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Interaction of furin in immature secretory granules from neuroendocrine cells with the AP-1 adaptor complex is modulated by casein kinase II phosphorylation

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The composition of secretory granules in neuroendocrine and endocrine cells is determined by two sorting events; the first in the *trans*-Golgi complex (TGN), the second in the immature secretory granule (ISG). Sorting from the ISG, which may be mediated by the AP-1 type adaptor complex and clathrin-coated vesicles, occurs during ISG maturation. Here we show that furin, a ubiquitously expressed, TGN/endosomal membrane endoprotease, is present in the regulated pathway of neuroendocrine cells where it is found in ISGs. By contrast, TGN38, a membrane protein that is also routed through the TGN/endosomal system does not enter ISGs. Furin, however, is excluded from mature secretory granules, suggesting that the endoprotease is retrieved from the clathrin-coated ISGs. Consistent with this, we show that the furin cytoplasmic domain interacts with AP-1, a component of the TGN/ISG-localized clathrin sorting machinery. Interaction between AP-1 and furin is dependent on phosphorylation of the enzyme's cytoplasmic domain by casein kinase II. Finally, in support of a requirement for the phosphorylation-dependent association of furin with AP-1, expression of furin mutants that mimic either the phosphorylated or unphosphorylated forms of the endoprotease in AtT-20 cells demonstrates that the integrity of the CKII sites is necessary for removal of furin from the regulated pathway.

Keywords: AP-1/casein kinase II/furin/immature secretory granule

Introduction

Mammalian cells that exhibit regulated secretion sort and sequester a subset of their newly synthesized proteins into secretory granules which form from the *trans*-Golgi network (TGN). One consequence of this is the need for a further exit pathway from the TGN additional to the two ubiquitous TGN exit routes, one constitutively transporting proteins to the plasma membrane, the other transporting lysosomal enzymes to endosomes. Vesicles of the latter pathway are clathrin coated and arise via interaction of phosphorylated lysosomal enzymes with the mannose-6-phosphate receptor (MPR) (Hille-Rehfeld, 1995), while little is known about constitutive secretory vesicles (CSVs) which may have a lace-like coat (Ladinsky *et al.*, 1994). The molecular mechanisms giving rise to the third exit route from the TGN and the formation of the immature secretory granule (ISG) in endocrine and neuroendocrine cells are also not well understood.

The existence of three exit pathways from the TGN in cells with a regulated secretory pathway implies that tertiary sorting mechanisms exist in the TGN. However, no experiments have been performed in cells with a regulated pathway which have measured the efficiency of sorting into the three exit pathways from the TGN. Sorting in the TGN of a constitutively secreted heparan sulfated proteoglycan (hsPG) and the regulated secretory protein secretogranin II (SgII) into CSVs and ISGs, respectively, has been demonstrated in PC12 cells (Tooze and Huttner, 1990). On the other hand, in β-cells of the endocrine pancreas, lysosomal enzymes have been shown to enter ISGs with proinsulin, suggesting that there is little or no sorting of these molecules in the TGN during ISG formation (Kuliawat and Arvan, 1994). From these data, and additional data obtained in other regulated secretory cell lines and tissues (Castle *et al.*, 1987; Grimes and Kelly, 1992; J.Tooze *et al.*, 1991; Carnell and Moore, 1994), it seems most likely that the sorting efficiency in the TGN varies with the cell type or even with the cell clone. Post-TGN sorting mechanisms must then operate to remove non-secretory granule proteins from the ISG to ensure that the final mature secretory granule (MSG) has the correct composition. The extent of the post-TGN sorting mechanisms will be determined by the level of missorting in the TGN. In addition, mechanisms must operate to retain TGN-resident proteins in the TGN during the formation of TGN-derived vesicles. Morphological evidence implicates clathrin-coated vesicles (CCVs) in the removal of non-secretory granule membrane components (Orci *et al.*, 1985; Tooze and Tooze, 1986), and it is anticipated that the sorting of the soluble content will accompany the membrane remodelling event mediated by the putative CCVs.

As an ISG matures to an MSG, several events occur which alter the composition of the short-lived ISG such that it becomes an MSG, the most abundant population of secretory granules at steady-state (Tooze *et al.*, 1993). Both the size of the secretory granule and the structure of the dense core change during maturation (Farquhar *et al.*, 1978; Huang and Arvan, 1995). The change in secretory granule size in some cell types has been postulated to be a result of homotypic fusion of ISGs: between three and five ISGs on average fuse in PC12 cells to form an MSG (S.Tooze *et al.*, 1991). During homotypic fusion, excess membrane will be generated to the extent that up to seven CCVs could be formed from the ISGs. This would allow post-TGN sorting mechanisms dependent upon CCV formation to occur. Other changes in the ISG commence

during maturation, for example a decrease in the lumenal pH from pH 6.3 (Urbé *et al.*, 1997) to pH 5.5 (Johnson and Scarpa, 1975), and processing of prohormones by prohormone convertases. Intragranular pH changes and processing of prohormones result in a physical change in the behaviour of the soluble content proteins (Gerdes *et al.*, 1989) and the structure of the dense core (Michael *et al.*, 1987; Huang and Arvan, 1994). These physical changes have also been implicated in the post-TGN sorting mechanisms (Arvan and Castle, 1992).

We are interested in the maturation of ISGs in PC12 cells, in particular the nature and function of the clathrin coat on the ISGs. In general, the formation of a clathrin coat on membranes requires an initial association of the adaptor complex with the membrane, followed by clathrin binding and polymerization. Using an assay that reconstitutes the formation of a clathrin coat on the ISG, we have demonstrated that the clathrin coat on the ISG is composed of the adaptor complex AP-1, and recruitment of AP-1 onto ISG membranes requires ADP-ribosylation factor (ARF) (Dittié *et al.*, 1996). AP-1, the TGN-specific adaptor, is a heterotetramer of two large (γ and β1) and two small (μ1 and σ1) subunits (for recent reviews, see Robinson, 1997; Traub, 1997). Interaction of AP-1 with the membrane is postulated to be mediated through interaction of specific signals in the cytoplasmic tail of transmembrane receptors with μ1 (Ohno *et al.*, 1995) and additional docking proteins (Traub *et al.*, 1993; Mallet and Brodsky, 1996; Seaman *et al.*, 1996). These AP-1 docking proteins, which have not yet been identified, are thought to bind AP-1 in an ARF-dependent fashion. Furthermore, AP-1 has been shown to interact with the MPR in a phosphorylation-dependent reaction: the binding of AP-1 to TGN membranes is increased by phosphorylation of the cytoplasmic domain of MPR by casein kinase II (CKII) (Le Borgne *et al.*, 1993; Mauxion *et al.*, 1996).

The question we have addressed is whether TGN-resident proteins that recycle to and from the plasma membrane, e.g. furin and TGN38, are also sorted into ISGs of the regulated exocytotic pathway, and, if so, do they interact with AP-1? Both furin, a TGN-resident endoprotease belonging to the family of subtilisin-related serine proteases (Roebroek *et al.*, 1986; van de Ven *et al.*, 1990) and TGN38 (Luzio *et al.*, 1990) are found in TGN membranes and in most post-Golgi compartments including endosomal membranes and the plasma membrane (Molloy *et al.*, 1994). By immunolabelling techniques, both furin and TGN38 have been shown to co-localize with clathrin-coated membranes (Schäfer *et al.*, 1995) or AP-1 (Reaves and Banting, 1994), respectively. However, there is evidence that TGN38 may associate in the TGN with a lace-like coat complex distinct from the clathrin coat (Jones *et al.*, 1993; Ladinsky *et al.*, 1994). To determine whether furin or TGN38 are sorted into ISGs, we examined the distribution of these two proteins in neuroendocrine cells. We show here that while TGN38 is not detected in ISGs, furin is found in ISGs, but not in MSGs, suggesting that furin is removed from ISGs during maturation. We have examined the role of the cytoplasmic domain of furin in the association of AP-1 with the ISG. Phosphorylation of the cytoplasmic domain of furin by CKII is required for AP-1 association, and CKII-phosphorylated furin tails can inhibit AP-1 association with ISGs. Finally, we show that furin mutants in which the CKII site is destroyed remain in MSGs. Thus, these observations provide evidence that the clathrin coat on the ISG functions to remove proteins from the ISG during maturation.

