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The effect of limited proteolysis on GTP-dependent Ca^{2+} efflux and GTP-dependent fusion in rat liver microsomal vesicles

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1. Limited proteolytic digestion of rat liver microsomes (microsomal fractions) with trypsin (5 μg/ml), proteinase K (1.0 μg/ml) and Pronase (20 μg/ml final concns.) resulted in abolition of GTP-dependent vesicle fusion. 2. Vesicle fusion could be partially restored to microsomes which had undergone limited tryptic digestion, by the addition of untreated microsomal vesicles. 3. GTP-dependent Ca^{2+} efflux from rat liver microsomes was also observed to be inhibited by limited proteolysis with trypsin and proteinase K. 4. Limited proteolysis of rat liver microsomes had no effect on subsequent GTP-dependent phosphorylation of polypeptides of M_r 17000 and 38000, and thus it is unlikely that the phosphorylation of these proteins is involved in GTP-dependent Ca^{2+} efflux and GTP-dependent vesicle fusion. 5. GTP binding by Gn proteins [proteins which bind GTP after transfer to nitrocellulose, as defined by Bhullar & Haslam (1986) Biochem. J. **245**, 617–620] was inhibited by pre-treatment of microsomes with trypsin, proteinase K and Pronase at concentrations similar to those which abolished GTP-dependent Ca^{2+} efflux and vesicle fusion. 6. We suggest that one or more of the Gn proteins may be involved in the molecular mechanisms of GTP-dependent vesicle fusion and Ca^{2+} efflux in rat liver microsomes and that limited proteolytic digestion may be a useful tool in further investigation of these processes.

INTRODUCTION

Previously, we have reported that in rat liver microsomal fractions (microsomes) InsP_3-stimulated Ca^{2+} release is greatly increased by micromolar concentrations of GTP, in the presence of PEG (Dawson, 1985; Dawson *et al.*, 1986). Under these conditions GTP mediates slow Ca^{2+} mobilization, which at high free extravesicular Ca^{2+} concentration (pCa 6.4–6.5) can be extensive (Dawson *et al.*, 1987). These observations have been supported by results obtained by Thomas (1988) using permeabilized hepatocytes. Other workers have also reported GTP-dependent Ca^{2+} mobilization from other cell types and microsomes derived from them. However, in some of these systems the effects of GTP differ from those observed in rat liver microsomes. The most important differences between GTP-dependent Ca^{2+} mobilization in rat liver microsomes and the other systems investigated are the speed and extent of Ca^{2+} efflux (Gill *et al.*, 1986; Ueda *et al.*, 1986; Henne & Söling, 1986; Mullaney *et al.*, 1987; Allan *et al.*, 1989), lack of enhancement of InsP_3-dependent Ca^{2+} efflux by GTP (Gill *et al.*, 1986; Ueda *et al.*, 1986; Henne & Söling, 1986; Wolf *et al.*, 1987), the requirement for PEG (Wolf *et al.*, 1987) and the promotion of uptake of Ca^{2+} caused by GTP in conditions where intravesicular Ca^{2+} is precipitated by oxalate (Hamachi *et al.*, 1987; Chueh *et al.*, 1987; Mullaney *et al.*, 1987, 1988).

Paiement *et al.* (1987) have shown that, at relatively high (millimolar) concentrations, GTP causes fusion of stripped endoplasmic-reticulum vesicles prepared from rat liver. We have demonstrated that low concentrations of GTP (micromolar) 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 between microsomal vesicles differentially labelled with the fluorescence probes R18 and F18 (Comerford & Dawson, 1988). GTP-dependent fusion of rat liver microsomal vesicles is observed to have characteristics very similar to GTP-dependent Ca^{2+} release in relation to PEG requirement, Mg^{2+} requirement and the effect of GTP concentration. We have suggested that GTP-dependent Ca^{2+} mobilization, in rat liver microsomes at least, may be due to a change in vesicle permeability to Ca^{2+}, accompanying vesicle fusion (see also Nicchitta *et al.*, 1987).

Studies in our laboratory (Dawson *et al.*, 1986) have shown that, under conditions which promote GTP-dependent fusion and GTP-dependent Ca^{2+} efflux, two polypeptides (M_r 17000 and 38000) are phosphorylated by [γ-^{32}P]GTP, as detected by SDS/polyacrylamide-gel electrophoresis and autoradiography. We tentatively suggested that one or both of these proteins phosphorylated by GTP might be important in the process of vesicle fusion (Dawson *et al.*, 1986) and it is generally agreed that splitting of the γ-phosphate of GTP is required. However, Lukács *et al.* (1987) and Kiesel *et al.* (1987) have subsequently provided evidence that these phosphorylated proteins might not be involved in GTP-dependent Ca^{2+} efflux.

Paiement *et al.* (1987) found that fusion of rough-endoplasmic-reticulum membranes was, in their system, very sensitive to proteolysis. Godelaine & Beaufay (1987) have also found that limited proteolysis of rough endoplasmic reticulum leads to loss of GTP-binding sites. In this paper we use limited proteolysis of rat liver microsomes to show, firstly that GTP-dependent Ca^{2+}

Abbreviations used: InsP_3, inositol 1,4,5-trisphosphate; PEG, poly(ethylene glycol), average M_r 8000; R18, octadecyl-rhodamine B (chloride salt); F18, 5-(*N*-octadecanoyl)-aminofluorescein; GTP[S], guanosine 5'-[γ-thio]triphosphate; Gn proteins, GTP-binding proteins detected on nitrocellulose blots.

release, promotion of InsP_3-dependent Ca^{2+} release and membrane fusion are all lost on limited proteolytic digestion, and secondly, that the extent of phosphorylation of the polypeptides (M_r 17000 and 38000) is unaffected, making it very unlikely that these polypeptides are involved in these processes.

