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Dileucine Motif Is Sufficient for Internalization and Synaptic Vesicle Targeting of Vesicular Acetylcholine Transporter

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Efficient cholinergic transmission requires accurate targeting of vesicular acetylcholine transporter (VAChT) to synaptic vesicles (SVs). However, the signals that regulate this vesicular targeting are not well characterized. Although previous studies suggest that the C-terminus of the transporter is required for its SV targeting, it is not clear whether this region is sufficient for this process. Furthermore, a synaptic vesicle-targeting motif (SVTM) within this sequence remains to be identified. Here we use a chimeric protein, TacA, between an unrelated plasma membrane protein, Tac, and the C-terminus of VAChT to demonstrate the sufficiency of the C-terminus for targeting to synaptic vesicle-like vesicles (SVLVs) in PC12 cells. TacA shows colocalization and cosedimentation with the SV marker synaptophysin. Deletion mutation analysis of TacA demonstrates that a short, dileucine motif-containing sequence is required and sufficient to direct this targeting. Dialanine mutation analysis within this sequence suggests indistinguishable signals for both internalization and SV sorting. Using additional chimeras as controls, we confirm the specificity of this region for SVLVs targeting. Therefore, we suggest that the dileucine-containing motif is sufficient as a dual signal for both internalization and SV targeting during VAChT trafficking.

Key words: dileucine motif, internalization, synaptic vesicles (SVs), synaptic vesicle-like vesicles (SVLVs), trafficking, vesicular acetylcholine transporter (VAChT), vesicular targeting

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Synaptic transmission requires the efficient vesicular packaging of neurotransmitters, a process mediated by a group of vesicular neurotransmitter transport (VNT) proteins (1). Thus, targeting of these functional transport proteins to synaptic vesicles (SVs) is highly regulated in order to maintain synaptic efficacy. Despite this, the molecular mechanisms underlying the SV targeting of VNTs remain elusive. Evidence suggests that clathrin-mediated internalization plays an important role in the targeting of both newly synthesized and recycling VNTs to SVs. From the cell surface, SV-bound proteins require internalization and sorting that is thought to occur through at least two distinct pathways. Synaptic vesicle proteins may undergo selective internalization to directly form SVs, or proteins may be indiscriminately internalized to an endosomal intermediate from which SV proteins can be selectively sorted (2,3). The regulation of these distinct sorting processes, although not well understood, is believed to be mediated through specific cytoplasmic sorting motifs. These motifs execute their role in membrane trafficking through interaction with corresponding cytosolic machinery that direct the protein accordingly. Sorting motifs for internalization or synaptic vesicle targeting have been identified in several SV proteins (4–8). For example, synaptophysin has been shown to contain a C-terminal repeating tyrosine-based internalization motif that recruits dynamin to the membrane and directs clathrin-independent internalization (6,9,10). Another SV protein, synaptotagmin 1, has a cytoplasmic dileucine motif that is required for adaptor protein (AP)-3-mediated SV targeting (8). In contrast, another study suggested a role for synaptotagmin's lumenal N-terminus in SV targeting (5). While specific motifs have been indicated to play a role in SV targeting, the sufficiency of these motifs as well as the generality of these signals is not known.

Vesicular acetylcholine transporter (VAChT) is a member of the VNT family that is responsible for packaging acetylcholine into vesicles for regulated release (11–13). Molecular manipulations to alter the vesicular level of VAChT on SVs, namely overexpression or knockdown, lead to corresponding alterations in cholinergic transmission (14,15). Although a specific synaptic vesicle targeting motif (SVTM) has yet to be identified, the C-terminus of VAChT has been found to be required for its vesicular localization (16,17). Interestingly, this region has been identified to be phosphorylated by protein kinase C, which was suggested to play a role in modulating its internalization or trafficking (18,19). Furthermore, a classical dileucine motif within this region has been identified to be required for the internalization of the transporter (16). Interaction of VAChT through its C-terminus with clathrin-associated machinery, including AP-1 and AP-2, further suggest a critical role of the cytoplasmic sequence in the regulated clathrin-mediated internalization and membrane trafficking of VAChT (20,21). Consistently, interference with internalization machinery, including clathrin and dynamin, or mutation of the internalization motif disrupt the

targeting of VAChT to SVs leading to an accumulation of the protein on the cell surface (16,20,22). Together, these findings suggest a correlation between the C-terminal-directed and regulated internalization of the transporter and its SV targeting. However, whether the C-terminus of VAChT is sufficient for SV targeting remains unknown. Furthermore, it is unclear how the internalization of VAChT is involved in its vesicular localization and, most importantly, whether this SV targeting is determined by an SVTM within this region.