Results

Localization of furin to immature secretory granules

In neuroendocrine cells, the ISG formed from the TGN is a short-lived intermediate. We have developed several methods to study ISGs in PC12 cells using biosynthetic labelling and cell fractionation (S.Tooze *et al.*, 1991; Dittié *et al.*, 1996). Employing these methods, we asked whether we could detect TGN-resident proteins in the ISG in PC12 cells. Two TGN-resident proteins that are known to recycle to the plasma membrane are TGN38 and furin. The distribution of TGN38 and furin across two well-characterized sucrose gradients was assayed by immunoblotting with tail-specific antibodies to either TGN38 or furin (Figure 1). TGN38 was not found in fractions at the top of the velocity gradient whereas furin was detected in these fractions (fractions 3 and 4), where light vesicles, including ISGs, are found. Both TGN38 and furin were found in fractions 8, 9 and 10 (Figure 1A) which contain TGN membranes (Tooze and Huttner, 1990). Significant immunoreactivity for TGN38 was found in fractions 5–7: we believe this represents TGN38 in early endosomes which are detected in these fractions after a 7 min horseradish peroxidase (HRP) internalization (A.Dittié and S.Tooze, unpublished results). Subsequent fractionation on equilibrium sucrose gradients of the light vesicle pool demonstrated that a population of vesicles containing furin, but not TGN38, co-sedimented in fractions 7–9 with ISGs (Figure 1B).

To determine if furin is also detectable in mature secretory granules, we subjected a heavy vesicle pool from the velocity gradient to equilibrium centrifugation and, after fractionation, analysed the gradient fractions (Figure 1C). Vesicles containing furin from the heavy vesicle pool sedimented as a broad peak across fractions 5–9, and not in fractions 9–12 which contain MSGs. This indicates that the abundant MSGs which are highly enriched in this fraction do not have detectable amounts of the transmembrane form of furin. The distribution of furin and TGN38 in fractions 5–9 on the equilibrium gradient (Figure 1C) was similar to that of early endosomes identified by a 7 min internalization of HRP (A.Dittié and S.Tooze, unpublished results). Therefore, although furin and TGN38 co-sediment on sucrose gradients in fractions containing TGN membranes and endosomes, TGN38 is not found in the fractions containing post-Golgi vesicles and ISGs.

Although the co-sedimentation of proteins in vesicles through two sucrose gradients is strong evidence that the proteins are in the same vesicle, we used an immunoisolation approach to demonstrate this point directly. Immunoisolation of membranes containing a compartment-specific marker protein with antibodies raised against the cytoplasmic domain of another protein can be used to demonstrate co-localization of the two proteins. We used the TGN-specific modification of tyrosine residues by

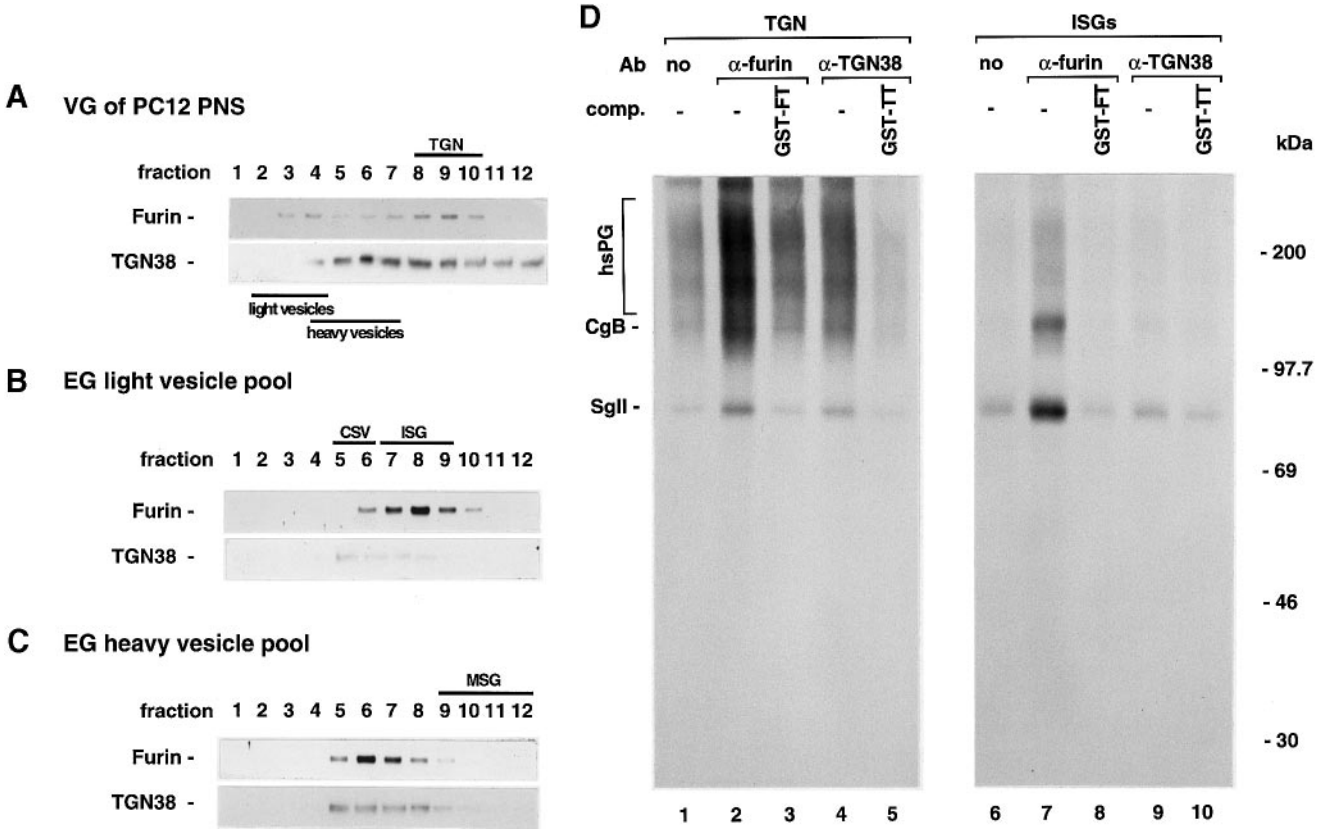

Fig. 1. Furin but not TGN38 is found in PC12 ISGs. (A–C) Furin and TGN38 distribution on sucrose gradients after fractionation of a PC12 cell PNS by sucrose gradient centrifugation using (**A**) velocity sucrose gradient centrifugation (VG) or (**B**) and (**C**) equilibrium gradient centrifugation (EG). The PNS was loaded onto the VG and the light vesicle pool (fractions 2–4) or the heavy vesicle pool (fractions 4–7) from the VG were fractionated further by EG. Equal volumes of each fraction were analysed by immunoblotting with a polyclonal α-furin and a monoclonal α-TGN38 antibody. The positions of the TGN, CSVs, ISGs and MSGs on the gradients, as previously described (S.Tooze *et al.*, 1991), are indicated. (**D**) Immunoisolation of membranes with α-furin and α-TGN38 antibodies. [^{35}S]sulfate-labelled TGN (5 min pulse, no chase) or ISGs (5 min pulse, 15 min chase) from PC12 cells were prepared as described in Materials and methods. For each immunoisolation, an equal volume of starting material was incubated with Staph A alone (lanes 1 and 6), Staph A pre-coated with α-furin antibody (lanes 2 and 7) or α-TGN38 antibody (lanes 4 and 9), Staph A pre-coated with α-furin antibody and pre-incubated with GST–furin tail (GST–FT) fusion protein (lanes 3 and 8) (competition), or Staph A pre-coated with α-TGN38 antibody and pre-incubated with GST–TGN38 tail (GST–TT) fusion protein (lanes 5 and 10) (competition). The immunoisolated material was analysed by 7.5% SDS–PAGE and subsequent fluorography. The positions of the sulfate-labelled molecules hsPG, CgB and SGII are indicated.

sulfate (Baeuerle and Huttner, 1987) followed by a short chase to label the soluble regulated secretory proteins chromogranin (CgB) and SgII in the ISG (Tooze and Huttner, 1990). ISGs were isolated by velocity and equilibrium sucrose gradient centrifugation from PC12 cells in which CgB and SgII had been labelled with [^{35}S]sulfate in a 5 min pulse followed by a 15 min chase. We then asked if it was possible to immunoisolate these labelled ISGs with antibodies directed against the cytoplasmic domain of TGN38 or furin. As seen in Figure 1D, lane 7, ISGs could be immunoisolated specifically with an anti-furin antibody: >80% of ISGs were bound under these conditions. No signal was detected in the absence of any antibody (Figure 1D, lane 6) or after pre-incubation of the anti-furin antibody with the GST–furin fusion protein (Figure 1D, lane 8). In contrast, antibodies to the cytoplasmic domain of TGN38 were not able to immunoisolate sulfate-labelled ISGs (Figure 1D, lane 9) above background levels obtained either with no antibody or after competition with the antigen (Figure 1D, lanes 6 and 10). As a control for the efficiency of the TGN38 antibody, we showed that it, as well as the anti-furin antibody, could specifically immunoisolate TGN membranes from PC12 cells labelled with a 5 min pulse of sulfate (Figure 1D, lanes 2 and 4). The short sulfate pulse without a 15 min chase labels the soluble proteins both of the regulated secretory pathway, CgB and SgII, and of the constitutive secretory pathway, a hsPG, present in the TGN (Tooze and Huttner, 1990).