Bhullar & Haslam (1986) have described a class of low-M_r GTP-binding proteins (Gn proteins) which were detected by high-affinity and high-specificity binding of GTP on to nitrocellulose blots of SDS/polyacrylamide-gel electrophoresis gels. We have found (Drøbak *et al.*, 1988) that some proteins of this type are present in our microsomal preparations. In contrast with the polypeptides phosphorylated by GTP, we show here that one or more of these Gn proteins are very sensitive to proteolysis under conditions which block vesicle fusion, and thus appear to be promising candidates to be involved in the membrane fusion process.

EXPERIMENTAL

Materials

Trypsin (type XIII: 10000–30000 BAEE units/mg of protein), trypsin inhibitor (type I-S) and proteinase K (type XI: 10–20 units/mg of protein) were obtained from Sigma. Pronase (45000 PUK/g) was obtained from Calbiochem. Dithiothreitol, ATP, GTP and GTP[S] were supplied by BCL, Lewes, Sussex, U.K. Fluorescent probes R18 and F18 were obtained from Molecular Probes Inc., Eugene, OR, U.S.A. [γ-^{32}P]GTP (30 Ci/mmol) was from New England Nuclear, DuPont (U.K.) Ltd., Stevenage, Herts., U.K. 'Rainbow Markers' and ^{14}C-labelled M_r standard proteins were obtained from Amersham International, Amersham, Bucks., U.K. InsP_3 was generously given by Dr. R. F. Irvine, A.F.R.C., Babraham, Cambridge, U.K.

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).

Incubation of microsomal membranes with proteolytic enzymes

Microsomal membranes (final concn. of 1.1 mg of protein/ml) were suspended in 150 mM-sucrose/50 mM-KCl/10 mM-Hepes/KOH (pH 7.0)/2 mM-dithiothreitol/0.2 mM-EGTA and proteolytic enzymes at the final concentrations indicated in the individual Figure legends. After incubation for 5 min at 30 °C, microsomes pretreated in this manner were used in experiments to investigate the effects of the proteolytic enzymes on GTP-dependent protein phosphorylation and GTP binding by Gn proteins. Modifications to this general protocol for investigations of the effects of proteolytic enzymes on GTP-dependent vesicle fusion and Ca^{2+} efflux are detailed below.

Effect of proteolytic enzymes on GTP-dependent microsomal vesicle fusion

The procedures used for probe incorporation and fluorescence measurement were as detailed by Comerford & Dawson (1988). Briefly, this was as follows: microsomal membranes (5.5 mg of protein) were suspended in a total volume of 2.5 ml of 150 mM-sucrose/50 mM-KCl/10 mM-Hepes/KOH (pH 7.0)/2 mM-dithiothreitol/0.2 mM-EGTA. The suspension was then divided into two equal halves. R18 (4 μl of 3.4 mM stock solution) was added to one half and F18 (2 μl of 4.1 mM stock solution) was added to the other. In both cases, the probes were added while the suspension was mixed vigorously on a vortex mixer. The suspensions were incubated at 30 °C for 5 min to allow incorporation of probes, after which time the two halves were recombined with thorough mixing and added to the fluorimeter cell, which already contained 2.5 ml of 150 mM-sucrose / 50 mM-KCl / 10 mM-Hepes / KOH (pH 7.0)/10% (w/v) PEG/2 mM-$MgCl_2$. After mixing in the fluorimeter cell, ATP (final concn. 5 mM) was added, and after a further 3 min vesicle fusion was initiated by the addition of GTP (40 μM). Energy transfer from F18 to R18 was measured, by monitoring the fluorescence increase at 600 nm at an excitation wavelength of 460 nm. Proteolysis was carried out by addition of the proteolytic enzymes 5 min before the addition of GTP. In experiments where only one half of the microsomes used was pre-treated with trypsin, trypsin was added to the half of the incubation mixture containing F18 and the action of trypsin was terminated, after 5 min incubation, by addition of trypsin inhibitor (40 μg/ml final concn.).

Effect of proteolytic enzymes on GTP-dependent and InsP_3-dependent Ca^{2+} efflux from microsomal vesicles

Ca^{2+} uptake and release by microsomal vesicles were measured by using a Ca^{2+}-sensitive electrode as described previously (Dawson *et al.*, 1987; Comerford & Dawson, 1988). In experiments to determine the effect of proteolytic enzymes on Ca^{2+} mobilization, the proteolytic enzymes were added 5 min before addition of GTP (40 μM). The final steady-state pCa was about 6.95 in all experiments.