Here we report, by using a chimeric protein between an unrelated plasma membrane-localized protein, Tac, and the VAChT C-terminus, the sufficiency of this sequence in synaptic vesicle-like vesicle (SVLV) targeting in PC12 cells. We further characterize the dileucine-containing motif of VAChT as an SVTM and examine its role as a dual signal for both internalization and SVLV targeting.

Results

The C-terminus of VAChT is sufficient for SVLV targeting

The C-terminus of VAChT has been shown to be required for its preferential localization to SVLVs in PC12 cells (16). Chimeric analysis between VAChT and a closely related, but preferentially large dense core vesicle (LDCV)-localized VNT, vesicular monoamine transporter 2 (VMAT2) has suggested that the VAChT C-terminus is able to direct VMAT2 to SVLVs (16). However, these results do not distinguish whether the VAChT C-terminus is sufficient for SVLV targeting. To test this possibility, we took advantage of Tac protein, the α-subunit of the interleukin-2 receptor (IL-2R), which has a single membrane-spanning domain whose internalization requires the presence of additional IL-2R subunits (16,23,24). Not endogenously expressed in PC12 cells, Tac protein localizes to the plasma membrane when exogenously introduced (Figure 1). Through fusing the C-terminus of VAChT to Tac, we created a chimeric protein, TacA (Figure 1A), which would allow us to independently examine the trafficking properties of the VAChT C-terminus in the context of an unrelated protein.

In order to determine whether the C-terminus of VAChT is sufficient to target Tac to SVLVs, stable PC12 transformants expressing Tac protein or the chimera TacA were examined. Through immunofluorescence staining, Tac protein showed diffuse plasma membrane localization with very little cytoplasmic distribution (Figure 1B). In contrast, the chimeric TacA showed localization to internal punctate structures enriched in both the perinuclear and tip regions of differentiated PC12 cells (Figure 1B). These TacA puncta were found to colocalize with endogenous synaptophysin, an SV marker, but not secretogranin II, a LDCV marker (Figure 1B). Furthermore, biochemical analysis of Tac and TacA through gradient sedimentation (25) showed that TacA localizes primarily to the light fractions of density sucrose gradients and cosediments preferentially with synaptophysin (72.17 $\pm$ 1.557%), but not secretogranin II (Figure 1D). In contrast, cells expressing wild-type plasma membrane-localized Tac protein sedimented to heavier fractions (8–10) than SVLV-containing fractions (9–12) (Figure 1C). Using velocity gradient fractionation, designed to isolate SVLVs by excluding other larger organelles such as endosomes (26–28), TacA is found in SVLV-containing fractions, strongly indicating its SVLV targeting (Figure 1F). As expected, Tac is excluded from these SVLV-containing fractions and localizes to the bottom of the gradient (Figure 1E). These findings were also confirmed with transiently transfected PC12 cells to avoid potential problems associated with stable lines (data not shown). Thus, three independent assays indicate the localization of TacA to SVLVs, confirming the sufficiency of the VAChT C-terminus to target an unrelated protein to SVLVs and suggesting that this region contains an SVTM.

Dileucine-containing motif is required for SVLV targeting

The cytosolic tail of VAChT consists of 60 amino acids (aa) which contain several previously defined motifs including a classical dileucine motif [E(XXX)LL], a classical tyrosine motif (YXXØ) and a nonclassical tyrosine motif (Figure 2A). In order to investigate which of these motifs determines the SVLV targeting of VAChT, a series of TacA chimera with deletions within the C-terminus of VAChT were constructed (Figure 2B). These chimeras were then examined for their subcellular localization in PC12 cells. Targeting to SVLVs was determined through two criteria: steady-state colocalization with synaptophysin through immunofluorescent staining and comigration with SVLV-containing fractions in velocity subcellular fractionation analysis. As shown in Figure 2B, constructs that met both these criteria are indicated with a symbol (+) for their SVLV targeting. In contrast, constructs unable to target to SVLVs are indicated with a symbol (−) for SVLV targeting. Analysis of deletion mutants clearly indicated the requirement of a small dileucine motif-containing region (Figure 2A, indicated in gray) for SVLV targeting. The deletion of this region disrupted SVLV targeting and led to the accumulation of the mutant on the cell surface, presumably because of a loss of dileucine motif-mediated internalization. This is demonstrated through representative immunofluorescent images in Figure 2C. Deletion mutant analysis suggested that the dileucine-containing motif is the only region required for SVLV targeting. Other regions, previously reported to play a role in VAChT trafficking, including tyrosine motifs (21), or the phosphorylation at serine 480 (18,19) were not found to be essential for SVLV targeting in our system. Thus, the 10-aa dileucine motif-containing sequence of the VAChT C-terminus is essential for the SVLV targeting of VAChT.

