Association of nucleoside diphosphate kinase with pancreatic zymogen granules: effects of local GTP generation on granule membrane characteristics

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It is well established that both GTP-binding proteins and phosphoproteins are involved in the control of exocytosis in the exocrine pancreas. Exocytotic membrane fusion is stimulated by guanosine 5'-[γ -thio]triphosphate, and the phosphorylation states of several proteins, including at least one on the zymogen granule membrane, are known to change during exocytosis. We show here that a nucleoside diphosphate kinase is associated with the cytoplasmic face of pancreatic zymogen granules. This enzyme behaves as a phosphoprotein of apparent molecular mass 21 kDa on SDS/polyacrylamide gels, and is able to produce GTP by using ATP to phosphorylate endogenous GDP. GTP production by nucleoside diphosphate kinase is stimulated by the

wasp venom peptide mastoparan, both through a direct action on the enzyme and through its ability to increase the availability of endogenous GDP. Two effects of the GTP produced by nucleoside diphosphate kinase are demonstrated: phosphorylation of a 37 kDa zymogen granule protein on histidine residues, and stimulation of the fusion of zymogen granules with pancreatic plasma membranes *in vitro*. These results suggest that granule-associated nucleoside diphosphate kinase is able to maintain local GTP concentrations, and raise the possibility that it might be involved in the control of exocytosis in the pancreatic acinar cell.

INTRODUCTION

Nucleoside diphosphate kinase (NDP kinase) has been known for many years as the major source of cellular nucleoside triphosphates. The enzyme catalyses the transfer of high-energy phosphate from a nucleoside triphosphate (usually ATP because of its abundance in the cytosol) to a nucleoside diphosphate. The transfer is effected by a classic Ping-Pong mechanism involving a high-energy phosphohistidine intermediate [1,2]. Renewed interest in NDP kinases has stemmed from the identification of the products of the genes *nm23*, involved in mammalian tumour malignancy [3], and *awd*, crucial for *Drosophila* development [4,5], as NDP kinases. It is also known that NDP kinase-dependent production of GTP is able to regulate G-protein-controlled effector systems, such as K⁺ channels in cardiac membranes [6] and adenylate cyclase in various tissues [7].

GTP-binding proteins play a major role in the control of intracellular membrane traffic [8]. Most attention has focused on the small, monomeric, proteins of the rab family. These proteins are widely distributed within cells, and each organelle is enriched in particular members of the family [9]. More recently, however, it has become clear that heterotrimeric G-proteins are also involved in the control of membrane traffic along the secretory pathway. For example, they play a role in vesicle budding from the *trans*-Golgi network [10] and in regulated exocytosis in both chromaffin cells [11] and mast cells [12]. In the exocrine pancreas, guanosine 5'-[γ -thio]triphosphate (GTP[S]) has been shown to stimulate exocytosis in permeabilized acini [13,14] and also fusion between zymogen granules and plasma membranes *in vitro* [15,16]. Furthermore, both monomeric and heterotrimeric

GTP-binding proteins are present on the zymogen granule membrane [17,18]. The granule membrane also has protein kinase activity, and the phosphorylation of a 45 kDa protein has been shown to be stimulated by GTP[S] [19]. The evidence that both GTP-binding proteins and phosphoproteins are involved in the control of exocytotic membrane fusion in the exocrine pancreas is therefore very persuasive. In this study, we address the possibility that NDP kinase is responsible for maintaining GTP levels in the vicinity of the zymogen granules. We demonstrate the association of NDP kinase with isolated pancreatic zymogen granules, and show that GTP generated in response to provision of ATP can be used both to phosphorylate a granule protein of molecular mass 37 kDa and to stimulate fusion between granules and plasma membranes. Our results raise the possibility that NDP kinase is involved in supporting exocytosis in the exocrine pancreas.

EXPERIMENTAL

Materials were obtained from the following sources: [γ -³²P]ATP (3000 Ci/mmol), [γ -³²P]GTP (> 5000 Ci/mmol) and enhanced chemiluminescence kit, Amersham International (Amersham, Bucks., U.K.); GTP[S], pyruvate kinase and lactate dehydrogenase, Boehringer (Mannheim, Germany); octadecylrhodamine B-chloride (R18), Molecular Probes (Eugene, OR, U.S.A.); molecular mass markers, protein assay reagent and horseradish peroxidase-conjugated goat anti-rabbit IgG, Bio-Rad (Hemel Hempstead, U.K.). All other reagents were from Sigma Chemical Co. (Poole, Dorset, U.K.).