Effect of proteolytic enzymes on GTP-dependent protein phosphorylation

Protein phosphorylation by [γ-^{32}P]GTP was investigated after pre-treatment of microsomal proteins with proteolytic enzymes. The latter was carried out as described above, except that 50 mM-NaCl replaced 50 mM-KCl in the buffer. This modification was introduced because of the high protein concentration used in these experiments. Solubilization by SDS was found to be much easier if K^+ concentration was kept to a minimum. Substitution of 50 mM-NaCl for 50 mM-KCl did not affect the extent of inhibition of GTP-dependent vesicle fusion, or Ca^{2+} efflux, by limited proteolysis (results not shown). Incubation conditions were otherwise as described by Dawson *et al.* (1986), with use of 4 μCi of [γ-^{32}P]GTP in a final volume of 100 μl and omitting PEG from the incubation mixture. After 1 min at 30 °C the reaction was stopped by removing 75 μl of the reaction mixture and adding to 25 μl of sample buffer, containing (final concns.) 0.1 M-dithiothreitol, 2% SDS, 0.08 M-Tris/HCl (pH 6.8), 15% glycerol and 0.006% Bromophenol Blue. Samples were heated (97 °C, 2 min), briefly centrifuged, and aliquots (15 or 40 μl) were applied to SDS/polyacrylamide gels (12.5% or 15%) and electrophoresed by the method of Blattler *et al.* (1972), modified from the original procedures of Laemmli (1970). Gels obtained were either dried down under vacuum or electroblotted on to nitrocellulose by using an LKB Novablot dry-blotter apparatus (70 mA

for 2.5 h at room temperature). Autoradiograms of dried gels or air-dried nitrocellulose blots were made by autoradiography using preflashed films and Ilford intensifier screens (5–7 days, −70 °C). Polypeptides on the nitrocellulose blots, including protein M_r markers, were detected by staining with Amido Black B (Hancock & Tsang, 1983).

Effect of proteolytic enzymes on GTP binding by Gn proteins

Microsomal proteins pre-treated with proteolytic enzymes were prepared for, and resolved by, SDS/polyacrylamide-gel electrophoresis (12.5% or 15% gels) as described by Drøbak *et al.* (1988). The resolved polypeptides were transferred from gels to nitrocellulose by electroblotting with a LKB Novablot dry-blotter apparatus (70 mA for 2.5 h at room temperature) or by the procedure described by Drøbak *et al.* (1988). Incubation of nitrocellulose blots with [γ-^{32}P]GTP and detection of labelled polypeptides were as previously described (Drøbak *et al.*, 1988).

Enzyme assays

Glucose-6-phosphatase activity of the microsomes was assayed as described by Dawson & Irvine (1984). Mannose-6-phosphatase activity was assayed under identical conditions, by substituting 5 mM-mannose 6-phosphate for 5 mM-glucose 6-phosphate in the assay. Latency was assessed by comparison of activities in the absence and presence of 0.15% taurocholate.

RESULTS

All proteolytic treatments used below were limited, in that in no case was there any major change in appearance of protein band distribution on SDS/polyacrylamide gels (stained with Coomassie Blue) or on nitrocellulose blots stained with either Amido Black or Indian Ink. In particular there was no apparent increase in the low-M_r peptide regions of the gels. Furthermore, the trypsin digestion conditions used have caused no change in glucose-6-phosphatase activity or, in agreement with Paiement *et al.* (1987), in the latency of mannose-6-phosphatase. After trypsin digestion (20 μg/ml for 5 min at 30 °C, stopped by trypsin inhibitor) the glucose-6-phosphatase activity was 2.2 ± 0.05 μmol of P_i/10 min per mg of protein ($n = 4$), compared with control values of 2.1 ± 0.04 μmol/10 min per mg. Mannose-6-phosphatase activity was 0.24 ± 0.03 and 0.20 ± 0.03 μmol of P_i/10 min per mg of protein after and before trypsin treatment respectively (84% and 85% latent respectively). This suggests that proteolysis produces no gross non-specific effects on membrane integrity.

Fig. 1 shows the inhibitory effect of limited proteolysis by trypsin, proteinase K and Pronase on GTP-dependent fusion of rat liver microsomes as determined by the fluorescence-energy-transfer procedure of Comerford & Dawson (1988). In each case the results shown are typical of several experiments and show that the GTP-dependent fusion process in rat liver microsomes was totally inhibited by pre-treatment of the microsomal vesicles at 30 °C for 5 min, with 5 μg of trypsin/ml, 1.0 μg of proteinase K/ml or 20 μg of Pronase/ml (all final concns. in the incubation mixture). Half-maximal inhibition of GTP-dependent fusion was seen with trypsin (1–2 μg/ml), proteinase K (0.15 μg/ml) and Pronase (8–9 μg/ml) (all final concns.), although with Pronase inhibition of GTP-dependent fusion showed a more complex biphasic response with respect to concentration. Fig. 2 shows the effect of limited tryptic digestion of GTP-dependent fusion of microsomal vesicles in experiments in which only half of the microsomal vesicles had been pre-treated with trypsin. Fig. 2(*a*) shows a typical trace of fluorescence change in microsomal vesicles pre-labelled with R18 and F18, on the addition of 40 μM-GTP ('G'), without pre-treatment with trypsin. Fig. 2(*b*) shows results obtained under similar conditions, but in this case the microsomal membranes had been pre-treated with trypsin (6 μg/ml final concn.) before GTP addition. Fig. 2(*c*) shows the effect of pre-treatment of only one half of the microsomal vesicles (F18-labelled) with trypsin (6 μg/ml final concn.), the other half (R18-