Dileucine-containing motif is sufficient for SVLV targeting

As the results from our deletion analysis suggested that the dileucine-containing motif in VAChT plays an essential

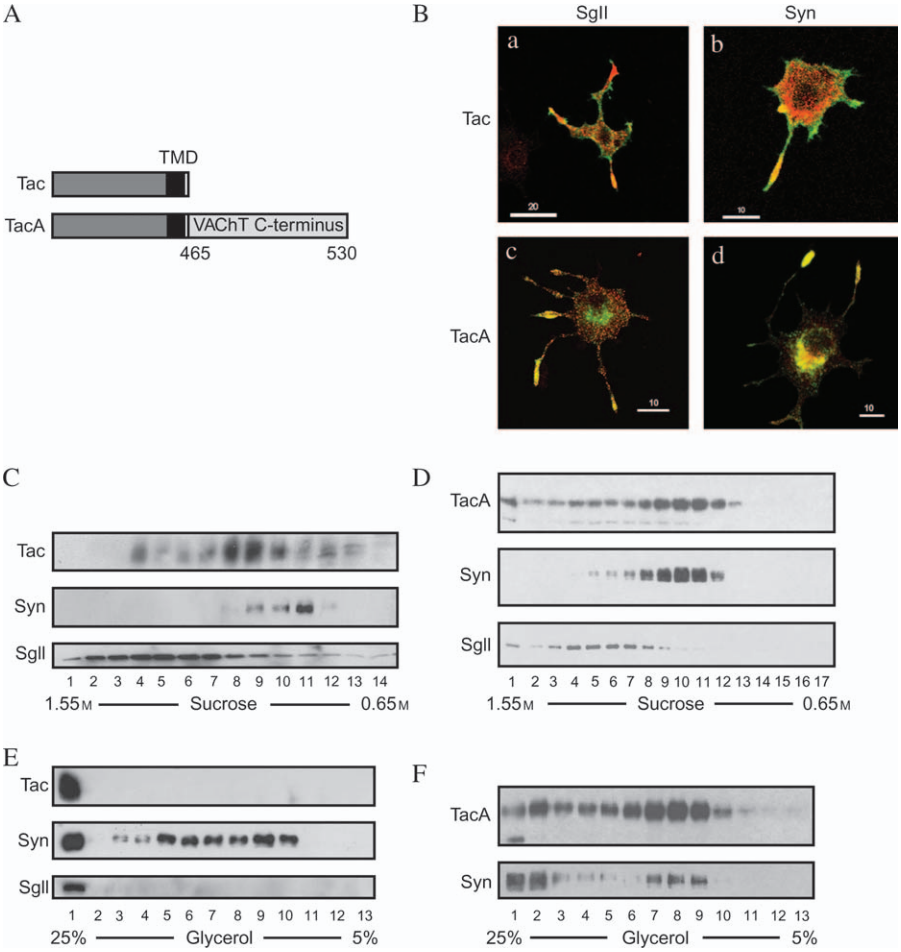

Figure 1: Vesicular acetylcholine transporter C-terminus is sufficient for SVLV targeting in PC12 cells. A) Schematic diagram of TacA construct. The VAChT C-terminus was fused to the C-terminal end of Tac protein. B) Targeting of TacA to SVLV using imaging analysis. PC12 cells stably expressing Tac and TacA were visualized through immunofluorescence (green). Both SVLVs and LDCVs were identified with antibodies against endogenous synaptophysin (Syn) or secretogranin II (SgII), respectively (red). C) Tac does not target to SVLVs in PC12 cells. Overexpressed Tac proteins in transiently transfected PC12 cells were fractionated through a sucrose density gradient (0.65–1.55 M). D) Targeting of TacA to SVLVs using density sedimentation. Postnuclear supernatants from TacA stable PC12 cells were fractionated through a sucrose density gradient. E) Exclusion of Tac from SVLV isolating velocity gradient. The PNS from transiently transfected cells were fractioned through a glycerol velocity gradient (5–25%). F) Targeting of TacA to SVLVs using velocity sedimentation. Glycerol velocity gradient was used for fractionation of TacA stable PC12 cells.

