Differential Effects of a Rab6 Mutant on Secretory Versus Amyloidogenic Processing of Alzheimer’s β-Amyloid Precursor Protein*

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The Ras-related GTP-binding protein, Rab6, is localized in late Golgi compartments where it mediates intracellular vesicular trafficking. Herein we report that coexpression of Alzheimer’s β-amyloid precursor protein (βAPP751) with a dominant-negative Rab6 mutant (Rab6N126I) in human embryonal kidney 293 cells causes an increase in secretion of the soluble amino-terminal exodomain (s-APPα) derived from non-amyloidogenic processing of βAPP751 by α-secretase. The effect was specific to Rab6N126I, since the corresponding mutation in Rab8 (i.e. Rab8N121I), which has been implicated in protein transport to the plasma membrane, caused a modest reduction in s-APPα secretion. While Rab6N126I stimulated secretion of APPα, the accumulation of amyloid β-peptide (Aβ) in the medium was either moderately reduced or unaffected. Similar differential effects of Rab6N126I on secretion of s-APPα versus Aβ were observed in cell cultures that were overproducing Aβ after transfection with a plasmid encoding the Swedish variant of βAPP751. Moreover, assays of medium from the latter cultures revealed a marked increase in secretion of s-APPα relative to s-APPβ (the immediate product derived from cleavage of βAPP by β-secretase). The results indicate that vesicular transport events controlled by Rab6 occur at or near a critical juncture in the trans-Golgi network where βAPP is sorted into either the constitutive α-secretase pathway or the amyloidogenic β-secretase pathway.

The 4-kDa amyloid β-peptide (Aβ) has been implicated in the pathogenesis of Alzheimer’s disease (1, 2). Aβ is formed as the result of intracellular proteolytic processing of β-amyloid precursor proteins (βAPP695, βAPP751, and βAPP770). The biosynthesis of Aβ begins when βAPP is cleaved by an enzymatic activity termed β-secretase, releasing a soluble NH₂-terminal exodomain (s-APPβ), which is secreted from the cell, and leaving a membrane-anchored COOH-terminal fragment containing the intact Aβ sequence (3-40). Aβ is released when the latter fragment is further trimmed by another proteolytic activity currently referred to as γ-secretase (7, 8). While amyloidogenic processing of βAPP is known to occur at low levels ubiquitously, cells that produce βAPP direct the substantial proportion of the precursor protein to the constitutive secretory pathway, where it is processed via a non-amyloidogenic mechanism. The latter entails initial cleavage of βAPP within the Aβ domain by an enzymatic activity termed α-secretase, releasing a soluble exodomain (s-APPα) containing part of the Aβ sequence (3, 9-11). Because the residual COOH-terminal stump lacks an intact Aβ domain, it cannot give rise to Aβ when cleaved by γ-secretase.

The mechanisms by which cells control the flux of βAPP into the amyloidogenic versus non-amyloidogenic pathways are poorly understood. It has been particularly difficult to determine the precise subcellular sites of the various processing events because the relevant proteases have not yet been isolated. However, there is sufficient evidence to suggest that α-secretase acts on the mature form of βAPP at the cell surface or in a late compartment of the default secretory pathway, after it has undergone tyrosine sulfation (10, 12-15). Less is known about the subcellular sites of the amyloidogenic processing of βAPP by β-secretase and γ-secretase. A number of studies have indicated that events occurring in acidic compartments (e.g. endosomes or vesicles of the trans-Golgi network) are involved in the biogenesis of Aβ (16-19). In particular, a recent study suggests that β-secretase cleavage of βAPP containing the Swedish mutation occurs within transitional vesicles between the trans-Golgi compartment and the cell surface (20).

