *et al.*, 1987). We have shown that these levels of GTP can induce fusion of rat liver microsomes, using the techniques of electron microscopy, light-scattering analysis (Dawson *et al.*, 1987) and fluorescence-resonance energy transfer (Comerford & Dawson, 1988, 1989). Similarities between GTP-dependent Ca^{2+} release and vesicle fusion in relation to PEG requirement, Mg^{2+} requirement, GTP requirement, sensitivity to guanosine 5'-[γ -thio]triphosphate (GTP[S]) and to limited proteolysis have led us to suggest that GTP-dependent Ca^{2+} mobilization in rat liver microsomes may be due to a change in vesicle permeability to Ca^{2+} , accompanying vesicle fusion (Dawson & Comerford, 1989).

We have suggested that one or more of the small- M_r GTP-binding proteins of rat liver microsomes are likely candidates to be involved in the mechanism of fusion of rat liver microsomal vesicles (Comerford & Dawson, 1989). These proteins are termed Gn proteins and, as defined by Bhullar & Haslam (1987), are polypeptides which, after SDS/PAGE and Western transfer to nitrocellulose, retain the ability to bind GTP with high specificity and affinity. In the context of the present paper, we include in the definition of Gn proteins all the small- M_r GTP-binding proteins (M_r 20000–30000) in the *ras* and *ras*-related families. We have shown that at least two of these Gn proteins, of M_r 21900 and 24100, are sensitive to proteolytic digestion (Comerford & Dawson, 1989) and that the sensitivity to proteolysis correlates well with the inhibitory effects of proteolysis on GTP-dependent Ca^{2+} release and fusion.

Much work, which has made use of mammalian and yeast cell-free systems or mutations in the *sec* or *ypt* genes of yeast, has shown that GTP binding and/or hydrolysis is required in intracellular membrane-trafficking processes (Melançon *et al.*, 1987; Beckers & Balch, 1989; Baker *et al.*, 1988). Further, the

demonstration that the products of the *sec 4* and *ypt 1* genes are Gn proteins (Goud *et al.*, 1988; Bacon *et al.*, 1989) has led to the suggestion that these proteins may be involved in the mechanisms of intracellular membrane trafficking. Various models have been proposed to describe how these GTP-binding proteins may be involved (Bourne, 1988; Rothman & Orci, 1990; Bourne *et al.*, 1990), which envisage GTP-bound proteins as markers for the recognition and/or initiation of fusion between 'donor' and 'acceptor' compartments of the secretory pathway. Further, these models envisage a cycle of GTP/GDP exchange which switches the GTP-binding protein between active and inactive states.

It is possible that the GTP-dependent fusion and Ca^{2+} release which we have observed in rat liver microsomes may be functionally and mechanistically similar to the involvement of GTP in intracellular membrane-trafficking processes. In a variety of systems, treatment with $AlCl_3$ and KF has been shown to inhibit intracellular membrane trafficking (Melançon *et al.*, 1987; Beckers & Balch, 1989; Baker *et al.*, 1988). We have therefore investigated the effects of AlF_4^- on GTP-dependent fusion in rat liver microsomal vesicles. In this paper we present evidence which demonstrates that, under experimental conditions which have been found to favour interactions of AlF_4^- with other GTP-binding proteins (i.e. relatively high free Mg^{2+} concentration and the presence of non-inhibitory levels of GDP), AlF_4^- is able to inhibit GTP-dependent fusion in rat liver microsomes, can inhibit high-affinity PEG-stimulated GTPase activity and can also inhibit GTP[^{35}S] binding by Gn proteins after transfer to nitrocellulose.

EXPERIMENTAL

Materials

Dithiothreitol, ATP and GTP were supplied by BCL, Lewes, Sussex, U.K. Fluorescent probes R18 and F18 were obtained from Molecular Probes, Eugene, OR, U.S.A. Adenosine 5'-[β -imido]triphosphate (p[NH]ppA) was obtained from Sigma Chemical Co., Poole, Dorset, U.K. GTP[^{35}S] (1320 Ci/mmol) was obtained from New England Nuclear, Du Pont (U.K.) Ltd.,

Abbreviations used: PEG, poly(ethylene glycol); average M_r 8000; GTP[S], guanosine 5'-[γ -thio]triphosphate; R18, octadecyl-rhodamine B (chloride salt); F18, 5-(*N*-octadecanoyl)-aminofluorescein; Gn proteins, small- M_r GTP-binding proteins of the *ras* or *ras*-like families detected on nitrocellulose blots after SDS/PAGE and electrotransfer; p[NH]ppA, adenosine 5'-[β -imido]triphosphate; AlF_4^- , used here to describe the active fluoroaluminate complex under the experimental conditions of $AlCl_3$ (30 μ M) and KF (5 mM).