role in SVLV targeting, we next examined the sufficiency of this motif to target the unrelated protein, Tac, to SVLVs. We therefore designed a construct, Tac8ADLM, in which the 10-aa (479–488) dileucine motif-containing region of VAChT, preceded by an eight-alanine peptide, was fused to Tac (Figure 3A). It has been shown that the internalization machinery associated with dileucine motifs works most efficiently when this motif is at least eight amino acids away from the last transmembrane domain of a membrane protein (29). Therefore, in order to avoid any possible disruption of internalization, we inserted an eight-alanine peptide between Tac and the short dileucine-containing motif of VAChT. Density gradient fractionation of PC12 cells transiently expressing Tac8ADLM (Figure 3 B) showed the chimeras' targeting primarily to synaptophysin-containing fractions. Further examination of this targeting using SV isolating velocity gradients showed consistent results. Tac8ADLM was enriched in SVLV fractions as marked by synaptophysin (Figure 3C). Together, these data strongly suggest that the dileucine-containing motif of VAChT alone is able to target Tac to SVLVs in PC12 cells.

The dileucine-containing motif serves as an internalization motif as well as an SVTM

The above results demonstrate that the VAChT dileucine-containing motif is able to target Tac to SVLVs. The identified region contains a classical dileucine motif, E(XXX)LL, which has been extensively characterized for its role in internalization (16,29). Therefore, it was unclear whether the trafficking signal that mediates internalization is different than the one that mediates SVLV targeting. To address this, we generated a series of pairwise alanine mutations within this region (Figure 4A) and analyzed their effects on internalization as well as SVLV targeting. To examine internalization, a semiquantitative, enzyme-linked immunosorbent assay (ELISA)-based biotin-conjugated system was used. The assay detected a fraction of internalization of TacA dialanine mutants over a 30-min time period. Confirming the efficacy of our assay, wild-type Tac protein remained primarily on the cell surface with only 25% internalization, whereas the majority (92%) of TacA was internalized. Truncation of TacA to TacA490 did not significantly alter internalization. Using pairwise alanine mutations of this dileucine-containing region, we showed