To elucidate the steps involved in intracellular trafficking and processing of βAPP, we have adopted a novel strategy, which is based on mutagenesis and expression of GTP-binding proteins encoded by the rab gene family. There are currently more than 30 distinct Rab proteins, which are localized in discrete organelles and vesicles, where they play key roles in protein trafficking between specific donor and acceptor compartments along the exocytic or endocytic routes (21-23). Like other Ras-related proteins, Rab proteins are active when they are in the GTP-bound state. Hence, mutant Rab proteins with reduced affinity for GTP exert a dominant negative effect over their endogenous counterparts when overexpressed in mammalian cells (24-26). We recently showed that when βAPP751 was coexpressed with a dominant-negative Rab1B mutant (i.e. Rab1B(N121I)) in HEK 293 cells, the maturation of βAPP751, and the secretion of both s-APP and Aβ were impaired (27), consistent with the established role of Rab1B in ER → Golgi transport (24, 28, 29).

In the present study we focused on Rab6, which is known to...
reside in trans-Golgi cisternae, the trans-Golgi network, and post-Golgi secretory vesicles (30–32), and has been implicated in both intra-Golgi transport (33) and the budding of exocytic vesicles from the trans-Golgi network (34). We found that when βAPP was coexpressed with a GTP-binding-defective Rab6 mutant (Rab6N126I), secretion of s-APPα was stimulated, while deposition of Aβ and s-APPβ into the medium was either modestly reduced or unaffected. These findings suggest that transport steps mediated by Rab6 occur at a branch point where amyloidogenic and non-amyloidogenic pathways for βAPP processing diverge.

**EXPERIMENTAL PROCEDURES**

Mutagenesis of Rab6 and Rab8—The cDNAs encoding human Rab6 (35) and Rab8 (36) were obtained by PCR amplification from first strand cDNA template, reverse-transcribed from total Hela cell mRNA (37). Each construct was further modified by PCR to encode a 10-amino acid Myc epitope tag at the amino terminus of the expressed protein, as described previously for Rab1B (27). The myc-rab6WT cDNA was ligated into the EcoRI/BamHI sites of the mammalian expression vector pCMV5 (38), to obtain pCMVrab6WT. The myc-rab6WT cDNA was ligated into the EcoRI/Xbal sites of pCMV5neo (39) to obtain pCMVrab8WT (40). The latter vectors were used as templates to generate DNA vectors encoding Myc-Rab6N126I and Myc-Rab8N121I by site-directed mutagenesis, using the PCR-based megaprimer method (40) with the following mutator oligonucleotides: Rab6 N126I, 5'-CTAGTAGGATCAAACAGACCTTGGTGCAC; Rab8 N121I, 5'-CTTGGGATCAAGTG
tTTGAGTGGATTGTG. The resulting PCR products were subcloned into pCMV5 to obtain pCMVrab6126I and pCMVrab8121I. The DNA sequences of all constructs were verified by the dideoxy chain termination technique using the Sequenase 2.0 kit (United States Biochemical Corp.).

Coexpression of βAPP with Rab Proteins—Expression vectors encoding βAPP751 (phCK751) and the Swedish variant of βAPP751 (pohCK751sw) have been described previously (27). Human 293 cells were grown in 60-mm dishes and transfected exactly as described by Dungan et al. (27). For transient coexpression of βAPP (WT or Swedish) with Rab6 (WT or N126I) or Rab8 (WT or N121I) in 293 cells, 10 μg of the specified rab pCMV DNA was combined with 1 μg of phCK751 or pohCK751sw.