Stevenage, Herts., U.K. Other chemicals used were of the highest purity available.

Microsomal preparation

Rat liver microsomes ('36000 g' fraction) were prepared as described by Dawson & Irvine (1984) from fed male rats. Protein concentrations were determined by the method of Lowry *et al.* (1951).

Effect of AlF_4^- on GTP-dependent microsomal vesicle fusion

The basic procedures used for probe incorporation and fluorescence measurement were essentially as detailed by Comerford & Dawson (1988, 1989). Briefly, this was as follows: microsomal membranes (2–2.5 mg of protein) were suspended in a volume of 0.75 ml containing 150 mM-sucrose, 50 mM-KCl, 10 mM-Hepes/KOH (pH 7.0) and 2 mM-dithiothreitol. The suspension was then split into two equal halves: R18 (1.2 μl of 3.4 mM stock solution) was added to one half and F18 (1.2 μl of 2 mM stock solution) was added to the other. In both cases, the probes were added while mixing the suspension vigorously on a vortex mixer. The suspensions were incubated at 30 °C for 5 min to allow incorporation of probes into the membranes of the microsomal vesicles, after which time the two halves were recombined with thorough mixing and added to the fluorimeter cell, together with 0.75 ml of 150 mM-sucrose/50 mM-KCl/10 mM-Hepes/KOH (pH 7.0)/10% (w/v) PEG/1 mM-MgCl₂ to give a final volume of 1.5 ml containing 5% PEG and 0.5 mM-MgCl₂. The microsomes were then incubated at 30 °C for 10 min, after which vesicle fusion was initiated by addition of GTP to a final concentration of 0.67 mM. When required, GDP (17 μM), AlCl₃ (30 μM) and KF (5 mM) were present in the fluorimeter cell at the indicated final concentrations. Experiments carried out to determine the effect of AlF_4^- on microsomal vesicle fusion in the absence of PEG required quite different conditions. In the preincubation with probes, microsomes (20 mg of total protein) were incubated with R18 (10 μl of 3.4 mM) and F18 (10 μl of 2 mM) as described above and, after recombining and mixing, these were added into the fluorimeter cell together with 0.75 ml of 150 mM-sucrose/50 mM-KCl/10 mM-Hepes/3 mM-MgCl₂/123 μM -GDP, with or without 60 μM -AlCl₃/10 mM-KF (final concentrations in the fluorimeter cell were thus 1.5 mM-MgCl₂, 66 μM -GDP, 30 μM -AlCl₃ and 5 mM-KF). After incubation for 10 min, GTP at a final concentration of 2 mM was added. Variations in individual experimental conditions are detailed in the appropriate Figure legends. In all experiments energy transfer from F18 and R18 was measured, in a Shimadzu RF 5000 spectrofluorimeter system, by monitoring the fluorescence increase at 595 nm with an excitation wavelength of 460 nm.

Determination of GTPase activity

The basic procedure for determination of PEG-stimulated GTPase activity has been previously detailed by Nicchitta *et al.* (1986), and basically this was as follows. Microsomes (final concn. 1.5 mg/ml) were incubated in 150 mM-sucrose/50 mM-KCl/10 mM-Hepes/KOH (pH 7.0)/5% PEG/1 mM-dithiothreitol/1.4 mM-MgCl₂/20 μM -GDP, and when present 30 μM -AlCl₃ and 5 mM-KF, at 30 °C for 9 min, after which p[NH]ppA at a final concentration of 3 mM was added. GTPase activity was initiated 1 min after addition of p[NH]ppA, by adding 1 μM -GTP containing 0.5–1 μCi of [γ -³²P]GTP. The reaction was stopped by removal of samples (30 μl), which were added to Stop solution (270 μl) which contained 5% (w/v) charcoal/1% PEG/10 mM-H₃PO₄. The sample was vortexed-mixed and stored on ice. Samples were centrifuged (12000 g, 2 min), and portions of the supernatants (150 μl) were removed

and used for determination of [³²P]P_i by scintillation counting. Parallel blank incubations, which were as above, but without microsomes, were run for each condition and subtracted as appropriate.