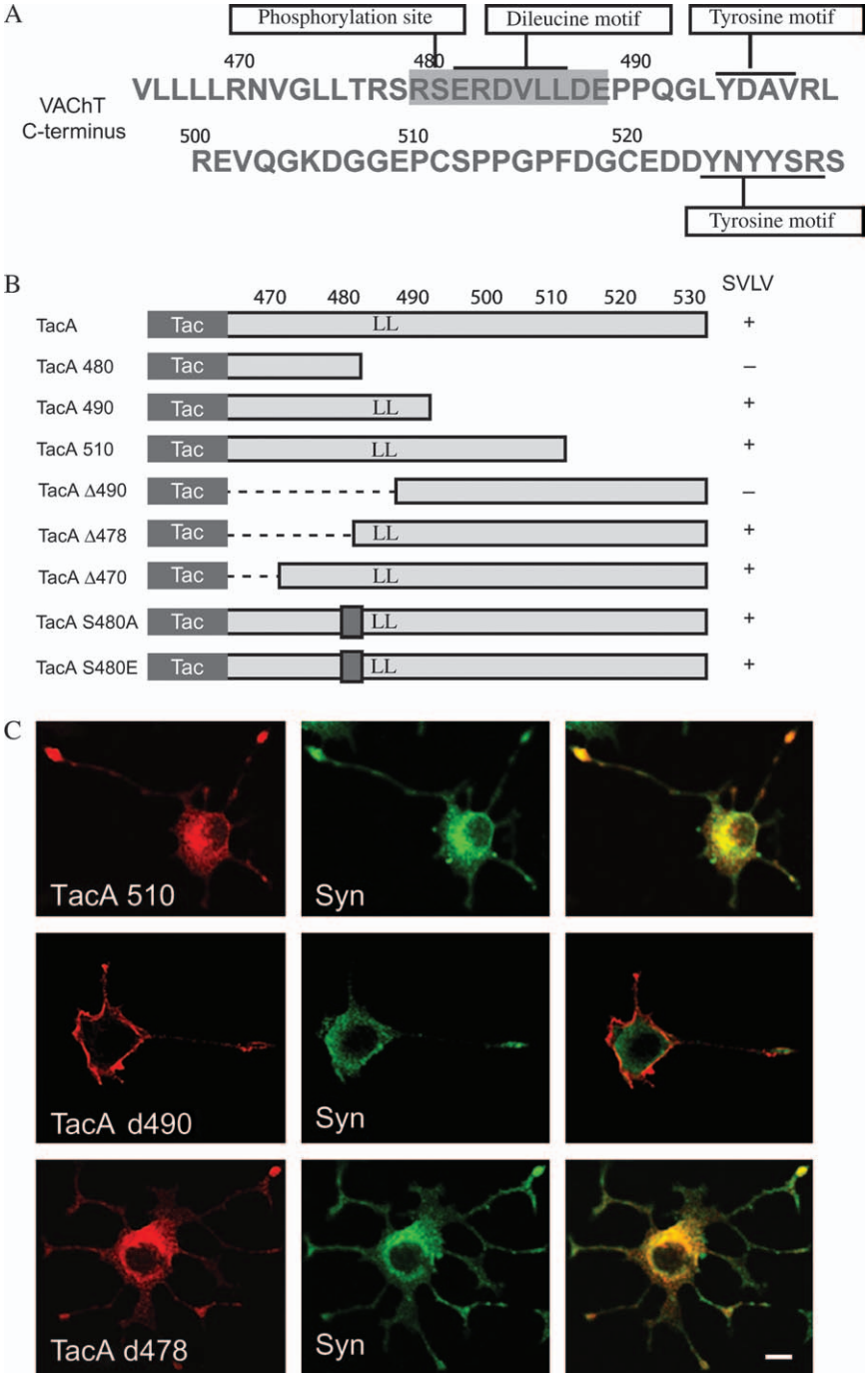

Figure 2: Dileucine-containing motif is necessary for SVLV targeting. A) Amino acid sequence of VAChT C-terminus (residues 465–530). Dileucine-containing motif is shaded in gray. B) Targeting of deletion mutants of TacA to SVLVs in PC12 cells. A series of deletion mutations were made within the C-terminus of VAChT using the TacA construct as a template. The SVLV targeting of the mutants were determined by both immunofluorescent co-localization with synaptophysin, as well as presence in SVLV isolating glycerol velocity gradients. Symbol (+) indicates the targeting of TacA mutant to SVLVs. Symbol (–) indicates nontargeting to SVLVs. C) Immunofluorescent analysis of subcellular localization of key deletion mutants. PC12 cells transiently transfected were stained for mutant TacA (red) and synaptophysin (green). Bar = 20 μm.

that the mutation of LL-485/486-AA disrupted internalization dramatically (31% internalization compared to control) (Figure 4B). This result is consistent with previous studies that demonstrate the requirement for the leucine residues for efficient VAChT internalization (16,29,30). Previously, the residues upstream of the leucines in many classical dileucine motifs have been indicated to regulate the efficiency of internalization. Not surprisingly, mutation of upstream residues DV-483/484-AA in the VAChT C-terminus led to a significant decrease in efficiency of internalization over 30 min (Figure 4B). Mutation of residues 479/480 led to a small but significant decrease in internalization. Neither downstream dileucine-flanking residues 487/488 nor residues 481/482 significantly contributed to the internalization of TacA as mutation of these residues did not significantly alter the internalization of these mutant TacA proteins (Figure 4B).

In parallel to the internalization assay, SVLV targeting of dialanine-scanning mutants was analyzed by using