Abbreviations used: ATP[S], adenosine 5'-[γ -thio]triphosphate; GTP[S], guanosine 5'-[γ -thio]triphosphate; NDP, nucleoside diphosphate; R18, octadecylrhodamine B-chloride.

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For the preparation of zymogen granules, male Sprague–Dawley rats (200–250 g) were fasted overnight and killed by cervical dislocation. The pancreas was removed and placed in Krebs–Henseleit solution (103 mM NaCl, 4.8 mM KCl, 1.2 mM MgCl_2 , 1.2 mM NaH_2PO_4 , 2 mM CaCl_2 , 2.5 mM NaHCO_3) at 4 °C. Adipose tissue was trimmed away and the remaining tissue was minced with scissors in ice-cold unbuffered 280 mM sucrose. The tissue was homogenized by six strokes in a glass/glass hand-driven homogenizer (Jencons) and centrifuged at 400 *g* for 10 min. The supernatant was centrifuged at 900 *g* for 10 min to produce a tight white granule pellet overlaid by a loose layer of mitochondria. The supernatant was removed (and on some occasions centrifuged at 100 000 *g* for 1 h to produce a cytosolic fraction), and the mitochondria were removed by washing with 280 mM sucrose/5 mM Mes buffer, pH 6.0. The granules were resuspended in 300 μl of the same buffer to a protein concentration of 5–10 mg/ml [20].

In some experiments, granules (150 μl of suspension) were washed by addition of 1 ml of a buffer containing 170 mM NaCl/200 mM NaHCO_3 (1:3, v/v), 1 mM EDTA, pH 7.8 (lysis buffer). The suspension was left on ice for 15 min, during which time most of the granules lysed. Unlysed granules were pelleted by centrifugation for 2 min in an Eppendorf Microfuge. Sucrose/Mes buffer, pH 6.5 (4 ml), was added to the supernatant, which was then centrifuged at 100 000 *g* for 30 min at 4 °C. The pellet, consisting of zymogen granule membranes, was resuspended in sucrose/Mes buffer, pH 6.5.

The NDP kinase assay exploits the fact that phosphorylation of dUDP using ATP as the phosphate donor leads to the generation of ADP. The ADP produced is assayed through the oxidation of NADH to NAD^+ in reactions catalysed by pyruvate kinase and lactate dehydrogenase in the presence of appropriate substrates and cofactors. The oxidation of NADH was followed through the decrease in absorbance at 340 nm. Reaction mixtures contained 7.3 mM ATP, 10 mM MgCl_2 (3 mM free Mg^{2+}), 10 mM KCl, 6.3 mM phosphoenolpyruvate, 1.92 mM NADH, 1.26 units/ml pyruvate kinase and 33 units/ml lactate dehydrogenase in sucrose/Mes buffer, pH 6.5. Reactions were initiated by addition of zymogen granules (5 μl ; approximately 25 μg of protein) to 170 μl of reaction mixture in a flat-bottomed 96-well plate, in the presence or absence of 0.86 mM dUDP. Reactions were carried out at room temperature. A series of absorbance measurements was made at 340 nm using a Titertek Multiskan MCC/340 microtitre plate-reader (Flow Laboratories Ltd., Rickmansworth, U.K.). NDP kinase activity was taken to be the difference between the rate of change of absorbance in the presence and absence of dUDP. The change in absorbance occurring in the absence of dUDP is likely to be due to the presence of ADP in the granule suspension. NDP kinase activity was expressed in units/mg of protein.

To detect NDP kinase-catalysed generation of GTP, granules were incubated at 37 °C for various times with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (0.1 μM). Reactions were stopped by denaturation of proteins with 5% (w/v) trichloroacetic acid on ice, and the trichloroacetic acid was then removed by extraction with diethyl ether. Briefly, an equal volume of water-saturated ether was added to the sample and mixed thoroughly. The ether was aspirated and the process repeated. Finally the sample was acidified with 10 mM HCl and a final ether extraction performed. Samples were then allowed to stand until any final traces of ether had evaporated. A 1 μl aliquot of the sample was diluted 50-fold in water containing 2 mmol of unlabelled ATP and GTP standards and analysed by HPLC, using a C18 column (Jones Chromatography, Hengoed, U.K.) with 5 mM tributylamine, 10% methanol and 10 mM diammonium orthophosphate, pH 7.4, as the mobile

phase. Fractions were collected, and radioactivity was determined by liquid scintillation counting.