Effect of AlF_4^- on GTP binding by Gn proteins

Microsomal proteins were prepared for, and resolved by SDS/PAGE (16%-acrylamide resolving gels) as previously described (Comerford & Dawson, 1989). Microsomal proteins were applied uniformly to the gel in a single well (5 cm in width). Polypeptides were transferred from gels to nitrocellulose by electroblotting with an LKB Novablot dry-blotter (100 mA, 1 h at room temperature) with 50 mM-Tris (pH 8.5)/384 mM-glycine/20% (v/v) methanol as the current carrying transfer buffer. Gels were pre-equilibrated in this buffer for 10–15 min before electrotransfer of the proteins. GTP binding by Gn proteins was determined by using GTP[³²S] and was essentially as described by Comerford *et al.* (1989) with the following modifications. The nitrocellulose corresponding to the 5 cm microsomal lane was cut into thin strips (5 mm wide) and preincubated in multipartitioned trays, containing incubation buffer [50 mM-Tris/HCl (pH 7.5)/1 mM-dithiothreitol/0.3% (v/v) Tween 20/12 μM -MgCl₂] containing GDP (20 pM final concn.), AlCl₃ (30 μM final concn.) or KF (5 mM final concn.), as required. After 9 min preincubation, ATP was added to a final concentration of 10 μM (ATP was added to block the non-specific binding sites, as detailed by Drøbak *et al.*, 1988); 1 min after ATP addition GTP[³²S] (1–2 $\mu\text{Ci}/\text{ml}$) was added.

Incubation of the nitrocellulose, washing, drying and detection of labelled polypeptides was as previously described (Comerford *et al.*, 1989). Even transfer of protein across the 5 cm lane was established by staining the individual strips with Amido Black (Hancock & Tsang, 1983), after determination of GTP binding.

RESULTS

Vesicle fusion has been measured in the experiments described here by the fluorescence-energy transfer technique which we have used previously (Comerford & Dawson, 1988, 1989). This method measures the initial rate of vesicle fusion, but does not give an accurate measurement of extent, since once the probes are randomly distributed in the membranes further fusion gives no further fluorescence change. Under normal experimental conditions (e.g. Fig. 1a) the final level of fluorescence reached is very similar to the maximum possible, obtained by random probe mixing with membranes. Fig. 1 shows a series of typical traces of fluorescence changes in microsomal vesicles pre-labelled with R18 and F18, which demonstrate the experimental conditions required for AlF_4^- inhibition of vesicle fusion. Initially we failed to observe AlF_4^- inhibition of GTP-dependent membrane fusion (e.g. Fig. 1a compared with Fig. 1f). However, since it has been suggested that fluoroaluminate interacts with the GDP-bound form of GTP-binding proteins (Chabre, 1990), it seemed possible that there might be a requirement for GDP for inhibition of vesicle fusion, if under normal circumstances the existence of the protein-GDP complex was not kinetically favoured. The remaining traces in Fig. 1 suggest this is indeed the case. These are experiments to investigate the effect of preincubation with: GDP (17 μM) (Fig. 1b); GDP (17 μM) and AlCl₃ (30 μM) (Fig. 1c); GDP (17 μM) and KF (5 mM) (Fig. 1d); and GDP (17 μM), AlCl₃ (30 μM) and KF (5 mM) (Fig. 1e). These data show clearly that preincubation with GDP alone or with GDP and AlCl₃ has little effect on the initial rate of GTP-dependent fusion. Preincubation of microsomes with KF and GDP brings about 30% inhibition of the initial rate of GTP-dependent fusion, whereas preincubation of microsomes with GDP, AlCl₃

Fig. 1. Requirements for AlF_4^- inhibition of GTP-dependent rat liver microsomal vesicle fusion

Microsomes (2 mg/ml) were labelled with R18 and F18 as described in the text. Labelled microsomes were combined and preincubated for 10 min with the following additions to the incubation conditions: (a) no addition; (b) 17 μM -GDP; (c) 17 μM -GDP and 30 μM - AlCl_3 ; (d) 17 μM -GDP and 5 mM-KF; (e) 17 μM -GDP, 30 μM - AlCl_3 and 5 mM-KF; (f) 30 μM - AlCl_3 and 5 mM-KF; after which GTP was added to 'G' to a final concentration of 0.67 mM. PEG (5%) was present throughout the incubations, after probe incorporation.