Immunoblot Analyses—For analysis of intracellular proteins, washed cell pellets derived from 60-mm dishes were solubilized in SDS sample buffer and subjected to SDS-PAGE and immunoblot analysis as described previously (41, 42). To confirm expression of the Rab proteins, one fourth of the cell lysate was subjected to SDS-PAGE on a 12.5% polyacrylamide gel and immunoblotted with 9E10 monoclonal antibody with specificity against residues 1–12 of the Aβ sequence, to detect s-APPα and s-APPβ. Anti-APP with Rab Proteins—Equal amounts of cell lysates from 3-to-5 fold over the endogenous Rab6, as determined by immunoblot analysis with an affinity-purified antibody against Rab6 (not shown). Expression levels of βAPPsw were approximately 40-fold over the endogenous βAPP in 293 cells, so that measurements of products derived from βAPP (i.e. s-APP) reflect almost exclusively the processing of the overexpressed βAPPWT in the subpopulation of cells expressing the Rab6 constructs. Upon harvesting the cultures, levels of s-APP (counts/min) and Aβ (pg/g) were expressed as a ratio to total levels of βAPP as determined by Western blot assay using 125IgG or ELISA, respectively (see “Experimental Procedures”). The values for total s-APP (panel A) and Aβ (panel B) in 2 ml of medium were normalized to the total intracellular βAPPsw in the cell monolayer. In panel C, the values for total extracellular Aβ were expressed as a ratio to total extracellular s-APP. The results shown are means (± S.E.) of separate determinations performed on three parallel cultures.

α-secretase, and detection with the monoclonal antibody 6C6, which is directed against the first 16 amino acids of Aβ (44). The ELISA used for determination of the concentration of Aβ1–42 also employed monoclonal antibody 266 for capture, but used for detection a polyclonal antibody 277-2, which was raised against a peptide including Aβ1 residues 33–42 (45). 647G or ELISA, respectively (see “Experimental Procedures”).

**RESULTS**

We recently showed that the inhibitory effects of a Rab1B mutation (N121I) on early steps in the posttranslational maturation of βAPPsw can be readily detected by transiently coexpressing both proteins in HEK 293 cells (27). In the present study, we used the same approach to examine the possible role of a Golgi-localized Rab protein, Rab6, in the secretory and amyloidogenic processing of βAPP. By analogy to other members of the Ras superfamily (46, 47), incorporation of the N126I substitution into Rab6 is predicted to drastically reduce the affinity of the protein for GTP. Accordingly, Myc-Rab6W126I immunoenriched from 293 cells with anti-Myc antibody failed to bind detectable 32P[GTP when compared to Myc-

[Fig. 1. Rab6WT has differential effects on secretion of s-APP versus Aβ. Cells were cotransfected with phCK751 alone or in combination with plasmids encoding Myc-tagged Rab6WT or Rab6N126I as indicated. All cultures were incubated without changing the medium for 48 h. Expression levels of the Myc-Rab6WT or Myc-Rab6N126I ranged from 3- to 5-fold over the endogenous Rab6, as determined by immunoblot analysis with an affinity-purified antibody against Rab6 (not shown). Expression levels of βAPPsw were approximately 40-fold over the endogenous βAPP in 293 cells, so that measurements of products derived from βAPP (i.e. s-APP) reflect almost exclusively the processing of the overexpressed βAPPWT in the subpopulation of cells expressing the Rab6 constructs. Upon harvesting the cultures, levels of s-APP (counts/min) and Aβ (pg/g) were expressed as a ratio to total extracellular s-APP. The results shown are means (± S.E.) of separate determinations performed on three parallel cultures.](https://example.com/fig1.png)
s-APP and Aβ were measured in samples of conditioned medium (Fig. 1). To exclude the possibility that differences in extracellular s-APP or Aβ might reflect variations in βAPP751 expression in the cell monolayers, the values for s-APP and Aβ were normalized to the total intracellular βAPP751 in each culture. Compared to cultures that were not transfected with Rab6 plasmid, the cultures that were coexpressing Rab6WT with βAPP751 showed a modest reduction in secreted s-APP. The opposite effect was observed in cultures expressing Rab6N126I, where levels of s-APP were markedly elevated (Fig. 1A). In contrast to the level of s-APP, the extracellular concentration of Aβ did not increase in the cultures that were expressing Rab6N126I (Fig. 1B). In fact, the Aβ levels were lower in these cultures than in the cultures expressing Rab6WT or no exogenous Rab6. The inverse effects of Rab6N126I on s-APP and Aβ in this study are underscored by the 5-fold decline in the direct ratio of extracellular Aβ to extracellular s-APP in the cultures expressing Rab6N126I versus Rab6WT (Fig. 1C).