Furin can be phosphorylated by CKII on ISGs

We and others have demonstrated that the phosphorylation of the cytoplasmic domain of furin by CKII is important for its localization to the TGN, and modulates retrieval of furin from the TGN (Jones *et al.*, 1995; Takahashi *et al.*, 1995). We were therefore interested in investigating whether the furin on the ISG could be phosphorylated by CKII. We incubated ISGs with [γ-^{32}P]GTP and either bovine brain cytosol, buffer or purified CKII, and asked if phosphorylated furin could be immunoprecipitated from these reactions (Figure 2). After incubation of ISGs with CKII, the anti-furin antibody was able to immunoprecipitate ^{32}P-labelled furin. Immunoprecipitation of the phosphorylation reaction performed with ISGs, [γ-^{32}P]GTP and purified CKII, using an antibody raised against SgII, resulted in no specific precipitation (Figure 2, compare

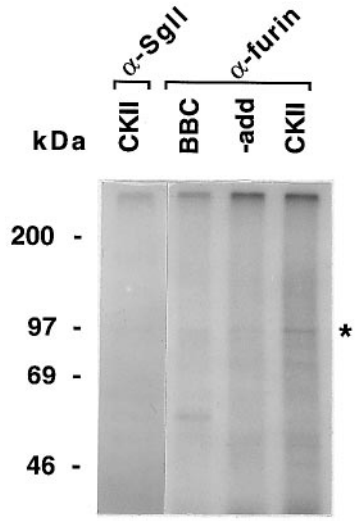

Fig. 2. Immunoprecipitation of furin after *in vitro* phosphorylation of ISG membranes. PC12 ISGs were incubated with [γ-^{32}P]GTP in the presence of bovine brain cytosol (BBC), buffer or CKII for 1 h at 37°C. The ISGs were sedimented and solubilized, followed by immunoprecipitation of furin with either an α-SgII antibody or an α-furin antibody. Bound proteins were analysed by 7.5% SDS–PAGE and fluorography. The position of furin is indicated by an asterisk.

α-SgII plus CKII and α-furin plus CKII). In addition, ^{32}P-labelled furin could also be immunoprecipitated after incubation with or without bovine brain cytosol although in reduced amounts compared with incubations with CKII. As CKII is a cytosolic enzyme, we anticipated that addition of bovine brain cytosol might be sufficient to allow phosphorylation of furin. Phosphorylation of furin in incubations containing only ISGs and [γ-^{32}P]GTP was unexpected, and we have obtained preliminary evidence which indicates that CKII is associated with ISGs (A.Dittié and S.Tooze, data not shown). The ISG-associated CKII may be responsible for the phosphorylation of the furin on ISGs in these *in vitro* reactions done in the absence of cytosol or purified CKII.

Phosphorylation of the cytoplasmic domain of furin regulates AP-1 association

AP-1 adaptor complex associates with phosphorylated GST–furin fusion protein. We have shown previously that the TGN localization of furin is dependent upon CKII phosphorylation (Jones *et al.*, 1995), and we now show that furin can be phosphorylated *in vitro* on ISG membranes by CKII. The observation that furin is found in ISGs but not MSGs (Figure 1) argues that furin is removed from ISGs during maturation. The ability of AP-1 to bind to ISGs but not MSGs (Dittié *et al.*, 1996) suggests that proteins which are required for AP-1 binding are either removed or inactivated in the MSG. Since interaction of the cation-independent (CI)-MPR with AP-1 adaptors in the TGN is dependent upon the phosphorylation of two serine residues by CKII in the receptor's cytoplasmic domain (Le Borgne *et al.*, 1993), it is tempting to speculate that furin also interacts with AP-1 in a CKII-dependent manner. Thus, furin in its phosphorylated state would promote association of clathrin coat components, in particular the AP-1 adaptor complex, for removal from the ISG.

To determine if AP-1 complexes can bind to the cytoplasmic domain of furin and if the binding is influenced by CKII phosphorylation, we used a GST fusion protein containing the cytoplasmic domain of wild-type furin (GST–FT) immobilized on glutathione–Sepharose. We then added bovine brain cytosol, containing tautomycin, a furin phosphatase inhibitor (Jones *et al.*, 1995), as the source of adaptor complexes and measured the amount of AP-1 bound to GST or GST–FT by immunoblotting with the bovine-specific anti-γ-adaptin antibody 100/3 (Ahle *et al.*, 1988). Prior to immobilization on glutathione–Sepharose, the GST or GST–FT was incubated with CKII in the presence of ATP. GST–FT, which bound low amounts of γ-adaptin, could, after phosphorylation by CKII, efficiently bind γ-adaptin (Figure 3A); phosphorylation by CKII resulted in a 4-fold increase in binding. Furthermore, similar results were obtained using a biotinylated peptide corresponding to the 56 amino acids of the human furin cytoplasmic tail. The peptide was incubated either with or without CKII and ATP, and then immobilized on streptavidin beads. The beads were then incubated with cytosol, washed, and the amount of γ-adaptin bound was quantitated using mAb 100/3 (Figure 3B). CKII phosphorylation of the peptide resulted in a 5-fold increase of γ-adaptin binding compared with the non-phosphorylated peptide. As a control for the the specificity of binding, we asked if other cytosolic coat protein subunits, such as β-COP (Coat Other Protein), could bind to either the non-phosphorylated or phosphorylated furin cytoplasmic domain. β-COP, a component of coatomer (Allan and Kreis, 1986), is not known to bind to signals which mediate clathrin adaptor binding. As expected, by immunoblotting with M3A5, an anti-β-COP antibody, no β-COP was detected bound to either the non-phosphorylated or phosphorylated furin cytoplasmic domains after incubation in bovine brain cytosol (data not shown). As a control for non-specific binding of AP-1, a biotinylated 73 amino acid peptide corresponding to the N-terminal domain of GM130, a Golgi-associated protein (Nakamura *et al.*, 1997), was incubated with CKII and ATP and then immobilized on streptavidin beads. Even at the highest concentration tested, in the presence or absence of CKII, the GM130 peptide was not able to bind significant amounts of γ-adaptin (Figure 3B).

Phosphorylated proteins are required for γ-adaptin binding to ISGs. Our previous experiments reconstituting AP-1 binding to ISGs have demonstrated that binding is sensitive to pre-treatment with trypsin, supporting the hypothesis that proteinacous components on the ISG are involved in coat formation (Dittié *et al.*, 1996). To test if the phosphorylation of these components is involved in AP-1 binding to ISGs, we used a similar approach and pre-treated the ISG membranes with alkaline phosphatase. To increase the efficiency of the alkaline phosphatase treatment, ISG-associated clathrin coats, which are present on the ISGs when isolated, were released from the ISGs by incubation at 37°C. The coat-depleted ISGs were sedimented and resuspended in a buffer containing increasing amounts of alkaline phosphatase, re-isolated and incubated with cytosol and GTPγS at 37°C to reconstitute coat formation (Figure 4A). Coat formation, as measured by γ-adaptin binding, was sensitive to increasing concentrations of alkaline phosphatase. At 0.5 U of alkaline