and KF results in 75% inhibition of the initial rate of GTP-dependent fusion, with also a great decrease in the extent of fusion as indicated by the decreased final fluorescence level. Thus the inhibition of GTP-dependent vesicle fusion requires the presence of AlCl_3 , KF and GDP. The substantial inhibition observed when the vesicles are preincubated with GDP and KF may be explained by Al^{3+} contamination from glassware and reagents (Chabre, 1990). Inhibition of AlF_4^- also required higher free Mg^{2+} concentrations than those which we have used previously (Comerford & Dawson 1988, 1989). Under the conditions of Fig. 1, decreasing the Mg^{2+} concentration from 0.5 to 0.3 mM substantially decreased inhibition (results not shown).

In all the experiments detailed above, PEG was present in incubations at a final concentration of 5% (w/v). We have previously demonstrated that PEG is required for GTP-dependent Ca^{2+} release and vesicle fusion (Dawson, 1985; Comerford & Dawson, 1988) and have suggested that the sole function of PEG is to increase the proximity of the vesicles to each other, facilitating vesicle fusion. However, a further possibility is that PEG may in some way directly stimulate GTP-dependent vesicle fusion, perhaps by increasing the GDP_{off} rate, thus facilitating GTP binding/hydrolysis and subsequent fusion. To test this, we have examined GTP-dependent vesicle fusion in the absence of PEG but in the presence of 10 times the amount of microsomal protein used in our standard (+PEG) fusion assay. Vesicle fusion under these conditions would support our initial proposal concerning the action of PEG and would eliminate the possibility of PEG having a direct stimulatory role on the mechanism of vesicle fusion. Fig. 2 shows fluorescent traces typical of resonance energy transfer between F18 and R18 when GTP (2 mM) is added. The increase in microsomal protein required an increase in the concentrations of F18, R18 and MgCl_2 as detailed in the Experimental section. Fig. 2(a) shows that GTP causes fusion under these circumstances, as is also the case (Fig. 2b) when microsomes are preincubated in the presence of GDP (66 μM) for 10 min before addition of GTP. Fig. 2(c) shows a similar experiment, but in this case GDP (66 μM), AlCl_3 (30 μM) and KF (5 mM) were present in the preincubation before the addition of GTP. These data indicate that by increasing

Fig. 2. Inhibition of rat liver microsomal vesicle fusion by AlF_4^- in PEG-free incubation conditions

Microsomes (20 mg/ml) were labelled with R18 (10 μl of 3.4 mM) and F18 (10 μl of 2 mM) as described in the text. Fluorescence energy transfer was determined in incubation buffer without PEG as detailed in the Experimental section. Microsomes were preincubated for 10 min with the following additions: (a) none; (b) 123 μM -GDP; (c) 123 μM -GDP, 60 μM - AlCl_3 and 10 mM-KF, after which GTP at a final concentration of 2 mM was added ('G').

Fig. 3. Effect of GDP concentration of AlF_4^- inhibition of initial rates of GTP-dependent rat liver microsomal vesicle fusion

Incubation conditions and fluorescence energy transfer were as previously described for Fig. 1(e), except that GDP concentration in the preincubation was varied as indicated. ●, Initial rates of vesicle fusion in the absence of AlF_4^- pretreatment; ■, initial rates of vesicle fusion after pretreatment with AlF_4^- . Results shown are typical of at least three independent experiments.

protein concentration in the fusion assay and also increasing various components of the assay it is possible to demonstrate GTP-dependent vesicle fusion of rat liver microsomes in the absence of PEG (Fig. 2a). Further, we have also demonstrated that the extent and rate of GTP-dependent vesicle fusion, in the absence of PEG, is also inhibited by preincubation with AlF_4^- in the presence of GDP (Fig. 2c), the extent of inhibition being similar to that obtained in the '+PEG' experiments. Again, as in the '+PEG' experiments, no inhibition with AlF_4^- is observed if GDP is omitted (results not shown).

