Expression of ARF6 mutants in neuroendocrine cells suggests a role for ARF6 in synaptic vesicle biogenesis

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Abstract ARF6 regulates membrane trafficking between the plasma membrane and endosomes. We investigated the role of ARF6 in synaptic vesicle biogenesis as this process occurs both at the plasma membrane and at endosomes. We used a synaptic vesicle marker protein, p-selectin-horseradish peroxidase (HRP), to follow the effects of ARF6 expression on synaptic vesicle biogenesis in PC12 neuroendocrine cells. Expression of a constitutively active ARF6 mutant increased, while expression of a nucleotide-free ARF6 mutant decreased, p-selectin-HRP levels in the synaptic vesicle peak. These results provide the first direct evidence for a role for ARF6 in synaptic vesicle biogenesis. © **2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: ARF6; Synaptic vesicle; Membrane trafficking; Protein targeting; p-Selectin; PC12 cell

1. Introduction

Synaptic vesicles are a hallmark of neurotransmission; by fusing with the plasma membrane they allow neurons to communicate through release of the neurotransmitter into the synaptic cleft. The biogenesis of these vesicles requires the coordination of multiple molecular processes that are not yet fully understood. New synaptic vesicles can form directly from the plasma membrane either through a kiss-and-run mechanism or by a clathrin-mediated process that requires dynamin and the adapter protein (AP)-2 [1]. Synaptic vesicles are also generated through an indirect pathway that requires clathrin-mediated endocytosis from the plasma membrane, as well as the adapter protein AP-3 and the small GTPase ARF1 for budding from the endosome [2,3]. Both pathways are thought to be important to maintain neuronal function and synaptic vesicle integrity, and the mechanisms that target synaptic vesicle proteins in each pathway appear to be different [1]. Data from a number of laboratories suggest that individual synaptic vesicle proteins may be targeted preferentially through either the direct or the indirect pathway, creating a heterogeneous population of vesicles with different ratios of synaptic vesicle proteins [1]. Furthermore, it is thought that targeting of different synaptic vesicle proteins may involve different adapter proteins and as yet unknown effectors of the endocytic pathway.

The ARF family of small GTPases regulates membrane trafficking pathways throughout the cell. ARF1, the prototypical member of this family, plays a major role in cargo selection, the recruitment of coat proteins (including COPI on the Golgi apparatus, AP-1 on the *trans*-Golgi network, and AP-3 on endosomes) and vesicle formation. Several years ago, a new and atypical member of the ARF GTPase family, ARF6, was demonstrated to play a role in trafficking of proteins between the plasma membrane and endosomes in non-neuronal cells [4]. Like ARF1, the distribution of ARF6 between membranes and cytosol appears to be regulated by its GTPase cycle, but in contrast to ARF1, this process is also affected by magnesium [5]. While ARF1 is predominantly associated with Golgi and to a lesser extent with endosomes, ARF6 is localized to peripheral membranes, including the plasma membrane and endosomes, and a variety of studies suggest it may participate in vesicle-mediated transport between these compartments [4,6]. Expression of ARF6 mutants has pronounced effects on trafficking of the transferrin receptor (TfR) between the plasma membrane and early endosomes. The GTPase-deficient mutant Q67L is localized to the plasma membrane, where it causes an accumulation of the TfR [7,8]. The T27N mutant, defective in GTP binding, is associated with the endosomal recycling compartment and, in some cell types, causes an accumulation of post-endosomal-coated vesicles [8,9] and blocks the recycling of the TfR from the endosome to the cell surface [7].

A recent study of ARF6 mutants expressed in the polarized epithelial MDCK cell line demonstrated that ARF6 is preferentially involved in apical, rather than basolateral, endocytosis [10]. In contrast to results obtained in fibroblasts, in which the Q67L and T27N mutants have opposite effects on TfR trafficking, these studies indicate that both ARF mutants Q67L and T27N enhance endocytosis from the apical membrane. These results suggest that ARF6 may play a unique role in protein trafficking in different cell types and provide a strong incentive for an independent determination of ARF6 function in neurons and neuroendocrine cells.

Because ARF6 regulates membrane trafficking between two compartments from which synaptic vesicles form, the plasma membrane and endosomes, we hypothesized that ARF6 might play a key role in synaptic vesicle biogenesis. ARF6 is highly expressed in brain [11] and PC12 cells (Waring, Deans and Buckley, unpublished data), yet there has been little investi-

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gation into a role for ARF6 in synaptic vesicle formation. We provide here, the first direct evidence that ARF6 is involved in synaptic vesicle biogenesis by demonstrating that expression of the ARF6 mutants T27N and Q67L in PC12 cells significantly affects the amount of a synaptic vesicle marker protein, p-selectin-horseradish peroxidase (HRP), targeted to synaptic vesicles.