Phosphorylation of zymogen granule proteins was achieved by incubation of granules at 37 °C for various times in a total volume of 20 μl of sucrose/Mes buffer, pH 6.5, at a protein concentration of 2.5 mg/ml, in the presence of 1 mM dithiothreitol, protease inhibitors (10 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor, 1 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ antipain, 5 $\mu\text{g}/\text{ml}$ benzamidin, 1 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ pepstatin) and either $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (0.1 μM) or $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (0.06 μM). Reactions were stopped by the addition of an equal volume of gel loading buffer containing 250 mM dithiothreitol, 5% SDS, 0.2 M Tris/HCl, pH 6.8, 25% glycerol and 0.02% Bromophenol Blue. Note that samples were not boiled. Proteins were separated by SDS/PAGE (12.5% acrylamide). Gels were stained with Coomassie Brilliant Blue and sealed in a polythene bag, prior to exposure to Kodak X-ray film overnight (typically 15 h) at –70 °C. Protein phosphorylation was quantified by densitometric scanning using a Molecular Dynamics 425E-130 PhosphorImager[®] with ImageQuant[®] software (Molecular Dynamics Inc., Sevenoaks, Kent, U.K.). Phosphorylation was expressed as a percentage of the maximum phosphorylation obtained in each experiment.

For acid/base treatment of phosphorylated proteins, granules were incubated at 37 °C with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, and identical samples from a single reaction were separated by SDS/PAGE. After staining with Coomassie Brilliant Blue, the gel was cut up into lanes, which were then incubated at room temperature for 1 h, with rocking, in water, 1 M HCl or 1 M NaOH. For hydroxylamine treatment, gel lanes were incubated at room temperature for 1 h, with rocking, in water, 250 mM hydroxylamine, pH 7.0 (freshly prepared from NaOH and hydroxylamine hydrochloride [21]), or 250 mM NaCl, as a control. After the various incubations, the gels were reassembled, sealed in polythene bags and autoradiographed overnight.

Fusion between zymogen granules and pancreatic plasma membranes was measured through the dilution-dependent de-quenching of the fluorescence of R18, which was loaded into the granule membrane [16]. Granules from a single rat (300 μl of suspension in sucrose/Mes buffer, pH 6.0; protein concentration approx. 5 mg/ml) were loaded with R18 (100 μM) by incubation at 37 °C for 5 min. Labelled granules were recovered by centrifugation at 900 *g* for 10 min and resuspended in the original volume of buffer. Plasma membranes were prepared from rat pancreas as described previously [15], stored in batches at –20 °C and thawed immediately before use. De-quenching assays were carried out using a Hitachi F-2000 (Wokingham, U.K.) fluorescence spectrometer. Wavelengths used were 560 nm (excitation) and 590 nm (emission). Samples of labelled granules (10 μl), together with test reagents, where appropriate, were added to 700 μl of sucrose/Mes buffer, pH 6.5, at 37 °C, and incubated for 100 s to achieve a steady baseline. Plasma membranes (20 $\mu\text{g}/\text{ml}$ protein) were then added, and the fluorescence signal was monitored for 200 s. In each experiment, the fluorescence signals were expressed as a percentage of the maximum signal obtained in each experiment. Errors are S.E.M., from *n* independent experiments. Statistical significance of differences was assessed using Student's *t* test, with *P* < 0.05 being taken as significant.

RESULTS

Association of NDP kinase with zymogen granules

The presence of NDP kinase in various fractions prepared from rat pancreas was investigated by immunoblot analysis, using an antiserum raised against the rat enzyme ([22]; kindly provided by Dr. G. Blevins, University of Arkansas, Little Rock, AR, U.S.A.).

Figure 1 Immunoblot analysis of NDP kinase associated with various pancreatic fractions

Samples (10 μ g of protein) of zymogen granules (lane 1), granule membranes (lane 2), granule membranes washed with 1 M NaCl for 2 h at 4 $^{\circ}$ C (lane 3), plasma membranes (lane 4) and cytosol (lane 5) were subjected to SDS/PAGE. The separated proteins were transferred to nitrocellulose and probed with an antiserum raised against rat NDP kinase, at a 1:2000 dilution. Bound antibody was visualized using horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1000) and enhanced chemiluminescence. The closed arrowhead identifies NDP kinase; open arrowheads identify the positions of molecular mass markers (kDa).

As shown in Figure 1, the mobility of the enzyme indicates a molecular mass of 21 kDa. Strong signals were given by cytosol, zymogen granules and plasma membranes (in decreasing order of intensity). Following lysis of the granules by treatment with lysis buffer containing 1 mM EDTA, the majority of immunoreactive NDP kinase was lost from the zymogen granule membranes. The small signal remaining after lysis, however, was not completely removed by washing the membranes with 1 M NaCl for 2 h at 4 $^{\circ}$ C. These results indicate that NDP kinase is found in the cytosol, but that it also co-purifies with zymogen granules. The association of NDP kinase with membrane fractions from the exocrine pancreas has been reported previously [23], and has been shown to survive extensive washing. Under the washing conditions used here, however, the binding of the bulk of the NDP kinase to the granules is relatively weak, although a small fraction of the enzyme appears to be very tightly bound.