Having established a requirement for GDP for maximal AlF_4^- inhibition of GTP-dependent fusion in rat liver microsomes, in both the presence and the absence of PEG, we investigated the concentration requirement for GDP in AlF_4^- -dependent inhibition of fusion. Fig. 3 shows the relationship between initial rate of vesicle fusion, as determined by resonance energy transfer

Fig. 4. Effect of preincubation time with AlF_4^- on inhibition of GTP-dependent vesicle fusion in rat liver microsomes

Microsomes (2 mg/ml) were pre-labelled with R18 and F18 and fluorescence energy transfer was determined as described in the text. In each experiment the incubation conditions were as described for Fig. 1(e). Fig. 4 shows typical experiments in which AlF_4^- was added 1 min (a), 2.5 min (b), 5 min (c) or 10 min (d) before GTP addition (0.67 mM) at 'G', with GDP being present throughout.

Fig. 5. AlF_4^- inhibition of rat liver microsomal vesicle fusion in the presence of p[NH]ppA

Microsomes (2 mg/ml) were preincubated as described for Fig. 1 in the absence (a) or presence (b) of AlCl_3 and KF, except that MgCl_2 concentration in the preincubation was 1.8 mM. After 9 min p[NH]ppA (3 mM) was added at 'A'. After a further 1 min GTP (0.67 mM) was added ('G').

between F18 and R18 in the presence of GTP (0.67 mM), MgCl_2 (1 mM), AlCl_3 (30 μM) and KF (5 mM) and varied GDP concentration. In each case the results shown are typical of at least three independent experiments and show that GTP-dependent fusion in rat liver microsomes was maximally inhibited by AlF_4^- in the presence of 17 μM -GDP (50% inhibition at 5 μM), and that up to this concentration GDP has no apparent effect on GTP-dependent fusion in the absence of AlF_4^- . Above this concentration GDP can substantially inhibit GTP-dependent vesicle fusion, probably by competing for the GTP binding site with GTP.

Fig. 6. AlF_4^- inhibition of PEG-stimulated GTPase activity in rat liver microsomes

Microsomes (1.5 mg/ml final concn.) were preincubated as described in the Experimental section. Rat liver microsomal GTPase activity was determined in the presence of 5% PEG, with AlF_4^- present (○) or absent (●). The results shown are typical of three independent experiments. Error bars are shown where the extent is greater than the diameter of the point.

Fig. 4 shows a series of typical traces, after GTP addition, of resonance energy transfer between F18 and R18, which show that the length of the preincubation time with AlF_4^- is important in determining the degree of inhibition of GTP-dependent vesicle fusion in rat liver microsomes. These data show that increasing length of the preincubation time with AlF_4^- from 1 to 10 min brings about increased inhibition of GTP-dependent vesicle fusion, with GDP being present throughout the entire preincubation period. A similar experiment (results not shown), where AlF_4^- was present throughout and GDP was added at various times, gave similar results, showing that inhibition depended on both AlF_4^- and GDP being present throughout the preincubation period.

In experiments to investigate the effect of AlF_4^- on PEG-stimulated GTPase assay, it was necessary to have ATP present to protect GTP from hydrolysis by non-specific nucleoside triphosphatases (Nicchitta *et al.*, 1986). However, since the microsomal preparation contained nucleoside-diphosphate kinase activity (Dawson *et al.*, 1986), inclusion of ATP in preincubation medium with GDP was impossible, since the GDP was rapidly converted into GTP. We therefore investigated the possibility of using the non-hydrolysable ATP analogue p[NH]ppA instead of ATP to block the non-specific hydrolysis of GTP by nucleoside triphosphatase activities and thus (i) preserve added GTP and (ii) block the possibility of GTP formation from nucleoside-diphosphate kinase activity. Fig. 5 shows typical traces of fluorescence changes observed from these experiments in which microsomes are incubated under standard (+PEG) conditions, with the alteration that, 1 min before GTP addition, p[NH]ppA (3 mM) is added. Fig. 5(a) shows that, in the presence of p[NH]ppA, microsomal vesicle fusion occurs when GTP is added to microsomes which have been preincubated with GDP. When AlF_4^- , GDP and p[NH]ppA are present in the preincubation (Fig. 5b), GTP-dependent microsomal vesicle fusion is substantially inhibited (about 80%). From these experiments it was concluded that p[NH]ppA could replace ATP in assays to determine GTPase activity associated with membrane fusion in rat liver microsomal vesicles.