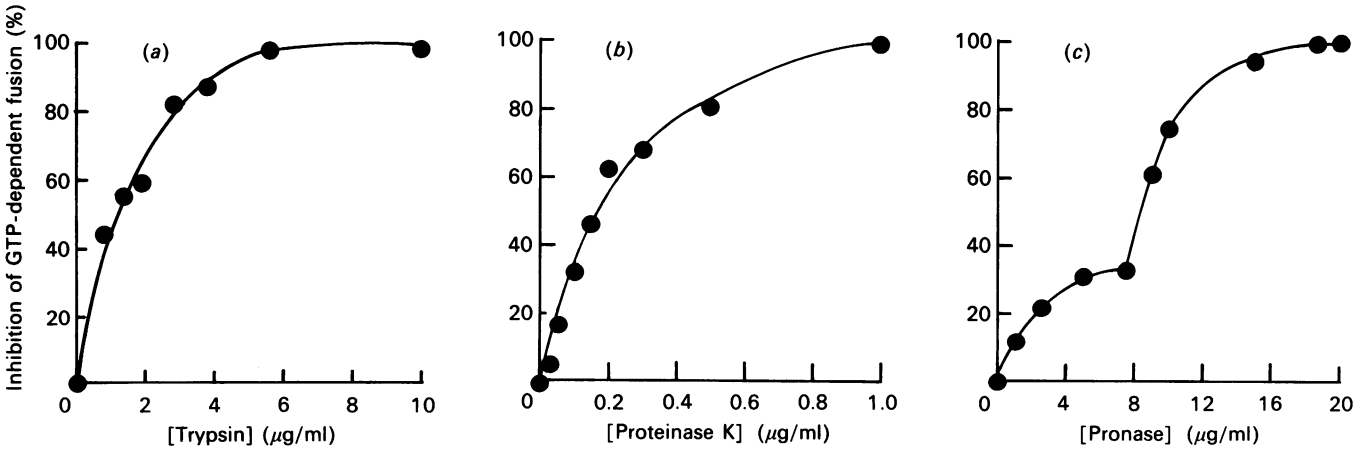

Fig. 1. Effect of limited proteolysis on GTP-dependent fusion

Microsomes (1.1 mg/ml final concn.) were pre-treated with the indicated final concentrations of trypsin (*a*), proteinase K (*b*) and Pronase (*c*). After 5 min incubation at 30 °C, GTP (40 μM final concn.) was added and subsequent GTP-dependent fusion was measured. Results are expressed as percentage inhibition of the initial rate of fusion [as determined from traces similar to Fig. 2(*a*)] after pre-treatment with the proteolytic enzymes, compared with untreated controls. Conditions of incubation and the measurement procedure used were as detailed in the Experimental section.

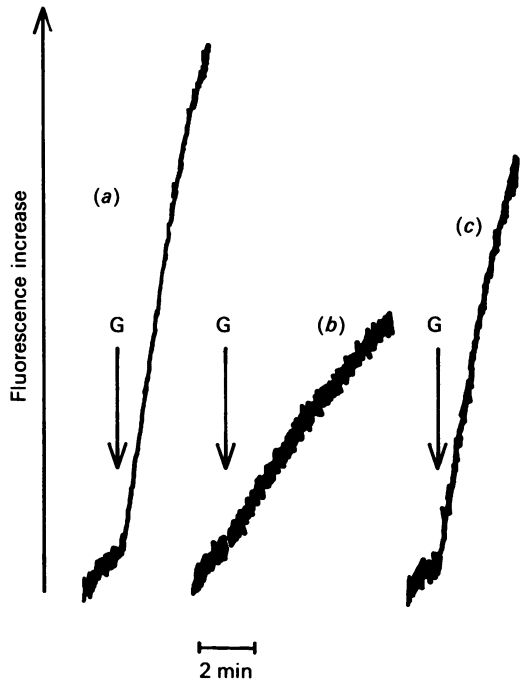

Fig. 2. Restoration of GTP-dependent fusion in microsomal vesicles after pre-treatment with trypsin

Microsomes were (*a*) untreated (control), (*b*) pre-treated with trypsin (6 μg/ml final concn.) for 5 min at 30 °C, after which trypsin inhibitor (40 μg/ml final concn) was added to terminate proteolytic digestion, or (*c*) differentially treated, such that the vesicles incubated with R18 were untreated, whereas the half of the vesicles treated with F18 were pre-treated with trypsin and proteolysis was terminated as in (*b*). Microsomes thus pre-treated were used in the GTP-dependent assay. GTP (40 μM final concn.) was added at 'G'.