Characterization of NDP kinase activity

NDP kinase activity associated with zymogen granules was assayed through the production of ADP during the transphosphorylation of dUDP with ATP. ADP production was measured through the decrease in absorbance at 340 nm as a consequence of the oxidation of NADH, in reactions catalysed by pyruvate kinase and lactate dehydrogenase. NDP kinase activity was found to be associated with zymogen granules. The granules remained largely intact during these incubations, and the measured enzyme activity was found to be unaffected by treatment with lysis buffer (results not shown). The possibility that NDP kinase is a granule contents enzyme can therefore be excluded. NDP kinase activity was absolutely dependent on the presence of Mg^{2+} ions, with peak activity at 3 mM Mg^{2+} . Interestingly, NDP kinase activity was found to be stimulated by the wasp venom peptide mastoparan, which is known to activate the heterotrimeric G-proteins G_i and G_o [24]. At a Mg^{2+} concentration that was optimal for enzyme activity (3 mM), the effect of mastoparan was relatively small (16% stimulation). However, at a sub-optimal Mg^{2+} concentration (0.3 mM), its effect was proportionally much larger (170% stimulation).

Figure 2 Phosphorylation of zymogen granule proteins

Granules were incubated for various times, as indicated, at 37 $^{\circ}$ C with either [γ - 32 P]ATP or [γ - 32 P]GTP. Proteins were then separated by SDS/PAGE and visualized by autoradiography. Closed arrowheads identify phosphorylated proteins; open arrowheads identify the positions of molecular mass markers (kDa).

NDP kinase-catalysed generation of GTP by phosphorylation of endogenous and exogenous GDP

The NDP kinase on the zymogen granules was able to produce GTP when provided with ATP and GDP. At zero time, the only nucleoside triphosphate detected by HPLC was ATP. After a 5 min incubation in the presence of exogenous GDP (100 μ M), however, the ATP peak was replaced by a GTP peak. GTP was also produced during incubation in the absence of exogenous GDP, which indicates that there is sufficient GDP associated with the isolated granules to act as a substrate for the NDP kinase. The presence of mastoparan (10 μ M) during a 1 min incubation caused a 1.2-fold increase in the size of the GTP peak and a corresponding decrease in the size of the ATP peak. Total nucleoside triphosphate levels fell by approx. 5-fold between 1 min and 5 min of incubation, although the size of the GTP peak relative to that of the ATP peak continued to rise. At 5 min, mastoparan (10 μ M) caused a 1.7-fold increase in the size of the GTP peak and again a decrease in the size of the ATP peak. The fall in total counts in nucleoside triphosphates over 5 min of incubation was much greater when exogenous GDP was absent. This result suggests that ATP is consumed rapidly by ATPases, but that any GTP produced is relatively stable.

Phosphorylation of granule proteins

When zymogen granules were incubated with [γ - 32 P]ATP, five granule-associated proteins (molecular masses 21, 37, 45, 67 and 125 kDa) became radiolabelled (Figure 2). Only three of these proteins, p21, p37 and p125, became phosphorylated during incubation of the granules with [γ - 32 P]GTP. p125 was labelled more weakly with [γ - 32 P]GTP than with [γ - 32 P]ATP, suggesting that the protein kinase responsible for its phosphorylation has a preference for ATP. In contrast, p37 was labelled much more strongly with [γ - 32 P]GTP than with [γ - 32 P]ATP, suggesting a preference of its protein kinase for GTP. p21 was labelled strongly in both cases. The phosphorylation of p45 has been studied previously, and shown to be modulated by both Ca^{2+} and GTP[S] [19]. In the present study, we have focused our attention on the phosphorylation of p21, which is rapid and transient, and

Figure 3 Effect of cromoglycate on p21 phosphorylation

Zymogen granules were phosphorylated with [γ - 32 P]ATP for 20 s at 37 °C in the presence of various concentrations of cromoglycate. Proteins were separated by SDS/PAGE and visualized by autoradiography. (A) Typical autoradiograph; (B) quantification of p21 phosphorylation. Phosphorylation is expressed as a percentage of the maximum phosphorylation obtained in each experiment. Values are means $\pm$ S.E.M. ($n = 3$).

of p37, which is prominent only when gels are exposed without heating/drying (note that all autoradiographs were produced without boiling the proteins in sample buffer and without drying the gels).

p21: identity with NDP kinase

p21 has features that indicate that it is the phosphorylated enzyme intermediate of NDP kinase. First, NDP kinase has already been shown to migrate on gels as a 21 kDa protein (see Figure 1). Secondly, analysis of zymogen granule phosphoproteins by gel filtration indicates that the molecular mass of the undenatured protein is approx. 100–140 kDa (results not shown). This indicates that p21 is a hexamer, as previously reported for NDP kinase [2]. Thirdly, labelling of p21 occurs efficiently even on ice (results not shown), a characteristic of phosphorylated enzyme intermediates (see [25]).