Fig. 6 shows that GTPase activity determined in the presence of PEG is inhibited by AlF_4^- pre-treatment. The inhibition of PEG-stimulated GTPase activity by AlF_4^- is about 70%, and this is comparable with the observed extent of AlF_4^- inhibition of GTP-dependent microsomal vesicle fusion under similar

Fig. 7. AlF_4^- inhibition of $\text{GTP}^{[35\text{S}]}$ binding to Gn proteins from rat liver microsomes

Gn proteins from rat liver microsomal vesicles were obtained as described in the Experimental section. Typical densitometer traces obtained from scanning autoradiograms of Gn proteins from rat liver microsomes are shown: —, autoradiogram of $\text{GTP}^{[35\text{S}]}$ binding to Gn proteins after preincubation of the nitrocellulose strips containing the Gn proteins with GDP (20 μM), AlCl_3 (30 μM) and KF (5 mM); ----, autoradiogram of $\text{GTP}^{[35\text{S}]}$ binding to Gn proteins after preincubation of the nitrocellulose strips containing the Gn proteins with GDP (20 μM) only. The results shown are typical of three experiments. Values on peaks show the M_r ($\times 10^{-3}$).

conditions. Also, in agreement with Nicchitta *et al.* (1986), GTPase activity in rat liver microsomes is stimulated (in our experiments by about 80%) in the presence of PEG compared with the activity in its absence (results not shown). Further, very little inhibition of GTPase activity by AlF_4^- is observed in incubation conditions without PEG (results not shown).

Fig. 7 shows typical densitometry traces derived from scanning autoradiograms of Gn proteins, which have been detected by $\text{GTP}^{[35\text{S}]}$ binding to nitrocellulose. Nitrocellulose strips containing the proteins were preincubated with either GDP (20 μM) (broken line) or GDP (20 μM), AlCl_3 (30 μM) and KF (5 mM) (continuous line) before incubation with $\text{GTP}^{[35\text{S}]}$. The results clearly show that GDP and AlF_4^- pretreatment inhibits $\text{GTP}^{[35\text{S}]}$ binding to all the Gn proteins, except for the protein of M_r 17400, and there is apparently some differentiation between sensitivity of individual bands to AlF_4^- /GDP pre-treatment. The densitometer traces of Gn proteins which were preincubated with GDP (20 μM) were identical with those of strips containing Gn proteins which were preincubated in the absence of any additions or in the presence of AlF_4^- but in the absence of GDP (results not shown).

DISCUSSION

In the present paper we have shown that pretreatment of rat liver microsomal vesicles with AlCl_3 and KF and GDP results in inhibition of GTP-dependent vesicle fusion. The requirement for GDP to obtain inhibition by AlF_4^- explains why under our normal incubation conditions (Comerford & Dawson, 1988, 1989) no inhibition was observed. However, the current model describing the effect of fluoroaluminate on GTP-binding proteins (Bigay *et al.*, 1985; Chabre, 1990) envisages that the fluoroaluminate binds to the protein-GDP complex, mimicking the γ -phosphate group. Inhibition by fluoroaluminate therefore requires that there is a finite concentration of the protein-GDP complex present during the catalytic cycle. Under our experimental conditions, it would appear that the 'off' rate for GDP

is sufficiently high for this not to be the case, so that AlF_4^- only inhibits if the concentrations of the protein-GDP complex is increased by GDP addition. GDP-bound forms of *ras* and *ras*-like proteins (Hall & Self, 1986; Garret *et al.*, 1989) and the recombinant form of Gz, purified from *Escherichia coli* (Casey *et al.*, 1990), are favoured in the presence of relatively high free Mg^{2+} concentration, and increasing the free Mg^{2+} concentration in our experiments also favoured inhibition by AlF_4^- . Also, in agreement with others (Sternweis & Gilman, 1982; Bigay *et al.*, 1985; Kanaho *et al.*, 1985; Stadel & Crooke, 1988; Magnusson *et al.*, 1989; Wakelam, 1989; Bengtsson *et al.*, 1990; Shuttleworth, 1990) we have shown that there is a time-dependence for AlF_4^- inhibition of GTP-dependent events. In microsomal vesicle fusion, preincubation with both GDP and AlF_4^- for 10 min before GTP addition is required for inhibition of vesicle fusion (Fig. 4).