2. Materials and methods

2.1. Cell culture and transfection

PC12 cells (ATCC, catalog # 1721-CRL) were cultured and transfected by electroporation as described [12]. Our transfection efficiency was approximately 10%, as assessed by immunofluorescent detection of the FLAG epitope tag on the ARF6 constructs two days after transfection.

2.2. cDNA constructs

The human ARF6 wild-type, T27N and Q67L C-terminal FLAG-tagged constructs were obtained from Dr. Victor Hsu [9]. The ARF6 cDNAs were subcloned from the pXS vector into pcDNA3.1+ (Invitrogen, Carlsbad, CA, USA), using the *Eco*RI and *Xba*I restriction sites. The p-selectin-HRP cDNA in the vector pRK34 [13] was a gift from Dr. Daniel Cutler (Medical Research Council, University College, London, UK).

2.3. Subcellular fractionation and glycerol velocity gradients

Homogenization of PC12 cells, subcellular fractionation and glycerol velocity gradients were performed as described [12,14]. Eight fractions were collected and membranes were pelleted by centrifugation at 85 000 rpm (301 427 $\times g_{avg}$) for 90 min in a Beckman TLA100.4 rotor. Each pellet was resuspended in 400 μl citrate buffer, pH 5.5 (50 mM citrate, 150 mM NaCl), with 0.1% Triton X-100 by shaking for 30 min at 4°C and spun at 20 000 $\times g$ for 10 min in a microcentrifuge to remove insoluble material.

2.4. Quantitation of p-selectin-HRP targeting

Gradient fractions, homogenate and S2 aliquots were used for a peroxidase assay as described [13]. The amount of p-selectin-HRP in synaptic vesicles was determined by adding the peroxidase activity of all fractions in the synaptic vesicle peak. The specific activity of p-selectin-HRP in the synaptic vesicle peak was normalized to the specific activity of p-selectin-HRP in the homogenate or the S2 to normalize for p-selectin-HRP expression levels. Finally, the normalized p-selectin-HRP in the synaptic vesicle peak of PC12 cells co-transfected with ARF6 and p-selectin-HRP cDNAs were expressed as a percent of p-selectin-HRP in the synaptic vesicle peak of PC12 cells transfected with p-selectin-HRP cDNA alone.

2.5. Synaptophysin immunoblotting

Immunoblotting and quantitation of synaptophysin and of FLAG ARF6, using the murine anti-FLAG M2 antibody (Sigma, St. Louis, MO, USA), were performed as described [12].

3. Results

To determine whether ARF6 plays a role in synaptic vesicle biogenesis, we have used a marker protein, p-selectin-HRP, to quantitatively examine the effect of ARF6 expression on the formation of synaptic vesicles containing p-selectin-HRP. To confirm the results of Norcott et al. [13], who demonstrated that p-selectin-HRP is targeted to synaptic vesicles, we expressed p-selectin-HRP in PC12 cells and analyzed the distribution of peroxidase activity on a glycerol velocity gradient. The gradient fractions were also assayed for immunoreactivity to the synaptic vesicle protein synaptophysin by Western blotting to determine the localization of synaptic vesicles in relation to the peroxidase activity. A peak of synaptophysin immunoreactivity was present at 14% glycerol, precisely corresponding with a peak of peroxidase activity representing p-selectin-HRP (Fig. 1). This co-localization indicates that p-selectin-HRP is targeted to synaptic vesicles in PC12 cells, as described by Norcott et al. [13], and therefore can be used as a marker for synaptic vesicles to investigate the effects of ARF6 expression on synaptic vesicle biogenesis. Peroxidase activity was also present at the top of the gradient; this peak may represent p-selectin-HRP in small membrane fragments.

We co-expressed p-selectin-HRP with each of the following ARF6 constructs by transient transfection: wild-type ARF6, the GTP-binding deficient ARF6 T27N mutant, or the GTPase deficient ARF6 Q67L mutant. We first examined whether ARF6 expression – wild-type or mutant – would alter the size of the synaptic vesicles and therefore, their migration in the glycerol velocity gradient. Synaptic vesicles containing p-selectin-HRP migrated at the same position in the gradient regardless of ARF6 expression (p-selectin-HRP alone = 13.5 ± 0.56% glycerol, wild-type ARF6 = 13.2 ± 0.56%, T27N = 13.5 ± 0.47%, Q67L = 13.3 ± 0.28%). In addition, the position of the synaptic vesicles in transfected cells (~10% of total), as measured by the position of p-selectin-HRP in the gradient, was identical to the position of the synaptic vesicles in all the cells using the endogenous synaptic vesicle protein synaptophysin as a marker.