To test further the possibility that p21 is NDP kinase, pharmacological modulation of its phosphorylation was investigated. Cromoglycate has been shown to inhibit the autophosphorylation of NDP kinase *in vitro* [26]. Zymogen granules were incubated with various concentrations of the drug on ice for 20 min prior to addition of [γ - 32 P]ATP. Phosphorylation was then carried out at 37 °C for 20 s. Cromoglycate was found to inhibit labelling of p21, with an IC_{50} of 1.8 mM (Figure 3). This value is in close agreement with reported values (e.g. 2 mM [26]).

Since the mechanism of action of NDP kinase involves transphosphorylation of nucleoside diphosphates, it has been proposed that competitive inhibition can be produced by the addition

Figure 4 Effect of UDP on p21 phosphorylation

Zymogen granules were phosphorylated with [γ - 32 P]ATP for 20 s at 37 °C in the presence of various concentrations of UDP, as indicated. Proteins were separated by SDS/PAGE and visualized by autoradiography. A typical autoradiograph is shown.

Figure 5 Chemical cross-linking of ATP and GTP to zymogen granule proteins

Zymogen granules were incubated with [γ - 32 P]ATP (lanes 1 and 2) or [γ - 32 P]GTP (lanes 3 and 4) for 10 min at 37 °C. Nucleotides were then cross-linked to proteins to which they were bound by the sequential addition of NaIO₄, NaCNBH₃ and NaBH₄. In controls (lanes 2 and 4), NaIO₄ was omitted. Proteins were separated by SDS/PAGE and cross-linked nucleotides were visualized by autoradiography. Labelled proteins (kDa) are indicated by closed arrowheads. Molecular mass markers (kDa) are indicated by open arrowheads.

of UDP at high concentrations [27]. As shown in Figure 4, UDP decreased phosphorylation of p21 in a concentration-dependent manner, a result which supports the suggestion that p21 is NDP kinase.

If p21 is indeed NDP kinase, then it should bind both ATP and GTP. To test this, an affinity labelling experiment was carried out using a modification of the method of Löw et al. [28]. Zymogen granules were incubated with [α - 32 P]ATP or [α - 32 P]GTP for 10 min at 37 °C. Nucleotides were oxidized by the addition of 4 mM NaIO₄ for 1 min at 37 °C and then reduced by addition of 80 mM NaCNBH₃ for 1 min at 37 °C. Finally, 100 mM NaBH₄ was added for 1 h on ice. In controls NaIO₄ was replaced by water. Proteins were analysed by SDS/PAGE and autoradiography. As shown in Figure 5, a 21 kDa band was labelled by both ATP and GTP, consistent with its being NDP kinase.

By using p21 as a marker of NDP kinase, the nature of the attachment of the enzyme to the zymogen granule membrane was investigated. Granule membranes were prepared with labelled p21 bound by incubating granules with [γ - 32 P]ATP on ice for 10 min, with Mg²⁺ at 10 mM, followed by addition of granule lysis buffer. The membranes were then collected by centrifugation at 100 000 *g* for 30 min in the presence or absence of 10 mM EDTA. Equal amounts of protein in the various samples were then analysed by SDS/PAGE and autoradiography. It was found that, in the absence of EDTA, all of the labelled p21 was associated with the pelleted membranes,

Figure 6 Association of p21 with the zymogen granule membrane

Zymogen granules were phosphorylated with [γ - 32 P]ATP for 10 min on ice and a sample of the granules was taken (lane 1). The remaining granules were lysed in the presence of either 10 mM Mg^{2+} (lanes 2 and 3) or 10 mM EDTA (lanes 4 and 5) and the granule membranes were pelleted by centrifugation at 70 000 g for 1 h. Samples of pellets (lanes 2 and 4) and supernatants (lanes 3 and 5) were taken. Sample sizes were normalized to an equal volume of the original granule suspension. Proteins were separated by SDS/PAGE and visualized by autoradiography. T, total; P, pellet; S, supernatant.