Our earlier proposal (Dawson & Comerford, 1989) was that the PEG requirement for GTP-induced membrane fusion is due to membrane aggregation caused by PEG, causing locally very high membrane concentrations. If this is true, it should be possible to overcome the PEG requirement by working at very high membrane concentrations, and here we show this to be the case (Fig. 2). Furthermore, GTP-dependent fusion in the absence of PEG shows very similar properties, in terms of sensitivity to AlF_4^- , GTP and Mg^{2+} , to that in the presence of PEG. $\text{GTP}^{[35\text{S}]}$, which has been shown to inhibit GTP-dependent Ca^{2+} release (Dawson *et al.*, 1987) and GTP-dependent vesicle fusion in the presence of PEG (Comerford & Dawson, 1988), also inhibits fusion in the absence of PEG (results not shown).

We have also investigated the effect of AlF_4^- on high-affinity PEG-stimulated GTPase activity in rat liver microsomes and have shown, in agreement with Nicchitta *et al.* (1986), that endogenous high-affinity GTPase activity is significantly enhanced in the presence of PEG. Although the nature of this GTPase activity is not well defined, it is a plausible assumption that at least part of the PEG-stimulated GTPase activity is associated with PEG-stimulated GTP-dependent membrane fusion. This is supported by the observation that AlF_4^- can bring about 80% inhibition of PEG-stimulated GTPase activity, which is comparable with the extent of inhibition of GTP-dependent vesicle fusion brought about by AlF_4^- under similar conditions. Further, this high-affinity PEG-stimulated GTPase activity is inhibited by limited proteolysis by trypsin in a manner which is comparable with the inhibition of GTP-dependent vesicle fusion and Ca^{2+} release, as previously reported (Comerford & Dawson, 1989; results not shown).

In a previous paper, we suggested that one or more of the Gn proteins in rat liver microsomes may be involved in GTP-dependent vesicle fusion (Comerford & Dawson, 1989). Since then, much evidence has accrued to support the view that Gn proteins may be involved in the processes of intracellular membrane trafficking (Bourne *et al.*, 1990, 1991). Fluoroaluminate (and $\text{GTP}^{[35\text{S}]}$) have been shown to inhibit GTP-dependent intracellular trafficking processes in a mammalian cell-free system (Melançon *et al.*, 1987; Beckers & Balch, 1989) and have also been demonstrated to inhibit transport of the α -factor precursor from endoplasmic reticulum to Golgi in yeast (Baker *et al.*, 1988). Recently, Plutner *et al.* (1990), using synthetic peptides to the effector regions of Gn (*rab*) proteins, have demonstrated inhibition of vesicular transport in a cell-free system. In agreement with these ideas, we have found that pretreatment of Gn proteins on nitrocellulose with AlF_4^- and GDP, under conditions similar to those used to inhibit GTP-dependent vesicle fusion, results in inhibition of $\text{GTP}^{[35\text{S}]}$ binding to Gn proteins and that there may be some selectivity in sensitivity to AlF_4^- /GDP pretreatment. The M_r values obtained

for the Gn proteins in rat liver microsomes in these experiments (Fig. 7) differ somewhat from those that we have previously reported. This is because of better separation of the proteins obtained in these experiments by using a 16% (w/v) resolving gel, and also better resolution of individual Gn proteins was obtained, as previously reported (Comerford *et al.*, 1989; Ali & Evans, 1990), by using GTP[³⁵S] rather than [γ -³²P]GTP to probe the nitrocellulose.

We believe that the good correlation obtained between AlF_4^- inhibition of GTP-dependent fusion of microsomal vesicles, of inhibition of high-affinity PEG-stimulated GTPase activity and of inhibition of binding of GTP[³⁵S] to Gn proteins suggest that these processes are all part of the same phenomenon. We propose that relatively high free Mg^{2+} concentrations and long preincubation time with GDP and AlF_4^- facilitate the binding of these latter compounds to the binding site of the particular Gn protein involved in rat liver microsomal vesicle fusion. Occupancy of this binding site by GDP and AlF_4^- thus prevents GTP binding and hydrolysis, which is required for vesicle fusion. This model is in agreement with models proposed to explain the effect of AlF_4^- on signal-transduction G-proteins, and it may thus be that there is some similarity in mechanism between the two processes.

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