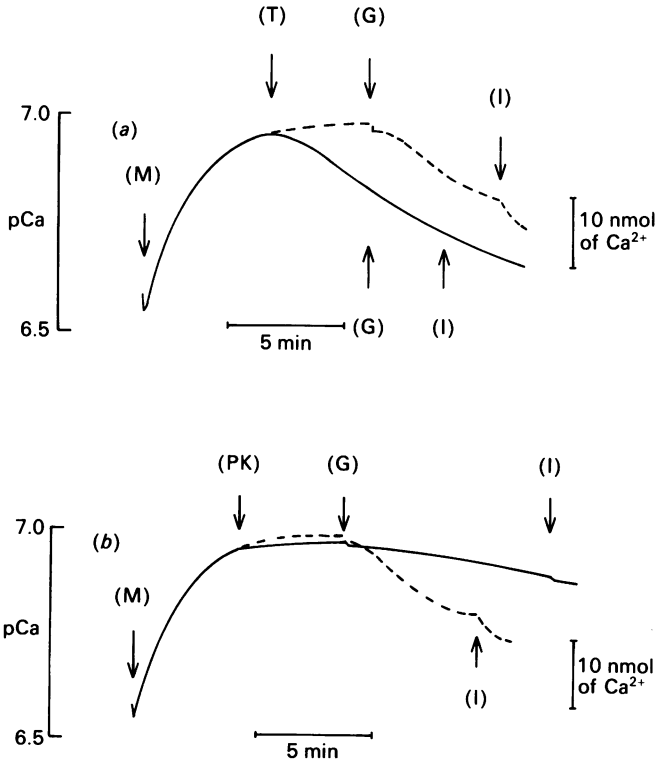

Fig. 3. Effect of limited proteolysis on GTP-dependent Ca^{2+} efflux

This Figure shows Ca^{2+} uptake and release by microsomes, and the effect of pre-treatment of vesicles with either trypsin or proteinase K on Ca^{2+} movements. Ca^{2+} uptake and efflux were determined by Ca^{2+} electrode as described in the Experimental section. Microsomes (1.1 mg/ml final concn.) were added at 'M' to initiate Ca^{2+} uptake. Additions were: (*a*) trypsin (final concn. 5 μg/ml; 'T'), (*b*) proteinase K (final concn. 1 μg/ml; 'PK'). 'G' and 'I' indicate the additions of GTP (40 μM final concn.) and $InsP_3$ (0.4 μM final concn.) respectively. In both (*a*) and (*b*) the continuous line shows Ca^{2+} efflux after addition of proteolytic enzymes, and the broken line represents Ca^{2+} movements in untreated controls. The results shown are typical of four separate experiments.

labelled) being untreated. As previously, trypsin pre-treatment (6 μg/ml final concn.) resulted in 100% inhibition of GTP-dependent fusion (Fig. 2*b* compared with Fig. 2*a*). However, Fig. 2(*c*) shows that, when only the half of the population of microsomal vesicles labelled with F18 was pre-treated with trypsin, GTP-dependent fusion was observed, albeit at a slower rate and to a lesser extent than in the absence of any pre-treatment with trypsin. Pre-incubation of the fluorescent probes with trypsin in the absence of microsomal vesicles, followed by termination of proteolysis with trypsin inhibitor, and then addition of the microsomal protein to the pre-treated probes, did not alter the extent of probe incorporation or the detection of GTP-dependent fusion of rat liver microsomes (results not shown). Thus there is no effect of the proteolytic enzymes on the fluorescent probes themselves.

Fig. 3 shows the effect of limited proteolytic digestion by trypsin (Fig. 3*a*) or proteinase K (Fig. 3*b*) on GTP-dependent Ca^{2+} efflux and $InsP_3$-dependent Ca^{2+} release from rat liver microsomal vesicles. Addition of trypsin (6 μg/ml final concn.) resulted in Ca^{2+} efflux from rat liver microsomal vesicles (Fig. 3*a*) which is due to either decreased Ca^{2+} accumulation by partial inhibition of the microsomal Ca^{2+}-ATPase or increased vesicular permeability to Ca^{2+} brought about by limited proteolytic digestion of the vesicles (probably the former, since mannose-6-phosphatase latency does not change). Addition of GTP to microsomal vesicles, pre-treated with trypsin, caused no stimulation of Ca^{2+} efflux compared with the rate of Ca^{2+} efflux solely due to proteolytic digestion by trypsin. Although the Ca^{2+} leakage caused by trypsin would deplete the intra-microsomal Ca^{2+} pool to some extent, the total Ca^{2+} released by trypsin after 5 min is only about 50% of that releasable by GTP (Fig. 3*a*), so that the lack of effect of GTP after trypsin treatment cannot be ascribed to depletion of the GTP-releasable Ca^{2+} pool. Furthermore, in the case of proteinase K, proteolytic digestion of the vesicles did not induce extensive Ca^{2+} efflux, but inhibition of GTP-dependent Ca^{2+} efflux was still observed (Fig. 3*b*). Inhibition of GTP-dependent Ca^{2+} efflux and of GTP-dependent fusion were found over similar ranges for each of the proteolytic enzymes. Pronase was found to be extensively contaminated with Ca^{2+}, and it was not possible to obtain Ca^{2+}-electrode traces for the effect of Pronase on GTP-dependent Ca^{2+} release.

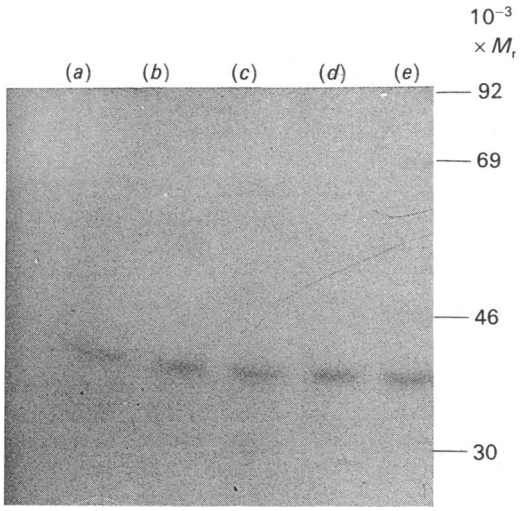

Fig. 4. Effect of limited proteolysis on GTP-dependent phosphorylation of microsomal proteins

This Figure shows the effect of pre-treatment of microsomes with Pronase (20 μg/ml; lane *a*), untreated (control; lane *b*), proteinase K (2 μg/ml; lane *c*), proteinase K (0.2 μg/ml; lane *d*) and Pronase (8 μg/ml; lane *e*) on subsequent GTP-dependent phosphorylation by using the treated microsomal proteins. The phosphorylation incubation mixture and conditions used were as described in the Experimental section. The autoradiogram shown is typical of three such experiments. ^{14}C-labelled M_r standard proteins were electrophoresed on the same gel, and their positions were as indicated.