To verify the specificity of the results observed with the Rab6 mutant, the equivalent mutation (N121I) was introduced into Rab8. The latter protein is localized to the cell periphery (48, 49) and is normally expressed in a wide variety of cells, including 293 cells.2 Rab8 has been implicated as a mediator of protein transport from the trans-Golgi network to the basolateral (50) or dendritic (51) plasma membrane in polarized epithelial and neuronal cells, respectively. However, it is unclear whether Rab8 plays a similar role in non-polarized cells. When βAPP751 was coexpressed with Rab8N121I, the relative amount of secreted s-APP was modestly reduced in comparison to parallel cultures expressing Rab8WT (Fig. 2A). The levels of Aβ were also slightly reduced in the cultures expressing Rab8N121I (Fig. 2B), and there was no change in the direct ratio of Aβ to s-APP in the culture medium (Fig. 2C). These findings contrast with the striking decrease in the ratio of Aβ/s-APP (mostly due to increased secretion of s-APP) observed in the cultures expressing Rab6N126I (Fig. 1C).

To determine whether the higher end point values for accumulated extracellular s-APP in cultures expressing Rab6N126I reflected an increased rate of secretion of s-APP, samples of medium from individual cultures overexpressing βAPP751 with either Rab6WT or Rab6N126I were collected at multiple time points over a 24-h period (Fig. 2A). The results of this analysis demonstrated an increased rate of s-APP accumulation in the cultures expressing Rab6N126I compared to cultures expressing Rab6WT. The increased deposition of s-APP into the medium could not be attributed to differential expression of βAPP751, since the intracellular levels of mature (fully glycosylated) βAPP751 (Fig. 3B) and immature βAPP751 (Fig. 3C) were comparable in cultures expressing both the wild-type and mutant Rab6 proteins. Consistent with the results obtained in the 48-h

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2 F. Castellano and W. A. Maltese, unpublished observation.
coexpression experiment (Fig. 1), the 24-h end point values for extracellular s-APP, normalized to total intracellular βAPP751, were increased approximately 2-fold in the cultures expressing Rab6N126I versus Rab6WT (Fig. 3D), while the values for Aβ were not increased (Fig. 3E).

The s-APP secreted by 293 cells expressing wild-type βAPP751 is predominantly the α form (i.e. s-APPα) released upon cleavage by α-secretase (S2). In light of the contrasting effects of Rab6N126I on levels of s-APP and Aβ, we were interested in determining how expression of this Rab6 mutant might affect secretion of the immediate product generated by β-secretase cleavage (i.e. s-APPβ). To facilitate this analysis, 293 cells were cotransfected with plasmids encoding either Rab6WT or Rab6N126I together with the Swedish variant of βAPP751 (i.e. SWβAPP751), which contains a dual amino acid change (Lv516 → Asp and I452 → Leu) known to promote increased processing of the protein along the Aβ pathway (S2–S4). Using antibodies that discriminate between α and β forms of s-APP, we observed that the amount of s-APPα (normalized to intracellular SWβAPP751) secreted into the medium was almost doubled in cultures expressing Rab6N126I (Fig. 4A). In contrast, the normalized value for extracellular s-APPβ was modestly reduced in the same cultures (Fig. 4B). These reciprocal changes were reflected in a 3-fold increase in the direct ratio of the α and β forms of s-APP in the medium from cultures coexpressing SWβAPP751 with Rab6N126I versus Rab6WT (Fig. 4C). Although Rab6N126I caused a small decline in the level of extracellular s-APPβ in this experiment, we were unable to detect a significant decrease in intracellular s-APPβ (referred to as c-APPβ in Fig. 4D). This suggests that expression of Rab6N126I does not interfere directly with transport steps required for SWβAPP751 to gain access to the subcellular compartment containing β-secretase.