We next examined whether ARF6 expression affects the amount of p-selectin-HRP in the synaptic vesicle peak on the glycerol velocity gradient. We co-expressed p-selectin-HRP with wild-type, T27N or Q67L ARF6. The amount of p-selectin-HRP in the synaptic vesicle peak was normalized to the HRP activity in the homogenate or in the S2 (supernatant loaded onto the gradient) to control for the expression level of p-selectin-HRP within each experiment. Relative ARF6 expression levels were determined by immunoblotting for the C-terminal FLAG tag. Although the expression levels of each construct were variable from experiment to experiment,

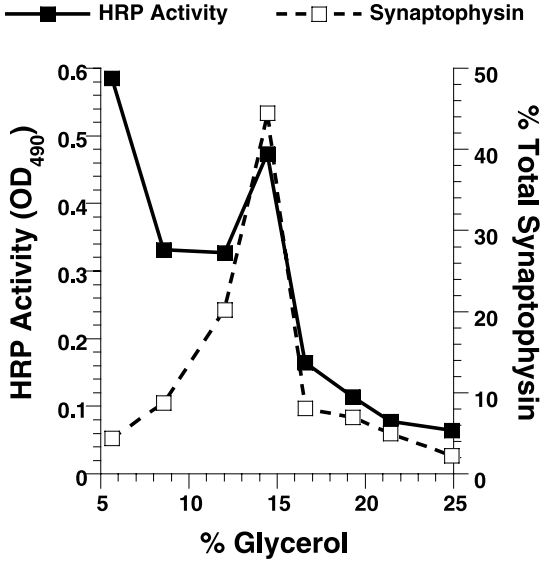

Fig. 1. p-Selectin-HRP is targeted to synaptic vesicles. PC12 cells were transiently transfected with p-selectin-HRP and a glycerol velocity gradient was run to enrich in synaptic vesicles. Each gradient fraction was spun at 240 000 $\times g$ to pellet all membranes and the resuspended pellets were analyzed for synaptophysin immunoreactivity and peroxidase activity.