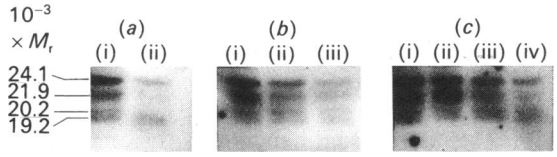

Fig. 5. Effect of limited proteolysis on GTP binding by Gn proteins

This Figure shows the effect of pre-treatment of microsomes with proteinase K, trypsin and Pronase, for 5 min at 30 °C, on GTP binding by Gn proteins. (*a*) Effect of proteinase K pre-treatment: (i) untreated (control) and (ii) 1 μg of proteinase K/ml. (*b*) Effect of trypsin pre-treatment: (i) untreated (control), (ii) 1 μg of trypsin/ml and (iii) 8 μg of trypsin/ml. (*c*) Effect of pre-treatment with Pronase: (i) untreated (control), (ii) 1 μg of Pronase/ml, (iii) 8 μg of Pronase/ml and (iv) 20 μg of Pronase/ml. All concentrations shown were the final concentrations of the proteolytic enzymes in the incubation mixture. M_r values of the polypeptides as shown were determined by reference to standard ^{14}C-labelled proteins of known M_r, which were electrophoresed on the same gel as the samples. The results shown are typical of five experiments.

Fig. 4 shows the effect of proteolytic digestion by Pronase and proteinase K on GTP-dependent phosphorylation of microsomal polypeptides. Pronase (20 μg/ml final concn.) and proteinase K (2 μg/ml final concn.) had very little effect on the phosphorylation of the polypeptides of M_r 38000 (lanes *a* and *c*) or 17000 (results not shown), although under these incubation conditions Pronase and proteinase K caused 100% inhibition of GTP-dependent fusion (Fig. 1). Further, proteinase K at this concentration had been shown to bring about significant inhibition of GTP-dependent

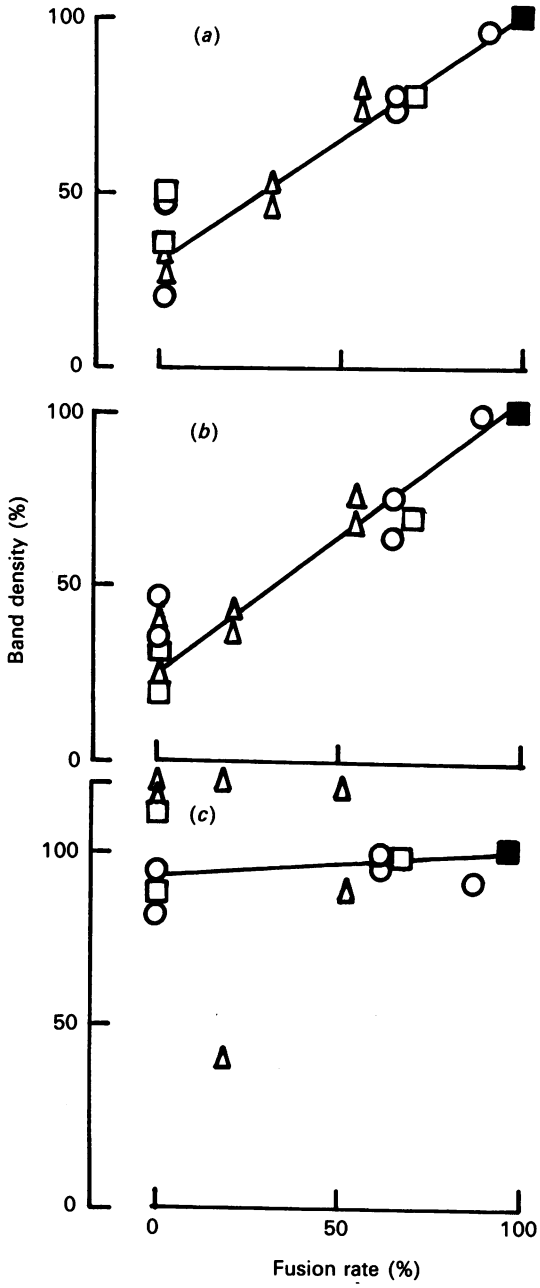

Fig. 6. Disappearance of GTP-binding ability owing to proteolytic digestion

Ordinates: the density of the bands on autoradiograms similar to those shown in Fig. 5 was assessed by measuring the peak height by scanning densitometry: (*a*) data for the bands migrating with M_r 24100, (*b*) for the M_r-21900 band, and (*c*) combined bands M_r 20200 and 19200, which were not resolved by the densitometer. Points are shown from five separate experiments, and in each case are referred to the peak height of the untreated control value as 100%. ■, Untreated control; ○, various concentrations of proteinase K; △, various concentrations of trpysin; □, various concentrations of Pronase. Abscissa: the fusion rates are taken from data shown in Fig. 1, for the appropriate proteolytic enzyme concentrations.