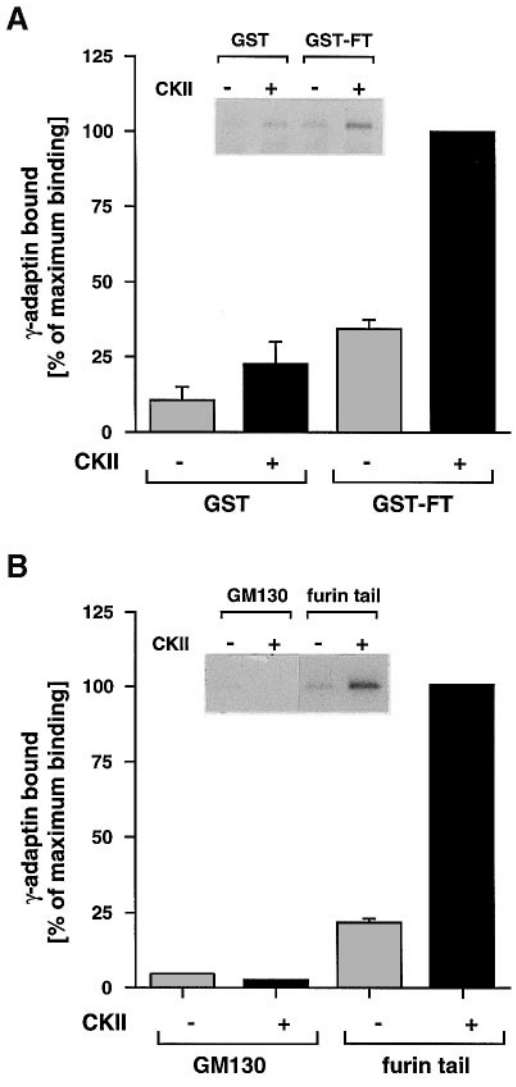

Fig. 3. Binding of AP-1 to immobilized furin tail. (**A**) Either GST or GST–FT was incubated with or without CKII and ATP and immobilized on glutathione–Sepharose 4B beads. (**B**) Biotinylated peptides representing the amino-terminus of GM130 or the human furin tail, incubated with or without CKII and ATP, were immobilized to streptavidin beads. After incubation of the glutathione or streptavidin beads with bovine brain cytosol, the bound material was separated by SDS–PAGE and the amount of AP-1 bound was quantitated by immunoblotting with γ-adaptin-specific mAb 100/3 followed by phosphorimager analysis. The amount of γ-adaptin bound is expressed as a percentage of maximum binding. The autoradiograph of a representative experiment is shown in (A) and (B). Error bars represent the standard deviation of the mean.

phosphatase/μg of ISG protein, ~50% of γ-adaptin binding was abolished compared with the control. To control for the specificity of the alkaline phosphatase preparation, we performed the phosphatase treatment in the absence of divalent cations, or at pH 7.2, conditions which should result in inactivation of the alkaline phosphatase but not of most proteases. Omission of both Mg^{2+} and Zn^{2+} ions or lowering the pH to 7.2 resulted in a control level of γ-adaptin binding (Figure 4B). Furthermore, incubation of the ISGs after alkaline phosphatase treatment with CKII before the addition of cytosol was sufficient to restore γ-adaptin binding nearly to control levels (Figure 4C).

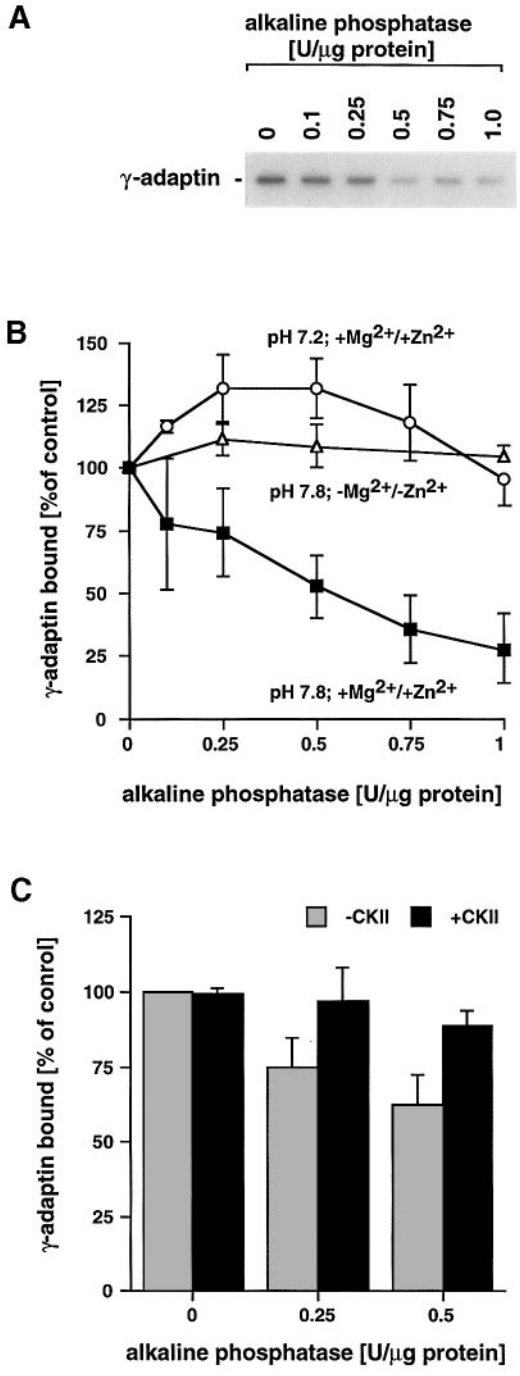

Fig. 4. Binding of AP-1 to ISGs is sensitive to alkaline phosphatase treatment and can be reconstituted by incubation with CKII. (**A**) PC12 ISGs were incubated with increasing amounts of alkaline phosphatase. (**B**) ISGs were either incubated with alkaline phosphatase as in (A) (pH 7.8; $+Mg^{2+}/+Zn^{2+}$), in a neutral buffer (pH 7.2; $+Mg^{2+}/+Zn^{2+}$) or in a buffer without divalent cations (pH 7.8;$-Mg^{2+}-Zn^{2+}$). ISGs in (A) and (B) were re-isolated and incubated with bovine brain cytosol and 100 μM GTPγS for 30 min at 37°C. A representative experiment is shown in (A). (**C**) ISGs were incubated with increasing amounts of alkaline phosphatase as in (A), re-isolated and incubated with or without CKII and ATP. After inhibition of CKII with heparin, the ISGs were incubated with bovine brain cytosol and GTPγS as above. The amount of γ-adaptin bound to the ISGs was determined after sedimentation and immunoblotting with mAb 100/3 followed by fluorography. In (B) and (C), the amount of γ-adaptin bound is expressed as a percentage of control (no alkaline phosphatase). Error bars: SD of mean.

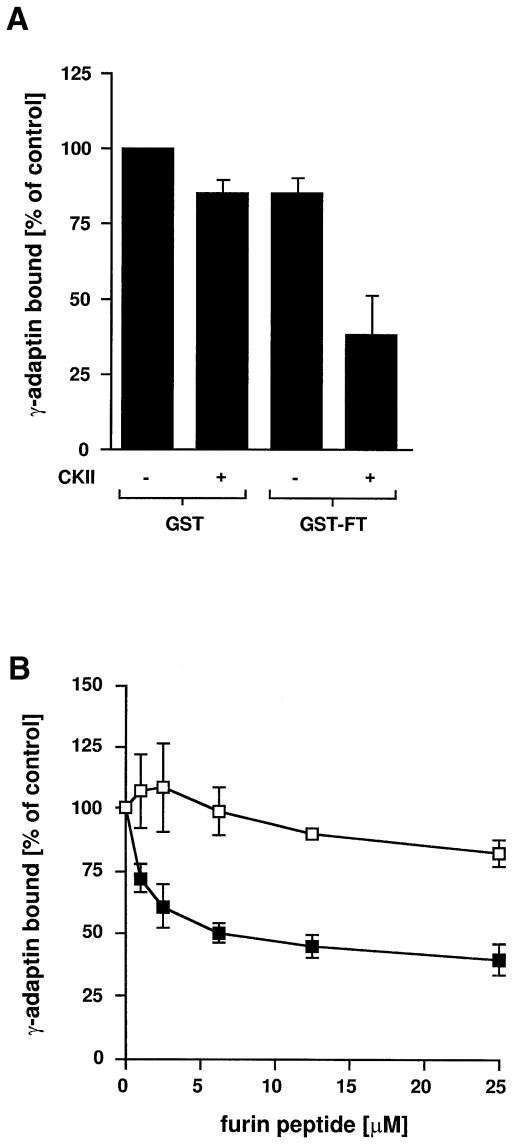

Fig. 5. AP-1 binding to PC12 ISGs can be competed by pre-incubation of cytosol with the CKII-phosphorylated furin tail. Bovine brain cytosol was pre-incubated for 15 min on ice with (**A**) 5 μM GST or GST–FT fusion proteins or (**B**) increasing amounts of a furin tail peptide, either in their non-phosphorylated forms (□) or after incubation with CKII and ATP (■). PC12 ISGs and 100 μM GTPγS were added to the pre-treated cytosol and incubated for 30 min at 37°C. The amount of γ-adaptin bound to the ISGs was determined after sedimentation and immunoblotting with mAb 100/3 followed by fluorography and phosphorimager analysis. The amount of γ-adaptin bound is expressed as a percentage of maximum binding (A) or control binding (–peptide) (B). Error bars: SD of mean.