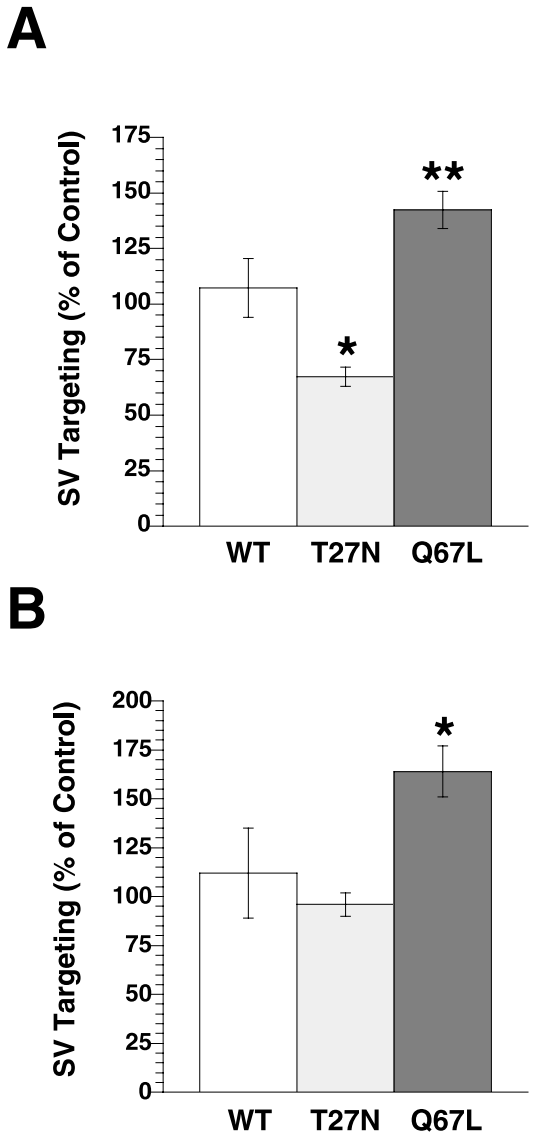

Fig. 2. Expression of ARF6 T27N and Q67L mutants affects the amount of p-selectin-HRP in gradient fractions containing synaptic vesicles. PC12 cells were transiently transfected with p-selectin-HRP alone (control) or co-transfected with p-selectin-HRP and wild-type ARF6, ARF6 T27N or ARF6 Q67L. Cellular homogenates were spun to pellet endosomes and large dense core vesicles, and the resulting supernatant (S2) was separated in a glycerol velocity gradient to enrich in synaptic vesicles. The amount of p-selectin-HRP in the synaptic vesicle peak, the cellular homogenate, and the S2 was determined by a peroxidase assay. To control for p-selectin-HRP expression levels the amount of p-selectin-HRP in the synaptic vesicle peak was normalized to (A) the cellular homogenate or to (B) the S2 and expressed as a percent of the control ± S.E.M. (A) *T27N, $P < 0.0003$, $n = 3$; **Q67L, $P < 0.002$, $n = 4$. (B) *Q67L, $P < 0.005$, $n = 4$, unpaired Student's t-test.

they were all expressed at equivalent levels (data not shown). Furthermore, the expression levels were not correlated with any effect on p-selectin-HRP targeting to synaptic vesicles (data not shown).

The amount of p-selectin-HRP in the synaptic vesicle peak with ARF6 expression was expressed as a percent of the control (cells expressing p-selectin-HRP alone). Expression of either ARF6 mutant significantly affected the level of p-selectin-HRP in the synaptic vesicle peak, while wild-type ARF6 expression did not alter the amount of p-selectin-HRP in the synaptic vesicle peak (Fig. 2A). Expression of the GTP-binding deficient ARF6 T27N mutant resulted in a significant decrease, while the GTPase-deficient ARF6 Q67L mutant caused a significant increase in the amount of p-selectin-HRP in the synaptic vesicle peak normalized to the homogenate.

A different effect was seen when the amount of p-selectin-HRP in the synaptic vesicle peak was normalized to the amount of p-selectin-HRP in the S2 (Fig. 2B). Expression of wild-type ARF6 or ARF6 T27N did not affect the amount of p-selectin-HRP in the synaptic vesicle peak, while ARF6 Q67L significantly increased the level of synaptic vesicle p-selectin-HRP. While the expression of the ARF6 Q67L mutant increased p-selectin-HRP in the synaptic vesicle peak when normalized to either the homogenate or S2, the expression of ARF6 T27N mutant decreased p-selectin-HRP targeting to the synaptic vesicle peak only when normalized to the homogenate. We calculated the specific activity of p-selectin-HRP in the S2 from cells co-transfected with ARF6 as a percent of the control. Expression of wild-type ARF6 or ARF6 Q67L with p-selectin-HRP had no effect, while expression of ARF6 T27N caused a significant decrease in the specific activity of p-selectin-HRP in the S2 (Fig. 3). Expression of any of the ARF6 constructs, including ARF6 T27N, did not change the specific activity of p-selectin-HRP in the homogenate (data not shown), indicating that total cellular p-selectin-HRP expression levels were not affected by ARF6 expression. This result suggests that ARF6 T27N expression inhibits the formation of mature synaptic vesicles or causes mistargeting of p-selectin-HRP.

4. Discussion

We have shown here that the expression of ARF6 mutants in PC12 cells has a profound effect on the biogenesis of synaptic vesicles. ARF6 could be playing several roles in synaptic vesicle formation including coat protein recruitment, cargo selection or vesicle budding. In non-neuronal cells, ARF6 Q67L expression results in a redistribution of the TfR, an

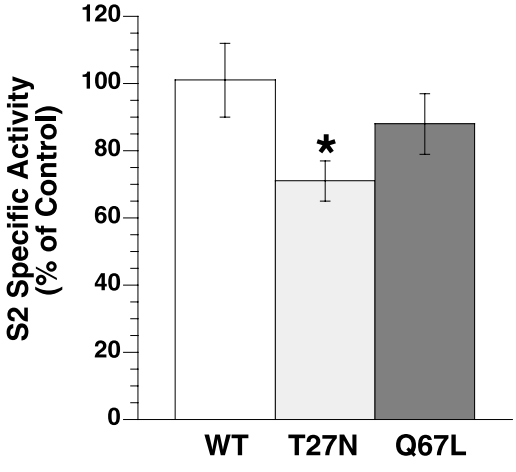

Fig. 3. Expression of ARF6 T27N decreases the specific activity of p-selectin-HRP in the S2. Cells were transfected and prepared as in Fig. 2. The specific activity of p-selectin-HRP in the S2 was calculated and expressed as a percent of the control. Expression of ARF6 T27N caused a significant difference, $*P < 0.0107$, unpaired Student's t-test ($n = 3$).