In accord with the earlier studies of wild-type βAPP (Fig. 1B), coexpression of SWβAPP751 with Rab6N126I resulted in a 35–40% decrease in the amount of Aβ that accumulated in the conditioned medium after 48 h (Fig. 4E). This decrease was similar in magnitude to the decline in s-APPβ in the same cultures (Fig. 4B). It is now recognized that Aβ exists in a number of isoforms that include alternate carboxyl termini: e.g. Aβ1–40 and Aβ1–42 (55, 56). Although Aβ1–40 is the more abundant species, Aβ1–42 has attracted particular attention since it is prone to form insoluble amyloid fibrils (57) and appears to be a major constituent in amyloid deposits in Alzheimer’s disease (58, 59). Thus, in addition to assaying total Aβ (mostly Aβ1–40), we employed an ELISA that specifically measures Aβ1–42 in medium from the cultures expressing SWβAPP751 (Fig. 4F). In contrast to the modest but significant decline in total Aβ observed in cells expressing Rab6N126I versus Rab6WT (Fig. 4E), there was no detectable difference in the extracellular Aβ1–42 in the same cultures (Fig. 4F).

Fig. 5 depicts the results of a separate time-course study in which we confirmed that the increased extracellular s-APP/s-APPβ ratio in medium from cultures coexpressing Rab6N126I and SWβAPP751 arises as a result of differential effects of the Rab6 mutant on the rates of secretion of the α and β forms of s-APP (Fig. 5, A–C). The marked increase in the rate of sAPPα secretion in the cultures expressing Rab6N126I versus Rab6WT was reflected in a 3-fold elevation in the total extracellular s-APPα, normalized to intracellular βAPP751, at the 12-h end point (Fig. 5D). In contrast, the cultures expressing Rab6N126I showed a slight decrease in the normalized value for s-APPβ (Fig. 5E) and no significant change in Aβ (Fig. 5F).

**DISCUSSION**

The results described in this report demonstrate that overexpression of a GTP-binding-defective Rab6 mutant in 293 cells results in a marked enhancement of secretion s-APPα, while extracellular accumulation of products derived from the amyloidogenic processing pathway (i.e. s-APPβ and Aβ) is either modestly inhibited or unaffected. Extensive studies have documented the localization of Rab6 in the trans-Golgi cisternae and trans-Golgi network in mammalian cells (30–32). Therefore, our findings indicate that intra-Golgi transport events regulated by Rab6 selectively influence the routing of βAPP751 into the α-secretase pathway. While previous studies have
show that s-APPα and s-APPβ are sorted differently in polarized MDCK cells (52) and that secretion of s-APPα and Aβ respond in opposite ways to stimulation of protein kinase C (60–64), the present report provides the first genetic evidence implicating a specific protein (Rab8) and a specific subcellular compartment (the trans-Golgi network) in the branching of the α and β-secretase pathways for βAPP processing.

Our studies with Rab8 underscore the specificity of the results obtained with the Rab6 mutant. In contrast to the stimulatory effect of Rab6N126I on s-APPα secretion, Rab8N121I had a small inhibitory effect on the same pathway. The decreased secretion of s-APPα caused by the Rab8 mutant in 293 cells is consistent with the proposed role of Rab8 in transport of proteins between the Golgi apparatus and the basolateral plasma membrane in polarized epithelial cells (50). However, because the effects of Rab8N121I were relatively minor in comparison to the complete block in s-APP secretion previously observed when ER → Golgi transport was disrupted by Rab1B N121I (27), we speculate that post-Golgi secretory transport in non-polarized cells probably involves members of the Rab family in addition to Rab8.

In addition to providing mechanistic insights into βAPP processing, the finding that the dominant-negative N126I mutation in Rab6 stimulates rather than inhibits secretion of s-APPα augments the only mutagenic analysis of Rab6 function performed in intact cells. Specifically, Martinez et al. (33) showed that overexpression of an activating Rab6 mutant, Rab6C27L, caused a delay in constitutive protein secretion, with a corresponding accumulation of transport markers in late Golgi compartments. The latter findings were interpreted as indicating that Rab6 acts either as an inhibitor of anterograde transport through the trans-Golgi compartment or as a positive regulator of retrograde transport from post-Golgi vesicles back to the trans-Golgi network or Golgi cisternae. Our results support this model of Rab6 function, since it would predict that a dominant-negative Rab6 mutant such as Rab6N126I might facilitate the anterograde flow of glycoproteins into the distal portion of the constitutive secretory pathway where α-secretase is thought to reside.