The cytoplasmic domain of furin can compete for AP-1 binding to ISGs in a phosphorylation-dependent reaction. We next tested the ability of GST fusion proteins containing the cytoplasmic domain of furin to inhibit the binding of AP-1 to ISGs in our binding assay (Dittié *et al.*, 1996) using bovine brain cytosol as the source of adaptors. GST and GST–furin were incubated with or without CKII. Heparin was added at the end of this incubation to stop the reaction. Following a pre-incubation of cytosol with GST and GST–FT, the amount of AP-1 from cytosol which was still able to bind to ISGs was determined (Figure 5A). Pre-incubation of 0.5 mg/ml cytosol with the phosphorylated GST–FT resulted in an ~60% reduction of the γ-adaptin binding to ISGs, whereas pre-incubation of cytosol with the same amount of unphosphorylated GST–FT, or GST incubated with CKII, resulted in no inhibition of AP-1 binding to ISGs.

To test directly if the cytoplasmic tail of furin alone can inhibit AP-1 association with ISG membranes in a CKII-dependent manner, we pre-incubated cytosol with increasing amounts of the phosphorylated 56 amino acid furin tail peptide before addition of ISGs (Figure 5B). With 5 μM phosphorylated peptide we observed a 50% inhibition of γ-adaptin binding to ISGs, while addition of the peptide without prior phosphorylation had little effect on γ-adaptin binding.

Furin is phosphorylated by CKII both *in vivo* and *in vitro* on Ser773 and Ser775, and mutations of both serines can inhibit CKII phosphorylation (Jones *et al.*, 1995). To test the ability of furin containing mutations at Ser773 and Ser775 to bind AP-1 and inhibit recruitment of AP-1 onto ISGs, we incubated cytosol with increasing concentrations of GST fusion proteins containing the wild-type (GST–FT) or furin mutants (Table I). The GST–furin fusion protein GST–FT or the furin mutants GST–DP, in which the phosphorylation site has been destroyed by mutation of Ser773 and Ser775 to alanine, and GST–TP, in which the sequence of furin has been truncated at amino acid 772, only had a slight effect on the γ-adaptin binding to ISGs. Interestingly, GST–MP, a GST–furin fusion protein in which Ser773 and Ser775 are mutated to aspartic acid to mimic the diphosphorylated state of furin, inhibited >50% of the binding of γ-adaptin to ISGs (Figure 6) compared with GST–DP.

Removal of furin from ISGs is dependent upon phosphorylation by CKII

Indirect immunofluorescence localization of furin in AtT20 cells. To confirm the biochemical data shown above, we examined the steady-state localization of furin in AtT20 cells. AtT20 cells are particularly useful for morphological analysis because under normal culture conditions they have discrete processes extending from the cell body which terminate in 'tips'. The tips, as well as the processes, have been shown to contain secretory granules as well as endosomal compartments, but not Golgi membranes (Tooze *et al.*, 1989; Tooze and Hollinshead, 1991). Therefore, it is relatively straightforward to distinguish between localization to the Golgi complex in the cell body and to secretory granules in the cell processes. After infection of AtT20 cells with recombinant vaccinia viruses encoding an epitope-tagged native furin (VV:fur/f), the majority of the epitope-tagged furin was localized to a well-defined paranuclear region in the cell body typical of the Golgi region in AtT20 cells and furin's demonstrated localization to the TGN (Molloy *et al.*, 1994): very little staining was detected in the tips or the processes with the mAb M1 (Figure 7A). The paranuclear localization presumably reflects the epitope-tagged furin present in TGN membranes as was anticipated because endogenous furin has been demonstrated to be present in TGN membranes (Figure 1 and L.Thomas and G.Thomas, unpublished observations). The level of furin present at the plasma membrane and in endosomal membranes in AtT20 cells

Table I. GST fusion proteins used in this study

Construct	Protein	Species	Sequence	CKII site	Reference
GST–furin tail	furin	rat	736–793	SDS	this study
GST–FT	furin	human	736–794	SDS	Jones *et al.* (1995)
GST–MP ($S_{773,775}D$)	furin	human	736–794	DDD	Jones *et al.* (1995)
GST–DP ($S_{773,775}A$)	furin	human	736–794	ADA	Jones *et al.* (1995)
GST–TP ($P_{772}t$)	furin	human	736–772	none	Jones *et al.* (1995)
GST-TGN38	TGN38	rat	308–340	none	this study
GST–CKIIα	CKIIα	human	1–392	none	this study

using the vaccinia virus expression system was very low and not easily seen by indirect immunofluorescence.

Furin DP mutant is localized in dense core secretory granules. To investigate if phosphorylation of the cytoplasmic domain of furin, previously implicated in furin recycling (Jones *et al.*, 1995), is involved in its localization in AtT20 cells to the regulated secretory pathway, we infected AtT20 cells with recombinant vaccinia virus containing epitope-tagged furin mutated at Ser773 and Ser775 in the CKII site. These mutants in the cytoplasmic domain of furin correspond to those made as GST fusion proteins (see Table I) and either mimic the diphosphorylated state (VV:fur/f MP) or destroy the CKII site (mimicking non-phosphorylated furin) (VV:fur/f DP). Infection of the AtT20 cells with VV:fur/f MP followed by immunofluorescence labelling with mAbM1 showed the VV:fur/f MP predominantly localized in the paranuclear, Golgi region similar to the wild-type furin (Figure 7B). In cells infected with VV:fur/f DP, the distribution of epitope-tagged furin mutant was dramatically different (Figure 7C and D, middle panel). In addition to the paranuclear staining pattern observed with the wild-type and MP mutant, the epitope-tagged DP mutant of furin was found in the processes and tips of the infected AtT20 cells (Figure 7C). Double labelling of the cells with mAb M1 and an antibody to the secretory granule content protein adrenocorticotrophic hormone (ACTH) and analysis by confocal microscopy revealed a co-localization in the cell processes and tips of epitope-tagged furin and ACTH to secretory granules (Figure 7D).

Discussion

In cells with a regulated secretory pathway, the content and composition of the nascent vesicles which form from the TGN is still not well resolved. We have identified the ISG as the TGN-derived vesicle containing the majority of the newly synthesized regulated secretory proteins in PC12 cells. ISGs have patches of clathrin coats on their membrane which are comprised of the AP-1 adaptor complex and clathrin (Tooze and Tooze, 1986; Orci *et al.*, 1987; Dittié *et al.*, 1996). It has been proposed that clathrin on the ISG is involved in a membrane remodelling event, implying that a vesicular pathway originates from the ISG which is mediated by CCVs (for review, see Tooze, 1991). To date, we have no way of estimating the total number of ISG-derived CCVs in the cell, although we would predict that the number would be extremely low. ISGs themselves are an intermediate with a $t_{1/2}$ of $<$40 min at steady-state, suggesting that the ISG-derived CCVs would have an even shorter lifetime. Finally, the ISG-derived

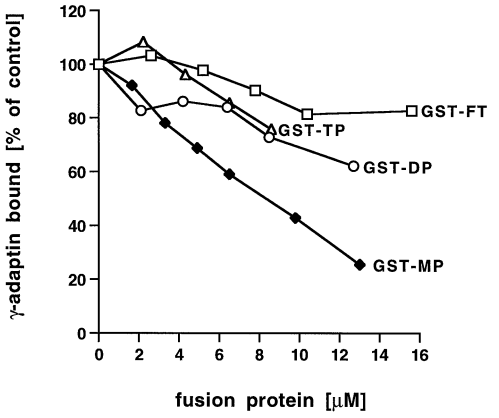

Fig. 6. Competition for AP-1 binding to PC12 ISGs using different furin tail mutants. Bovine brain cytosol was pre-incubated for 15 min on ice with increasing amounts of GST–FT or GST–furin tail fusion proteins mutated at the CKII site (see Table I) to either destroy (GST–DP) or truncate the furin before the CKII site (GST–TP), or to mimic the phosphorylated state (GST–MP). PC12 ISGs and 100 μM GTPγS were added to the pre-treated cytosol and incubated for 30 min at 37°C. The amount of γ-adaptin bound to the ISGs was determined after sedimentation and immunoblotting with mAb 100/3 followed by fluorography and phosphorimager analysis. The amount of γ-adaptin bound is expressed as a percentage of maximum binding. A representative experiment is shown done in duplicate.

CCVs most probably have a similar coat composition to TGN-derived CCVs (i.e. AP-1 and clathrin).