Figure 7 Characterization of the phosphorylation of p37

Granules were phosphorylated with [γ - 32 P]ATP for 10 min at 37 °C and the proteins were separated by SDS/PAGE. After electrophoresis, the gels were cut into lanes and subjected to drying at 80 °C for 2 h (lane 2), acid hydrolysis (1 M HCl for 1 h; lane 4), base hydrolysis (1 M NaOH for 1 h; lane 5), or treatment with either hydroxylamine (250 mM for 1 h; lane 7) or NaCl (250 mM for 1 h; lane 8). Lanes 1, 3 and 6 were the corresponding controls. After treatment, the gels were reconstituted and autoradiographed.

while in the presence of EDTA a large proportion of the p21 was released into the supernatant (Figure 6). This result indicates that bivalent cations are required for the interaction of p21 with the granule membrane. It also explains why NDP kinase was efficiently removed from the membrane by treatment with a buffer containing 1 mM EDTA (Figure 1).

p37: phosphorylation on histidine, and modulation by mastoparan

The phosphorylation of p37 was extremely heat-labile, being significantly decreased by gel drying with heat (80 °C for 2 h; Figure 7) and almost abolished by boiling the samples for 3 min prior to SDS/PAGE (results not shown). This is in contrast with p45, which we have shown to be phosphorylated exclusively on serine residues [19], which is resistant to heating. The labelling of p37 was also sensitive to acid (1 M HCl for 1 h), but not base (1 M NaOH for 1 h), hydrolysis, whereas p45 labelling was removed by base, but not by acid, hydrolysis. Incubation of labelled protein with hydroxylamine (250 mM for 1 h), known to remove phosphate groups bound to histidine residues [29], also caused a loss of label from p37. In contrast, p45 labelling was not sensitive to hydroxylamine (results not shown). These characteristics strongly indicate that phosphorylation of p37 is via a high-energy phosphoramidate bond to a histidine residue.

The results shown in Figure 2 indicated that p37 is phosphorylated by GTP. The presence of NDP kinase on the zymogen granule membrane provides a means of producing GTP by ATP-dependent phosphorylation of GDP. To see whether this mechanism actually operates, we examined the effects of GDP and ADP on p37 phosphorylation. It was found that GDP had a dual effect on the phosphorylation of p37 by [γ - 32 P]ATP, stimulating

Figure 8 Effects of GDP/ADP on phosphorylation of p37

Granules were phosphorylated with [γ - 32 P]ATP for 15 min at 37 °C in the presence of various concentrations of either GDP or ADP. Proteins were separated by SDS/PAGE and detected by autoradiography. (A) Typical autoradiographs. (B) Quantification of the effects of GDP (■) and ADP (●). Phosphorylation is expressed as a percentage of the maximum phosphorylation obtained in each experiment. Values are means $\pm$ S.E.M. ($n = 3$).

at concentrations up to 0.1 μ M and then inhibiting at higher concentrations (Figure 8). In contrast, ADP caused only a concentration-dependent decrease in the labelling of p37 over the entire range tested. The stimulatory effect of GDP is likely to be a consequence of an increase in [γ - 32 P]GTP generation, since it peaks at a similar concentration to that of the added [γ - 32 P]ATP.

Since mastoparan stimulates the NDP kinase-catalysed production of GTP from ATP and GDP, one would expect it to enhance the phosphorylation of p37 by [γ - 32 P]ATP. As shown in Figure 9, mastoparan caused a concentration-dependent stimulation of the phosphorylation of p37. At the optimal mastoparan concentration (30 μ M), p37 phosphorylation was increased more than 2-fold over control, in line with the stimulation of NDP kinase seen at the low concentration of Mg^{2+} present during phosphorylation. The stimulatory action of mastoparan was specific to p37, since phosphorylation of p45 was not significantly affected at concentrations up to 30 μ M. At a high concentration (100 μ M), mastoparan inhibited phosphorylation of both p37 and p45 (results not shown), presumably through a second, non-specific, effect. Phosphorylation of p37 by [γ - 32 P]GTP was inhibited by mastoparan over the same concentration range, presumably because the GTP was used by NDP kinase to phosphorylate other nucleoside diphosphates present in the granule fraction, such as ADP. Figure 9 also shows the effect of GTP[S] on the phosphorylation of p45 and p37. As reported previously [16], GTP[S] stimulated phosphorylation of p45 by

Figure 9 Effects of mastoparan and GTP[S] on the phosphorylation of p37 and p45

Zymogen granules were phosphorylated with either [γ - 32 P]ATP or [γ - 32 P]GTP for 10 min at 37 °C in the presence of various concentrations of mastoparan (MP), or GTP[S] at 100 μ M (lane 7). Proteins were separated by SDS/PAGE and detected by autoradiography. (A) Typical autoradiographs. Closed arrowheads identify labelled proteins; open arrowheads identify the positions of molecular mass markers (kDa). (B) Quantification of the phosphorylation of p37 (●) and p45 (■). Phosphorylation is expressed as a percentage of the maximum phosphorylation obtained in each experiment. Values are means $\pm$ S.E.M. ($n = 3$).