Ca^{2+} release (Fig. 3). Quantitative densitometry revealed no difference in density of any of the bands shown in Fig. 4, when allowance was made for the small variations in band width. Similar results have been obtained for trypsin. A trypsin concentration (6 μg/ml final concn., stopped by trypsin inhibitor after 5 min at 30 °C) which completely inhibited GTP-dependent fusion had very little effect (approx. 15% decrease in [γ-^{32}P]GTP labelling of the M_r-38000 band and no effect on labelling of the M_r-17000 band, determined by densitometric scan of autoradiograms) on GTP-dependent phosphorylation (results not shown).

Fig. 5 shows the effect of pre-treatment with various concentrations of the proteolytic enzymes on GTP binding by the Gn proteins. Pre-treatment of the microsomal vesicles with proteinase K, trypsin and Pronase, under standard incubation conditions, causes decreased GTP binding, particularly by the polypeptides migrating with M_r 24100 and 21900. The doublet migrating at M_r 20200 and 19200 is relatively much less affected. For example, 20 μg of Pronase/ml [Fig. 5(*c*)(iv)], which leads to complete inhibition of fusion, causes extensive removal of the M_r-24100 and -21900 bands, but has little effect on the M_r-20200 and -19200 doublet. Fig. 6 shows the combined results of several such experiments, where the disappearance of the various GTP-binding bands is correlated with inhibition of fusion. The bands at M_r 20200 and 19200 are too close together to be resolved by the densitometer, and have therefore been treated as a single species. It is clear that, for all three proteolytic enzymes, there is a very reasonable degree of correlation between the disappearance of the M_r-24100 and -21900 GTP-binding species and loss of fusion activity. In contrast, there is rather little effect of proteolysis on the intensity of the M_r-20200/-19200 doublet, in agreement with the data shown in Fig. 5.

DISCUSSION

In the present paper, we have shown that limited proteolytic digestion of rat liver microsomal vesicles can affect the GTP-dependent processes of vesicle fusion and Ca^{2+} efflux. Three proteolytic enzymes (trypsin, proteinase K and Pronase) have been used to investigate the inhibitory effect of limited proteolysis on GTP-dependent vesicle fusion. Our observation of limited proteolysis affecting fusion competence of rat liver microsomal vesicles is in concordance with the report by Paiement *et al.* (1987), who shows that trypsin pre-treatment (15 μg/ml final concn.) of stripped rough microsomes from dog pancreas totally abolished fusion in this microsomal system, as determined by morphological analysis using electron microscopy.

Paiement *et al.* (1987) and Godelaine & Beaufay (1987) have both provided evidence showing that there are cytosolically exposed GTP-binding sites, which are sensitive to limited proteolytic digestion, in microsomal vesicle preparations. The involvement of these GTP target sites in fusion of stripped microsomes has been suggested by Paiement *et al.* (1987), who have demonstrated that proteolytic digestion conditions which result in loss of GTP-dependent fusion competence maintained the latency of mannose-6-phosphatase, an intraluminal endoplasmic-reticulum marker enzyme. These data have been interpreted by Paiement *et al.* (1987) to show that there are cytosolically exposed protein(s) sensitive to limited proteolysis by trypsin, important in the GTP-mediated fusion process. We have shown that rat liver microsomal vesicles pre-treated with trypsin lose fusion competence. However, untreated vesicles appear to be able to fuse to trypsin- pre-treated vesicles (Fig. 2). This observation suggests that the fusion mechanism may be rather complex, possibly involving 'donor' and 'receptor' sites in the fusing membranes (see Bourne, 1988).

Previously we have provided evidence (Comerford & Dawson, 1988) that, in rat liver microsomes, the observations of GTP-dependent vesicle fusion and GTP-dependent Ca^{2+} efflux are related. Here we show that limited proteolysis of rat liver microsomal vesicles by pre-treatment with proteinase K resulted in inhibition of GTP-dependent Ca^{2+} efflux and GTP-dependent fusion at similar concentrations of this proteolytic enzyme (Fig. 3). These data provide support for the proposal that GTP-dependent Ca^{2+} efflux in rat liver microsomes is a function of altered vesicle permeability to Ca^{2+} as a consequence of GTP-dependent vesicle fusion.

In an attempt to understand further the molecular mechanisms involved in the GTP-dependent fusion/Ca^{2+}-efflux process in rat liver microsomes, we have investigated the effect of limited proteolysis on the phosphorylation of the M_r-38000 and -17000 bands and on the binding of GTP by the Gn proteins. None of the proteolytic digestion procedures used had any significant effect on the phosphorylation of the M_r-38000 and -17000 proteins under conditions where fusion was totally abolished. This casts severe doubt on any role for these proteins in the fusion process, in agreement with the observations of Lukács *et al.* (1987). However, there seems a very reasonable correlation (Figs. 5 and 6) between the decreased GTP binding by polypeptides of M_r 24100 and 21900 and inhibition of fusion. We therefore propose that one or both of these polypeptides may be involved in GTP-dependent fusion and Ca^{2+} efflux.