To understand the process of secretory granule maturation, it is essential to determine what cargo molecules are contained in the ISG-derived CCVs. Although it is theoretically possible to identify the ISG-derived CCVs by subcellular fractionation, in the absence of known cargo molecules or unique membrane marker proteins it is close to an impossible experimental task. As an alternative approach, we have (i) studied the distribution of two TGN-resident proteins which recycle to and from the plasma membrane via endosomes to determine if they can be detected in ISGs and MSGs and (ii) developed a cell-free assay which reconstitutes clathrin coat formation on the ISG (Dittié *et al.*, 1996). Using these approaches, we aim to identify proteins responsible for recruitment of clathrin on the ISG. We hypothesize that there is a population of ISG-resident, non-secretory granule proteins which are removed from the ISG during maturation in CCVs. These proteins may be present transiently in ISGs due either to missorting in the TGN during ISG formation or because the ISG is the compartment where clathrin coat-associated membrane proteins are sorted. Whether these proteins in fact have a function in the ISGs remains an open question.

We have demonstrated that furin, but not TGN38, is

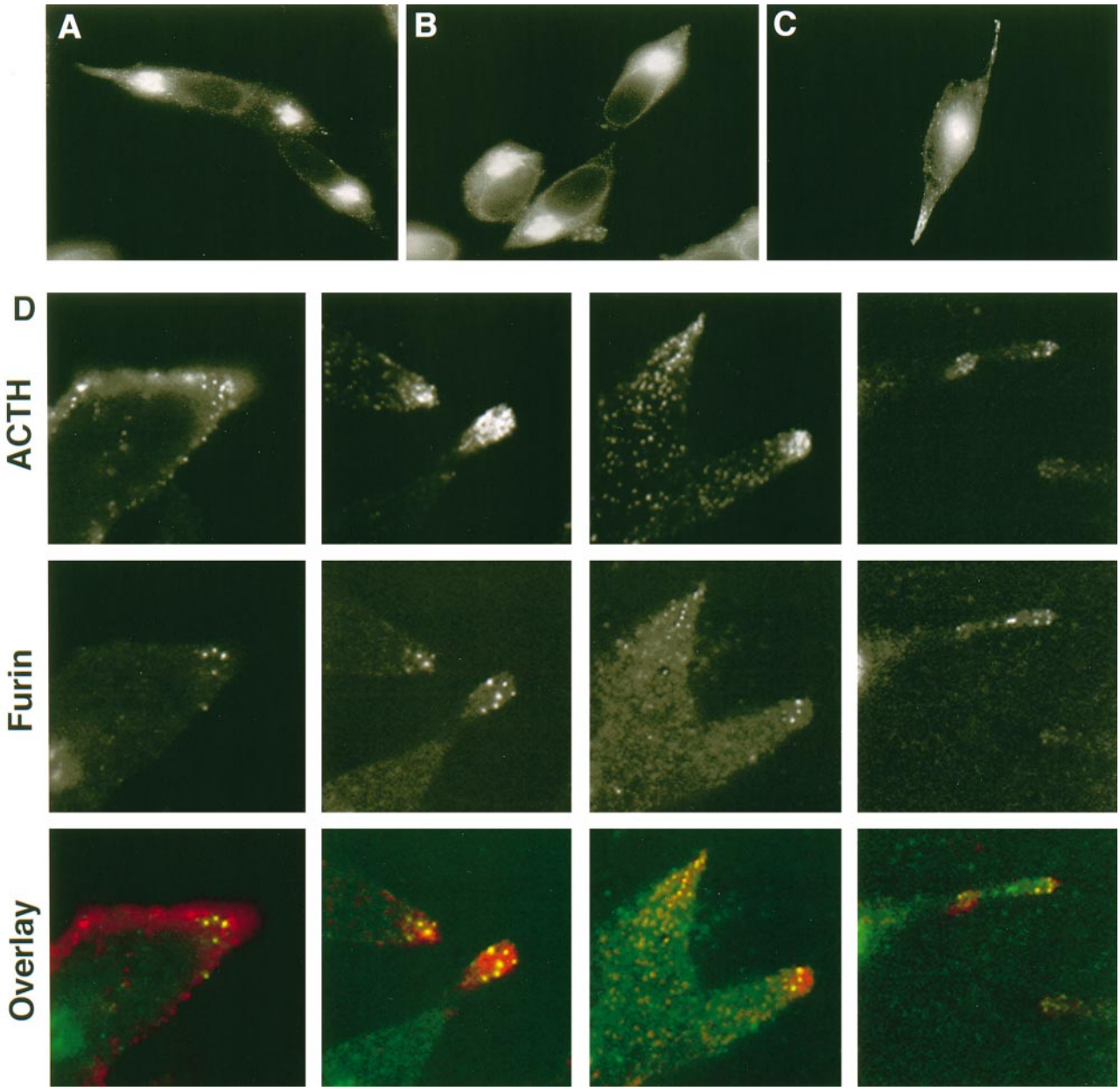

Fig. 7. Mutation of the CKII site changes the subcellular distribution of furin in AtT-20 cells. Replicate cultures of AtT-20 cells were infected with vaccinia virus recombinants expressing either Fur/f (**A**), Fur/f$S_{773,775}$D (furin MP mutant) (**B**) or Fur/f$S_{773,775}$A (furin DP mutant) (**C** and **D**). The cells were maintained at 37°C in the presence of 10 mM hydroxyurea. At 6 h post-infection, the cells were fixed, permeabilized and incubated with mAb M1 to visualize the epitope-tagged furin constructs (A–D) and an anti-ACTH antiserum (D only). The mAb M1 was visualized with a goat anti-mouse Cy2 and the ACTH antiserum with a goat anti-rabbit TxR. In (A–C), cells were visualized by immunofluorescence microscopy. In (D), the immunostaining of furin DP mutant and ACTH in cell tips was visualized by confocal microscopy. The top row of this panel shows ACTH staining, the middle row shows fur/f staining and the bottom row shows a colorized overlay of the two images. The images represent a single plane of the cell emphasizing the tip staining.

found in ISGs. At most, ~25% of the total furin is at steady-state in the ISG fraction after velocity gradient centrifugation. Although TGN38 trafficking via the plasma membrane is in many aspects similar to that of furin (Chapman and Munro, 1994; Molloy *et al.*, 1994; Schäfer *et al.*, 1995), TGN38 was not detected in ISGs. One possible explanation for this result could be that furin but not TGN38 interacts with the soluble regulated secretory proteins during aggregation in the lumen of the TGN. Furin is related to the prohormone convertase family of endopeptidases found in secretory granules and shares extensive regions of homology with these enzymes, in particular on the lumenal domain (van de Ven *et al.*, 1990; Smeekens *et al.*, 1991). However, because furin is a transmembrane protein, its interaction with the aggregating soluble regulated secretory proteins would be restricted and the bulk of furin would not be expected to be found in the ISGs. Recent data with a truncated furin mutant expressed in AtT20 cells (Creemers *et al.*, 1996) would suggest that this is not the case and that the lumenal domain of furin does not interact with aggregating regulated secretory proteins or contain a sorting signal. It would seem, therefore, that there may be signals in the transmembrane or cytoplasmic domain of furin which

allow for inclusion of furin but not TGN38 into nascent ISGs. TGN38, which is heavily glycosylated on its lumenal domain (Jones *et al.*, 1993), may in fact be actively excluded from the forming secretory granule core and therefore the ISG. Incorporation of furin into ISGs also confirms the idea that sequences in the cytoplasmic tail of furin may not function as a true retention sequence to keep the protein in the TGN. Our data also demonstrate that furin and TGN38 are sorted differently in the TGN, which supports the data obtained by Schäfer and colleagues (Schäfer *et al.*, 1995) that furin and TGN38 may have a different distribution in the TGN.

The cytoplasmic domain of furin has at least two targeting signals which mediate its intracellular localization: a tyrosine motif ($YKGL_{765}$) and an acidic cluster of amino acids ($CPSDSEEDEG_{783}$). The tyrosine-based endocytotic motif which is responsible for internalization of receptors at the cell surface has been shown to bind AP-2, and in particular the μ_2 subunit (Boll *et al.*, 1996). The acidic cluster, which encompasses two serine residues, both of which are phosphorylated by CKII (Jones *et al.*, 1995), has been demonstrated to mediate the TGN localization of furin (Jones *et al.*, 1995; Schäfer *et al.*, 1995; Takahashi *et al.*, 1995; Voorhees *et al.*, 1995). Acidic clusters of amino acids containing a CKII phosphorylation site have also been demonstrated to be involved in the TGN localization of gpI from varicella-zoster virus (Alconada *et al.*, 1996) and association of AP-1 with CI-MPR in the TGN (Le Borgne *et al.*, 1993). Furthermore, it has been shown that binding of AP-1 to CI-MPR in the TGN membrane is modulated by CKII phosphorylation of the acidic cluster of amino acids (Mauxion *et al.*, 1996). Interestingly, TGN38 does not have a cytoplasmic CKII site, suggesting that its TGN localization and association with the μ_1 subunit (Ohno *et al.*, 1995) require a different signal.