Based on the foregoing model of Rab6 function in intra-Golgi trafficking, we propose that by impairing retrograde transport or facilitating anterograde transport within the trans-Golgi compartment, Rab6N126I increases the flow of βAPP into the constitutive α-secretase pathway. The absence of a parallel stimulatory effect on secretion of s-APPβ and Aβ suggests that the pool of βAPP destined for processing by β-secretase is sorted into a distinct trans-Golgi or endosomal compartment prior to the transport steps mediated by Rab6. This proposal is consistent with studies indicating that β-secretase operates in an acidic compartment (16–19, 65). An alternative model of amyloidogetic processing envisions that βAPP is cleaved by β-secretase and γ-secretase in endosomes or lysosomes after it escapes cleavage by α-secretase and is reinternialized from the plasma membrane. Support for this model is derived from studies showing that intact βAPP exists at the cell surface and is reinternialized in dextrin-coated endocytic vesicles (12, 65). However, it seems unlikely that this is the major pathway for biogenesis of Aβ in 293 cells, since we failed to observe a coordinate increase in s-APPβ and Aβ production in cells where transport of βAPP to the cell surface was enhanced 2.5–3-fold.

The general experimental approach described herein might be particularly useful in future studies aimed at elucidating the molecular mechanisms underlying the biogenesis of distinct forms of Aβ. Specifically, the mutagenesis of various Rab proteins localized in different segments of the exocytic and endocytic pathways might provide insight into the question of whether Aβ1–40 and Aβ1–42 arise through the action of unique γ-secretase activities residing in separate subcellular compartments. While the differential effect of Rab6N126I on Aβ1–40 versus Aβ1–42 observed in the present study (Fig. 4) could be consistent with the latter possibility, the results are inconclusive because the effects of Rab6N126I on total Aβ were small and somewhat variable (e.g. no decrease was observed in the short term studies depicted in Figs. 3 and 5). Thus, it will be of considerable interest to see whether more definitive differences in production of the Aβ isoforms will be detected as these studies are expanded to include additional members of the Rab protein family.

REFERENCES
1. Selkoe, D. J. (1994) Annu. Rev. Cell Biol. 10, 373–403
2. Gandy, S., and Greengard, P. (1994) Int. Rev. Neurobiol. 30, 29–50
3. Haass, C., and Selkoe, D. J. (1993) Cell 75, 1039–1042
4. Haass, C., Schlossmacher, M. G., Hung, A. Y., Vigo-Pelfrey, C., Melson, A., Ostaszewski, B. L., Lieberburg, I., Koo, E. H., Schenk, D., Teplow, D. B., and Selkoe, D. J. (1992) Nature 359, 322–325
5. Shoji, M., Golde, T. E., Ghiso, J., Cheung, T. T., Estus, S., Shaffer, L. M., Cai, X. D., McKay, D. M., Tintner, R., Frangione, B., and Younkin, S. G. (1992) Science 258, 126–129
6. Seubert, P., Oltersdorf, T., Lee, M. G., Barbour, R., Blomquist, C., Davis, D. L., Bryant, K., Fritz, L. C., Galasko, D., Thal, L. J., Lieberburg, I., and Schenk, D. B. (1993) Nature 363, 260–263
7. Haass, C., Hung, A. Y., Selkoe, D. J., and Teplow, D. B. (1994) J. Biol. Chem. 269, 17741–17748
8. Higaki, J., Quay, D., Zhong, Z., and Cordell, B. (1995) Neuron 14, 651–659
9. Esch, F. S., Keim, P. S., Beattie, E. C., Blacher, R. W., Culwell, A. R., Oltersdorf, T., McClure, D., and Ward, P. J. (1990) Science 248, 1122–1124