Although this evidence for Gn-protein involvement is rather circumstantial, our proposal is reinforced by evidence from others. Very recently, several groups, working with yeast (Segev *et al.*, 1988; Schmitt *et al.*, 1988; Goud *et al.*, 1988) and CHO cells (Melançon *et al.*, 1987) have provided evidence that GTP binding and hydrolysis are of fundamental importance in the processes of membrane trafficking in secretion. A mechanism for membrane vesicle trafficking involving GTP-binding proteins has been proposed by Bourne (1988). The work using yeast has shown that the products of two genes, *Ypt1* and *Sec4*, are absolutely critical to the mechanisms of intracellular trafficking of membrane vesicles in the secretion process in yeast. Schmitt *et al.* (1988) have provided evidence which implicates the *Ypt1* gene product to be important in regulation of cytosolic Ca^{2+} concentration in yeast cells. Further, it has been demonstrated that these genes code for two different polypeptides, of M_r 23000 and 23500, which have been shown to be Gn proteins (Segev *et al.* 1988; Schmitt *et al.*, 1988; Goud *et al.*, 1988). Also of importance in relation to the present study is the observation by Melançon *et al.* (1987) that GTP[S] can inhibit GTP-dependent vesicular transport with a K_i of about 0.5 μM. We have observed (J. G. Comerford & A. P. Dawson, unpublished work) that GTP[S] inhibits GTP-dependent

fusion of rat liver microsomal vesicles with a K_i of about 0.7 μM. In our system we propose that one or both of the Gn polypeptides, which show sensitivity to limited proteolysis, may be analogous to the gene products of *Sec4* or *Ypt1* in yeast or the GTP-binding protein in CHO cells, and that GTP binding and hydrolysis by the Gn protein(s) may prepare vesicle membranes for binding and subsequent fusion.

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REFERENCES

Allan, E. F., Dawson, A. P., Drøbak, B. K. & Roberts, K. (1989) Cellular Signalling **1**, 23–29
Bhullar, R. P. & Haslam, R. J. (1986) Biochem. J. **245**, 617–620
Blattler, D. P., Garner, F., Van Slyke, K. & Bradley, A. (1972) J. Chromatogr. **64**, 147–155
Bourne, H. (1988) Cell **53**, 669–671
Chueh, S.-H., Mullaney, J. M., Ghosh, T. K., Zachary, A. L. & Gill, D. L. (1987) J. Biol. Chem. **262**, 13857–13864
Comerford, J. G. & Dawson, A. P. (1988) Biochem. J. **249**, 89–93
Dawson, A. P. (1985) FEBS Lett. **185**, 147–150
Dawson, A. P. & Irvine, R. F. (1984) Biochem. Biophys. Res. Commun. **120**, 858–864
Dawson, A. P., Comerford, J. G. & Fulton, D. V. (1986) Biochem. J. **234**, 311–315
Dawson, A. P., Hills, G. & Comerford, J. G. (1987) Biochem. J. **244**, 87–92
Drøbak, B. K., Allan, E. F., Comerford, J. G., Roberts, K. & Dawson, A. P. (1988) Biochem. Biophys. Res. Commun. **150**, 899–903
Gill, D. L., Ueda, T., Chueh, S.-H. & Noel, M. W. (1986) Nature (London) **320**, 461–464
Godelaine, D. & Beaufay, H. (1987) Biochem. Biophys. Res. Commun. **148**, 478–484
Goud, B., Salminen, A., Walworth, N. C. & Novick, P. J. (1988) Cell **53**, 753–768
Hamachi, T., Hirata, M., Kimura, Y., Ikebe, T., Ishimatsu, T., Yamaguchi, K. & Koga, T. (1987) Biochem. J. **242**, 253–260
Hancock, K. & Tsang, V. C. W. (1983) Anal. Biochem. **133**, 157–162
Henne, V. & Söling, H. D. (1986) FEBS Lett. **202**, 267–273
Kiesel, L., Lukács, G. L., Eberhardt, I., Runnebaum, B. & Spät, A. (1987) FEBS Lett. **217**, 85–88
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
Lukács, G. L., Hajnóczky, G., Hunyady, L. & Spät, A. (1987) Biochim. Biophys. Acta **931**, 251–254
Melançon, P., Glick, B. S., Malhotra, V., Weidman, P. J., Serafini, T., Gleason, M. L., Orci, L. & Rothman, J. E. (1987) Cell **51**, 1053–1062
Mullaney, J. M., Chueh, S.-H., Ghosh, T. K. & Gill, D. L. (1987) J. Biol. Chem. **262**, 13865–13872
Mullaney, J. M., Yu, M., Ghosh, T. K. & Gill, D. L. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 2499–2503
Nicchitta, C. V., Joseph, S. K. & Williamson, J. R. (1987) Biochem. J. **248**, 741–747
Paiement, J., Rindress, D., Smith, C. E., Poliquin, L. & Bergeron, J. J. M. (1987) Biochim. Biophys. Acta **898**, 6–22
Schmitt, H. D., Puzicha, M. & Gallwitz, D. (1988) Cell **53**, 635–647
Segev, N., Mulholland, J. & Botstein, D. (1988) Cell **52**, 915–924
Thomas, A. P. (1988) J. Biol. Chem. **263**, 2704–2711
Ueda, T., Chueh, S.-H., Noel, M. W. & Gill, D. L. (1986) J. Biol. Chem. **261**, 3184–3192
Wolf, B. A., Florholmen, J., Colca, J. R. & McDaniel, M. L. (1987) Biochem. J. **242**, 137–141

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