We have shown a correlation between phosphorylation of furin by CKII and the ability of AP-1 to bind to ISGs. Using the *in vitro* binding assay, in addition to γ-adaptin [also known as γ1 (Traub, 1997)], we can detect μ1 and σ1 (A.Dittié and S.Tooze, unpublished observations) on the ISGs. Our results suggest that the association of AP-1 with the cytoplasmic domain of furin in ISGs depends exclusively on the phosphorylation of the acidic cluster containing two CKII sites. Interaction of AP-1 with the Golgi membranes occurs via the N-terminal core domain or body of the AP-1 complex (Page and Robinson, 1995; Traub *et al.*, 1995), most likely by association of the μ1 subunit and transmembrane receptor tails (Ohno *et al.*, 1995). AP-1 interaction with the membrane also requires additional membrane-associated proteins, such as ARF (Traub *et al.*, 1993), and other unknown proteins (Mallet and Brodsky, 1996; Seaman *et al.*, 1996). The sequence of events in the binding and interaction of AP-1 with the membrane leading to stable association of AP-1 is not clear. It is possible that AP-1 is recruited to the ISG membrane by binding to one of these additional proteins and interacts with the phosphorylated cytoplasmic domain of furin just for the removal of furin. If furin was not already phosphorylated before exit from the TGN it may be phosphorylated on the ISG by the ISG-associated CKII-like activity, thus enabling AP-1 to bind to furin's cytoplasmic domain. It remains to be determined, during removal of furin, whether the cytoplasmic domain of furin and AP-1 interact directly or if additional factors are required.

Confirmation of these results from the *in vitro* binding and competition experiments was obtained by expression of epitope-tagged furin and furin mutants in AtT20 cells using a vaccinia virus expression system. Both the epitope-tagged native furin and the furin MP mutant were detected in the Golgi region of AtT20 cells. In contrast, the flag-tagged furin DP mutant, which cannot be phosphorylated by CKII, was detected in the tips of the AtT20 cells, in a compartment which co-localized with endogenous ACTH. As the ACTH detected in the tips of AtT20 cells is known to be in secretory granules, the furin DP mutant in the tips is also localized to the secretory granules. This result provides strong support for our hypothesis that furin is transiently present in ISGs, and removed from the ISG during maturation in a phosphorylation-dependent reaction, via the budding of CCVs.

We believe the furin DP mutant, which cannot be phosphorylated, cannot bind AP-1, and consequently cannot be incorporated into CCVs; instead it remains in ISGs as they mature into MSGs. However, localization of the furin DP mutant to the regulated pathway could suggest that this mutation causes furin to be sorted into the regulated pathway in AtT20 cells. The DP mutant was constructed by changing two serines to alanines in the cytoplasmic tail. This mutation is unlikely to constitute a sorting signal for targeting a membrane protein into secretory granules, and we do not favour the possibility that the furin DP mutant, but not furin or the furin MP mutant, is specifically sorted into secretory granules. Therefore, if we assume that furin and the furin MP and DP mutants are all found in ISGs, albeit at a low level, the presence of the furin DP mutant in MSGs could be explained in two ways: either furin and the furin MP mutant are removed from ISGs, and the furin DP mutant alone remains in ISGs which mature to MSGs, or the DP mutant is removed initially from the ISGs together with the furin and the furin MP mutant, but the DP mutant alone is sorted back into the regulated pathway after recycling from the plasma membrane via the endosome.

Previous studies have demonstrated that internalized fluid phase markers, such as cationized ferritin, can be detected after short chase periods in the ISG: incorporation of the marker into ISGs was most likely via the TGN (Komuro *et al.*, 1987). There is no evidence for a direct pathway from the endosome to the secretory granule and there is no reason to suppose that the furin DP mutant would recycle from the plasma membrane to the MSGs. The clear implication of our finding is that the furin DP mutant is sorted into newly formed ISGs in the TGN as are the native furin and the furin MP mutant, but that the latter molecules are removed rapidly from the secretory granule and are therefore undetectable in the MSG population whereas the DP mutant furin remains in the regulated pathway.

The CI-MPR is phosphorylated by a CKII-like activity concomitant with its exit from the TGN (Méresse and Hoflack, 1993). We speculate that furin is also phosphorylated in the TGN, and thus like the MPR binds AP-1 and is incorporated into CCVs. There is, however, a major difference in the steady-state localization of furin and

MPR: furin is typically found in the TGN whereas the CI-MPR and cation dependent (CD)-MPR both exhibit a steady-state localization in both the TGN and endosomes (for review, see Hille-Rehfeld, 1995). Escape of furin from the TGN into a recycling pathway may be mediated by AP-1 interaction and CCV formation, in which case one could imagine that CKII phosphorylation could be used to regulate this pathway. Removal of furin from an ISG must also be regulated by CKII phosphorylation in that only phosphorylated furin is removed from ISGs. Recently we have obtained results which demonstrate that CI-MPR is also present in ISGs but not MSGs, and that in competition assays AP-1 binding can be inhibited by addition of a CKII-phosphorylated GST fusion protein encoding the cytoplasmic domain of CI-MPR (A.Dittié and S.Tooze, in preparation). These results suggest that CKII phosphorylation is involved in the sorting of furin and other proteins, including the CI-MPR, from the ISG by CCVs containing AP-1. This mechanism would ensure that these membrane proteins do not remain in the secretory granules of endocrine cells. Further investigation of the CKII phosphorylation event may allow a better understanding of the process of secretory granule maturation in endocrine cells.

Materials and methods

Reagents

Carrier-free [^{35}S]sulfate, [^{125}I]protein A, [γ-^{32}P]ATP and [γ-^{32}P]GTP were from Amersham (Little Chalfont, UK). Nucleotides, creatine phosphate and creatine phosphokinase were from Boehringer Mannheim (Mannheim, Germany). CKII was prepared according to Litchfield *et al.* (1990). Alkaline phosphatase (P-7923), heparin and horseradish peroxidase (HRP type II) were from Sigma (Poole, UK). Tautomycin and microcystin LR were obtained from Calbiochem (Nottingham, UK). Fine chemicals were from Merck (Lutterworth, UK), Boehringer Mannheim, Life Technologies (Paisley, UK) and Sigma.

Cells and antibodies

PC12 cells (clone 251; Heumann *et al.*, 1983), originally obtained from Dr H.Thoenen (Martinsried, Germany), were maintained as described (Tooze and Huttner, 1990). AtT20 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/10% fetal calf serum (FCS) containing 25 μg/ml gentamicin. The mAb against bovine γ-adaptin (100/3) (Sigma) was used at a dilution of 1:200, mAb against the cytoplasmic domain of TGN38 (2F7.1) (obtained from Dr G.Banting, Bristol, UK) at a dilution of 1:5, mAb M1 against the FLAG-antigen (Kodak, Rochester, USA) at a dilution of 1:300 and mAb M3A5 against β-COP at a dilution of 1:1000 (Allan and Kreis, 1986). Polyclonal antibodies against the cytoplasmic domains of SgII (r175, Dittié and Tooze, 1995), TGN38 (r29) (obtained from Dr G.Banting), furin (Jones *et al.*, 1995) and ACTH (Tooze and Tooze, 1986) were used at dilutions of 1:500–1:1000. Affinity-purified anti-μ1 and anti-σ1 antibodies were obtained from Dr Robinson, Cambridge UK. Secondary antibodies were HRP-conjugated sheep anti-mouse or sheep anti-rabbit (Amersham) or Cy2-conjugated goat anti-mouse and TxR-conjugated goat anti-rabbit (Fisher Scientific, Loughborough, UK).

GST fusion proteins

The cytoplasmic domains of rat furin (Misumi *et al.*, 1990) and rat TGN38 (Luzio *et al.*, 1990) (see Table I) were cloned via RT–PCR from rat liver mRNA and ligated in-frame to the C-terminus of GST using the pGEX-3X system (Pharmacia, Milton Keynes, UK). The *Bgl*II–*Eco*RI fragment of human CKII α subunit cDNA (Lozeman *et al.*, 1990) (see Table I), comprising the entire coding sequence of the enzymatically active subunit of CKII was cloned in-frame to the C-terminus of GST using the pGEX-3X vector. All other constructs used in this study are in pGEX-3X (see Table I). The fusion proteins were expressed in BL21 cells and purified using glutathione–Sepharose 4B (Pharmacia). The GST–CKIIα fusion protein (0.25 mg/ml) was dialysed against 20 mM MOPS pH 7.0, 2 mM EDTA, 1 mM dithiothreitol (DTT) and 25% (w/v) glycerol and stored at 4°C.