[γ - 32 P]ATP. On the other hand, phosphorylation of p37, both by [γ - 32 P]ATP and by [γ - 32 P]GTP, was almost abolished by GTP[S], perhaps as a result of the binding of GTP[S] to the GTP-dependent protein kinase. Benzalkonium chloride, which activates G_o [24], also produced a concentration-dependent stimulation of p37 labelling, which was at least additive with the effect of mastoparan (results not shown).

An increase in the extent of conversion of ATP into GTP in the presence of mastoparan might be achieved either by direct stimulation of NDP kinase, or more indirectly by favouring the

Figure 10 Effect of GDP on the phosphorylation of p37 in the absence and presence of mastoparan

Proteins were phosphorylated with [γ - 32 P]ATP for 5 min in the presence of various concentrations of GDP, and in the absence or presence of mastoparan (MP; 10 μ M). Proteins were separated by SDS/PAGE and visualized by autoradiography. (A) Typical autoradiographs. (B) Quantification of the effects of GDP in the absence (●) and presence (■) of mastoparan (10 μ M). Phosphorylation is expressed as a percentage of the maximum phosphorylation obtained in each experiment. Values are means $\pm$ S.E.M. ($n = 3$).

release of GDP from G-proteins. If mastoparan acted by stimulating NDP kinase, it should enhance p37 labelling at all GDP concentrations. If on the other hand it acted by raising GDP levels, it should shift the optimal GDP concentration for stimulation of p37 phosphorylation to lower values, without having a significant effect on the absolute degree of stimulation. As shown in Figure 10, mastoparan (10 μ M) shifted the GDP optimum from 100 nM to 10 nM. However, the whole concentration–response curve was also raised significantly in the presence of mastoparan. This result indicates that mastoparan has a dual effect on the phosphorylation of p37 by ATP.

Promotion of exocytotic membrane fusion through NDP kinase activity

We have shown previously [15,16] that fusion between zymogen granules and pancreatic plasma membranes is stimulated by both GTP[S] and GTP. This raises the possibility that granule-associated NDP kinase might support this membrane fusion event, through the generation of GTP. To test this possibility, we examined the effects of ATP and GDP, alone and in combination, in the *in vitro* fusion system. The results are shown in Table 1. As expected, GTP[S] (100 μ M) stimulated fusion. Neither GDP (not shown) nor ATP had any significant effect when added alone. However, GDP in the presence of ATP caused a concentration-

Table 1 Effects of GDP/ATP on exocytotic membrane fusion *in vitro*

Fusion between zymogen granules and pancreatic plasma membranes was measured through the dilution-dependent de-quenching of the fluorescence of R18, which was loaded into the granule membrane. Plasma membranes were added to labelled granules, and the fluorescence signal was monitored at 37 °C for 200 s. Incubation mixtures contained no additives, GTP[S] (100 μ M) or ATP (1 mM) plus various concentrations of GDP. Fluorescence signals were expressed as a percentage of the maximum signal obtained in each experiment. Values are means $\pm$ S.E.M. ($n = 11$). * $P < 0.01$ compared with fusion in the absence of GDP.

Condition	Fusion (% of max.)
Control	66 $\pm$ 4
+ GTP[S]	94 $\pm$ 3
ATP	79 $\pm$ 5
ATP + GDP (1 μ M)	76 $\pm$ 7
ATP + GDP (10 μ M)	83 $\pm$ 5
ATP + GDP (100 μ M)	91 $\pm$ 4*
ATP + GDP (1 mM)	81 $\pm$ 4

dependent stimulation of fusion. At 100 μ M GDP, fusion was significantly greater than in the absence of GDP, but was not significantly different from that in the presence of GTP[S]. Fusion fell at higher GDP concentrations. This dual effect of GDP on fusion is reminiscent of its effect on the phosphorylation of p37 in the presence of [γ - 32 P]ATP.