integral membrane protein that recycles constitutively between the plasma membrane and the endosomal compartment, from the endosome to the plasma membrane. This result has been interpreted as the result of a decreased rate of internalization of TfR from the plasma membrane [7] and/or increased vesicle budding from the endosome [9] coupled with a relatively rapid, but unchanged, fusion rate for these transport vesicles ($t_{1/2} \sim 10$ min). The increase in the amount of p-selectin-HRP seen in the synaptic vesicle peak with ARF6 Q67L expression is consistent with these results. An increase in the rate of synaptic vesicle budding from endosomes coupled with a relatively slow rate of synaptic vesicle fusion ($t_{1/2} \sim 30$ min) would lead to an increase in the number of synaptic vesicles at the steady state. ARF6 could also regulate synaptic vesicle formation from the plasma membrane. In polarized epithelial cells, ARF6 Q67L expression stimulates clathrin-mediated endocytosis [10], an effect which has not been observed in non-neuronal cells, suggesting that the function of ARF6 may be dependent on cell type and cell-specific effectors and regulators. Interestingly, expression of an ARF6 guanine nucleotide exchange factor (GEF), msec7-1, in *Xenopus* neurons results in an increase in spontaneous synaptic currents and in the amplitude of evoked currents [15], suggesting that ARF6 expression in neurons can regulate the availability or number of synaptic vesicles. An alternate explanation of our results is that ARF6 expression regulates the amount of p-selectin-HRP in each vesicle without affecting the number of synaptic vesicles. While there is currently no experimental evidence that suggests ARF6 regulates the rate of cargo recruitment into coated vesicles, additional experiments will be necessary to determine the relative contributions of vesicle formation and cargo recruitment to the increased targeting we have observed.

Expression of ARF6 T27N in PC12 cells produced a decrease in the amount of p-selectin-HRP in the synaptic vesicle peak, as well as a decrease in the specific activity of the S2. These results suggest that ARF6 T27N expression inhibits synaptic vesicle formation or causes the mistargeting of p-selectin-HRP. Expression of ARF6 T27N in COS cells resulted in an accumulation of a novel type of coated endocytic vesicles [9]. These vesicles contain TfR, but none of the currently known coat proteins, including coatomer, clathrin or clathrin-associated proteins (Victor Hsu, personal communication). In PC12 cells, expression of T27N may result in formation of a synaptic vesicle precursor containing p-selectin and other synaptic vesicle proteins still enveloped in this novel coat structure. These coated vesicles sediment at relatively low velocities (Victor Hsu, personal communication), which could explain why p-selectin is depleted from the S2 in PC12 cells expressing the T27N mutant, but not in cells expressing ARF6 Q67L or wild-type ARF6.

Finally, expression of wild-type ARF6 in PC12 cells had no detectable effect on synaptic vesicle biogenesis. Since the GEFs for most small G proteins are thought to be rate-limiting, simply increasing the levels of wild-type ARF6 above endogenous amounts would be expected to have little or no effect on ARF6 function.

In addition to ARF6, another ARF family member is important for synaptic vesicle biogenesis in PC12 cells. Faundez et al. [2] demonstrated that formation of synaptic vesicles containing the synaptic vesicle protein synaptobrevin is sensitive both in vitro and in vivo to inhibitors of ARF1, and p-selectin targeting to synaptic vesicles is almost completely inhibited by treatment of PC12 cells with Brefeldin A, a fungal metabolite that specifically inhibits ARF1 [16]. Why would two distinct ARF proteins be involved in the same process? One possibility is that ARF1 and ARF6 regulate distinct steps in synaptic vesicle formation through different effectors. ARF1, for example, might regulate coat recruitment, while ARF6 regulates budding. Another possibility is that ARF1 and ARF6 regulate the formation of different types of synaptic vesicles, perhaps containing different ratios of synaptic vesicle proteins.

We have shown that expression of ARF6 mutants in PC12 cells affects the biogenesis of synaptic vesicles. The mechanisms by which ARF6 activation regulates coat recruitment, which itself can determine cargo selection, and/or vesicle budding, have yet to be determined. Of particular interest for future experiments is the role of ARF6 effectors, including adapter proteins and phospholipases, and regulators such as ARF6 GEFs and GAPs (GTPase activating proteins) in synaptic vesicle formation.

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