Vaccinia constructs and infection of AtT20 cells

Epitope-tagged furin (fur/f) was constructed as described previously (Molloy *et al.*, 1994). The generation of the point mutations (fur/f$S_{773,775}$A = fur/DP and fur/f$S_{773,775}$D = fur/MP) in the cytoplasmic tail of furin are described in Jones *et al.* (1995). Expression of the furin constructs using the recombinant vaccinia virus and indirect immunofluorescence was performed as described (Jones *et al.*, 1995). Confocal images were obtained with a Leica Confocal Laser Scanning Microscope equipped with a Leitz Fluorovert-FU Microscope and argon–krypton laser.

Preparation of PC12 TGN, post-Golgi vesicles (ISGs, CSVs and MSGs) and bovine brain cytosol

PC12 cells were pulse labelled with [^{35}S]sulfate and chased at 37°C [(Tooze and Huttner, 1990); see figure legends for details]. ISGs, CSVs, MSGs and TGN were prepared from either labelled or unlabelled PC12 cells after preparation of a post-nuclear supernatant (PNS) by velocity and equilibrium sucrose gradient centrifugation (Dittié *et al.*, 1996). Fractions (1 ml) were collected from the top of the gradient (fraction 1 = top). TGN was enriched in velocity gradient fraction 9, CSVs were found in equilibrium gradient (EG) fractions 5 and 6, ISGs in EG fractions 7–9, and MSGs in EG fractions 10–12. The ISGs used for the binding assay contained ~0.5 mg/ml protein.

Equal volumes of each fraction (100 μl from the velocity gradient fractions and 200 μl from the EG fractions) were used for Western blot analysis and the bound antibodies were visualized by ECL (Amersham). Bovine brain cytosol was prepared as described (Dittié *et al.*, 1996). Protein concentrations were determined by protein assay (Bio-Rad, Hemel Hempstead, UK) using IgG as a standard.

Horseradish peroxidase uptake

To determine the position of early and late endosomes after sucrose gradient centrifugation, PC12 cells were incubated with the endocytic fluid-phase marker HRP. Two 15 cm dishes of subconfluent PC12 cells each were washed twice in DMEM/0.1% horse serum/0.05% FCS (low serum DMEM) and incubated with 2 mg/ml HRP in low serum DMEM for 7 min at 37°C either without chase to label early endosomes or followed by a 30 min chase with normal growth medium to label late endosomes. A PNS was prepared from each and subjected to velocity and equilibrium centrifugation. The HRP activity in each fraction was determined using *o*-dianisidine (Marsh *et al.*, 1987).

In vitro phosphorylation of ISGs

ISGs were incubated with or without 2 mg/ml bovine brain cytosol or 1 U of purified CKII and 10 μCi of [γ-^{32}P]ATP or [γ-^{32}P]GTP in 50 mM Tris–HCl pH 7.4, 140 mM KCl, 10 mM $MgCl_2$, 1 μM microcystin LR and 0.1 μM tautomycin (CKII buffer) for 60 min at 30°C. The ISG pellets were lysed in TNTE (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.3% Tween-20, 5 mM EDTA) containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 5 μg/ml leupeptin. Furin was immunoprecipitated by addition of the polyclonal serum against furin at a dilution of 1:200, followed by addition of protein A–Sepharose 4B beads (Pharmacia). The beads were washed in TNTE and resuspended in sample buffer for SDS–PAGE. After separation of the proteins by SDS–PAGE, the ^{32}P-labelled proteins were visualized by autoradiography.

Immunoisolation

Immunoisolation of [^{35}S]sulfate-labelled TGN or ISGs using polyclonal antibodies against TGN38 and furin was carried out as described (Dittié *et al.*, 1996), using 100 μl of the TGN-enriched fraction 9 of the velocity gradient and ISG equilibrium fraction 8. For competition experiments, 0.1 mg/ml GST–TGN38 or GST–furin fusion proteins (Table I) were added before the immunoisolation and were also included at the same concentrations throughout the immunoisolation.

Cell-free assay to reconstitute γ-adaptin binding to PC12 ISGs

As previously described in detail (Dittié *et al.*, 1996), ISGs were incubated with varying amounts of bovine brain cytosol in 25 mM HEPES pH 7.2, 25 mM KCl, 2.5 mM MgOAc (binding buffer) and 100 μM GTPγS. After incubation for 30 min at 37°C, the reaction was stopped, the ISGs were sedimented and the γ-adaptin bound was quantitated after Western blotting using [^{125}I]protein A.

Alkaline phosphatase pre-treatment of ISGs. To release endogenous clathrin coats, ISGs were diluted 1:4 with binding buffer and pre-incubated for 30 min at 37°C, re-isolated by centrifugation for 30 min at 80 000 *g* at 4°C and resuspended in alkaline phosphatase buffer (50 mM HEPES pH 7.8, 50 mM KCl, 0.5 mM $ZnCl_2$, 3.3 mM MgOAc, 0.3 mM EDTA, 0.5 mM PMSF, 0.5 μg/ml leupeptin) containing 0.2 M sucrose and then treated for 30 min at 37°C with increasing amounts of alkaline phosphatase. To remove the alkaline phosphatase from the reaction mix, the ISGs were sedimented at 80 000 *g* for 30 min at 4°C and resuspended in 1.2 M sucrose, 10 mM HEPES pH 7.2. Controls were performed by lowering the pH of the alkaline phosphatase buffer to pH 7.2 or by omitting $ZnCl_2$ and MgOAc from the buffer. CKII phosphorylation after alkaline phosphatase treatment was performed as follows: ISGs were incubated in alkaline phosphatase buffer with alkaline phosphatase for 30 min at 37°C, sedimented of the ISGs at 80 000 *g*, the ISGs were resuspended in 25 mM HEPES pH 7.2, 130 mM KCl, 10 mM $MgCl_2$ and incubated for 30 min at 37°C in the presence of ATP and a regenerating system (Davey *et al.*, 1985) with or without GST–CKIIα fusion protein. The reaction was stopped by heparin and adjusted to a final concentration of 0.6 M sucrose, 25 mM HEPES pH 7.2, 25 mM KCl, 25 mM $MgCl_2$ prior to use in the binding assay.

Competition experiments using GST fusion proteins or peptides. Bovine brain cytosol (0.5 mg/ml) was incubated for 15 min at 4°C in the presence of 0.1 μM tautomycin and 1 μM microcystin LR with GST fusion proteins (0–15 μM, for details see figure legends and Table I) or a 56 amino acid peptide (0–25 μM) representing the entire human furin tail (Roebroek *et al.*, 1986), either non-phosphorylated or phosphorylated by CKII. When included, the CKII phosphorylation reaction was performed with 100 U of purified CKII/5 μg protein in CKII buffer for 30 min at 30°C. The reaction was stopped by the addition of heparin, and the GST fusion proteins were re-isolated in binding buffer by gel filtration on a Biogel P6DG (Bio-Rad) spin column.

Binding of adaptor components to immobilized furin tails

Fusion proteins were prepared from BL21 cells expressing GST, GST–FT, GST–MP or GST–DP (see Table I). After lysis of the bacteria by sonication and centrifugation for 10 min at 14 000 *g* to remove cell debris, the supernatant was incubated with or without 2 μl of GST–CKIIα in the presence of ATP and a regenerating system in CKII buffer for 1 h at 30°C, then stopped by the addition of heparin. The phosphorylated fusion proteins were isolated by incubation with glutathione–Sepharose 4B (50% w/v) for 2 h at room temperature. Alternatively, the 56 amino acid biotinylated human furin peptide or a 73 amino acid biotinylated peptide of the N-terminus of GM130 (Nakamura *et al.*, 1997) were incubated with or without GST–CKIIα as above. The peptides were then coupled to UltraLink™-immobilized streptavidin beads (Pierce, Rockford, USA) at 1 mg/ml beads for 2 h at room temperature.

The immobilized phosphorylated fusion proteins or peptides were resuspended in 100 μl of bovine brain cytosol (4 mg/ml) in binding buffer containing 0.1 μM tautomycin, 0.5 mM PMSF, 0.5 μg/ml leupeptin and 0.1 mg/ml heparin. After incubation for 30 min at 37°C, the resin or beads were washed with ice-cold binding buffer and resuspended in SDS sample buffer. The bound proteins were separated by SDS–PAGE, subjected to immunoblot analysis and the amount of bound AP-1 was quantitated as for the binding assay.

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