DISCUSSION

In this study we have shown that NDP kinase is associated with the cytoplasmic face of zymogen granules, and that both its membrane binding and its enzyme activity are Mg^{2+} -dependent. The enzyme behaves as a protein of molecular mass 21 kDa on SDS/polyacrylamide gels, and is almost certainly identical with the protein p21, which can be phosphorylated by both ATP and GTP. Mastoparan, which is known to activate heterotrimeric G-proteins of the G_i/G_o type by promoting GDP/GTP exchange [24], has been shown to stimulate zymogen granule-associated NDP kinase. A stimulatory effect of mastoparan on NDP kinase has been reported previously [30]. It was shown that mastoparan activated NDP kinase at concentrations at least an order of magnitude lower than those required to activate G_o , which was taken to indicate a direct effect on the enzyme. In our experiments, the EC_{50} for the effect of mastoparan on NDP kinase (approx. 3 μ M) was close to the reported value for activation of G_o . We found no direct evidence, however, for the involvement of a G-protein in the effect of mastoparan. AlF_{3-5} , another G-protein activator, was without effect on p37 phosphorylation (results not shown), although the possibility that this agent has additional effects on protein kinase activity cannot be ignored. The fact that the phosphorylation of p37 by ATP was stimulated both by mastoparan and by benzalkonium chloride does point towards the involvement of a G-protein, although it is also possible that both agents are able to affect NDP kinase activity directly. The observation that zymogen granule-associated NDP kinase may be under some form of control raises the interesting possibility that it might be involved in regulating exocytosis, through its ability to generate GTP close to the site of exocytotic membrane fusion. However, it should be borne in mind that the stimulatory effect of mastoparan was large only at submaximal Mg^{2+} concentrations, and relatively small at the millimolar concentrations known to exist in the cell.

GTP generated through the action of zymogen granule-associated NDP kinase is used to phosphorylate p37 on histidine residues. A role for the phosphorylation of proteins on

histidine residues in the regulation of cell function has been described previously. For example, it is involved in the control of chemotactic responses of bacteria [31] and in *nif* gene expression in *Escherichia coli* [32]. Similar regulatory systems have also been found in eukaryotes, such as that controlling the responses of yeast to osmotic changes [33]. Histidine phosphorylation has been described in mammalian cells in the form of protein histidine kinase activity in the nucleus [34], and the histidine phosphorylation of a 36 kDa protein associated with liver membranes [35,36]. Histidine phosphorylation of p36 is a regulated process; it is enhanced by stimulation with glucagon and guanine nucleotides, and also by activated ras protein *in vitro* [35,36]. p36 and its kinase have been shown to be peripheral membrane proteins, which can be extracted from the membrane by chelation of bivalent cations with EDTA [36]. The native protein has an apparent molecular mass of 70–75 kDa by gel filtration chromatography, suggesting that it may dimerize. It would be interesting to determine whether there is any relationship between p36 in the liver and p37 on the zymogen granule membrane.

It has been reported that the β -subunit of a heterotrimeric G-protein (molecular mass 35 kDa) can be transiently phosphorylated on histidine residues [37]. It was also suggested that the high-energy phosphate can then be used to phosphorylate GDP bound to the α -subunit. This idea of activation of GTP-binding proteins, both monomeric and trimeric, by phosphorylation of bound GDP has been pursued by several groups, although a recent careful study revealed no evidence for this mechanism [38]. It was concluded instead that GDP has to dissociate from the GTP-binding protein before it can be phosphorylated by NDP kinase. Nevertheless, we tested the possibility that p37 might be a β -subunit by attempting to immunoprecipitate it with a specific antiserum (results not shown). The results were negative. At present, therefore, we cannot identify this protein, although its location and properties suggest that it might play a role in exocytosis.

We have shown that the generation of GTP from ATP and GDP catalysed by NDP kinase can promote the fusion of zymogen granules with pancreatic plasma membranes. It is not yet clear, however, whether the generation of GTP by NDP kinase at the site of membrane fusion in the pancreatic acinar cell is crucial to the maintenance of exocytosis. Under normal physiological conditions, cellular GTP levels seem to be sufficient to maintain GDP/GTP exchange at GTP-binding proteins. It is possible, however, that NDP kinase comes into play under conditions of high demand for GTP, which might exist during stimulation of exocytosis. In addition, it might be able to phosphorylate GDP as soon as it dissociates from the GTP-binding protein, thus accelerating re-activation. Of relevance here is the demonstration that the stimulatory effect of adenosine 5'-[γ -thio]triphosphate (ATP[S]) on exocytosis in permeabilized PC12 cells is due to the NDP kinase-catalysed thiophosphorylation of GDP to produce GTP[S] [39]. Significantly, the time course of ATP[S]-stimulated secretion and the poor inhibition of this process by GTP pointed to a site of GTP[S] synthesis close to the target GTP-binding protein. The location of NDP kinase on the cytoplasmic surface of the zymogen granule could therefore allow it to play an important role in the provision of GTP at the site of exocytosis in the acinar cell.

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