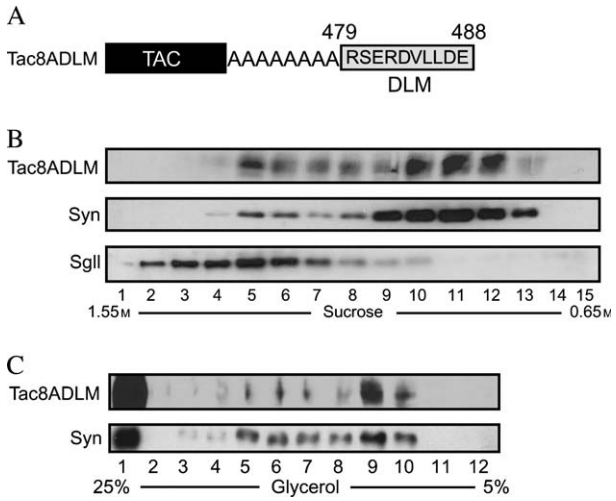

Figure 3: Dileucine-containing motif is sufficient for SVLV targeting in PC12 cells. A) Schematic diagram of Tac8ADLM. Eight alanines (8A), followed by the dileucine-containing motif (DLM) of VAChT, were fused to the C-terminal end of Tac protein. B) Tac8ADLM targets to SVLV in PC12 cells using density gradient fractionation. Postnuclear supernatants of PC12 cells transiently expressing Tac8ADLM were fractionated through sucrose density gradients. Tac8ADLM comigrated with synaptophysin-containing fractions. C) Tac8ADLM targets to SVLV in PC12 cells using velocity sedimentation. Tac8ADLM comigrates primarily with synaptophysin-containing fractions.

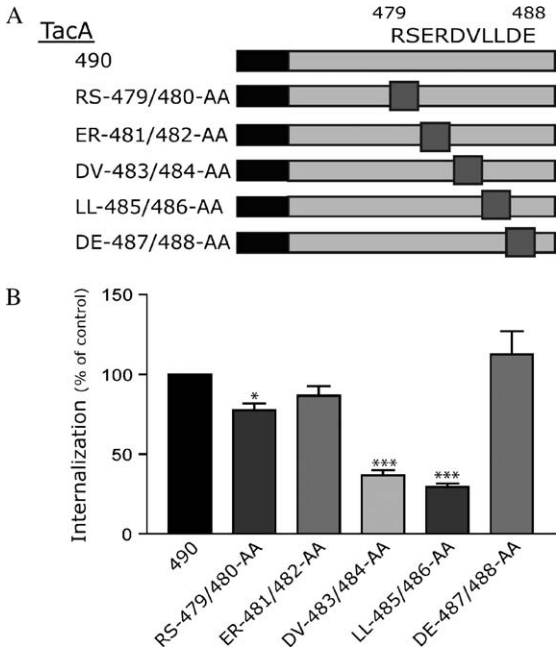

Figure 4: Dileucine-containing motif is essential for the internalization of TacA. A) Schematic diagram of dialanine-scanning mutants of TacA. Pairwise alanine mutations (gray box) were made using TacA490 as the template. B) Mutation of leucines and neighboring residues reduces internalization of TacA. PC12 cells transiently transfected with TacA dialanine mutants were analyzed semiquantitatively for their endocytosis for 30 min. Percent internalization of mutants was normalized to nonmutant TacA490 as control to determine effect on internalization (*$p < 0.05$, ***$p < 0.001$). Error bars depict standard deviation.

confocal immunofluorescent imaging and density fractionation. As shown in Figure 5A, the SVLV-staining pattern of TacA was disrupted by mutation of the leucines (LL-485/486-AA) but not by mutation of other residues in the dileucine-containing motif. Mutation of upstream residues DV-483/484-AA did not lead to significant accumulation of the chimera on the cell surface at steady state (Figure 5A), despite a decrease in its internalization at 30 min. This may suggest that, while these residues decrease the efficiency of internalization, steady-state internalization is not significantly affected as detected in our assay system.

Biochemical examination of dialanine mutants through density gradient fractionation confirmed our immunofluorescence data. As shown in Figure 5B, peak fractions for all mutant TacA proteins, except the dileucine mutant, correspond with peaks of SVLV-containing fractions. A shift in the peak of the dileucine mutant (LL-485/486-AA) toward heavier fractions, possibly containing endosomal and plasma membrane-derived vesicles, suggests that this mutant does not target to SVLVs, presumably trapped at the cell surface as indicated through staining (Figure 5A). Together with both internalization and fluorescent staining, these results suggest that the dileucine-containing region, identified as sufficient for SVLV targeting, does not harbor a separate SVTM. Rather, the dileucine motif may have dual properties for both internalization and SVLV targeting. Accordingly, the ability for the C-terminus of VAChT to direct trafficking toward SVLVs is influenced by its ability for internalization.

Specificity of the dileucine-containing motif for SVLV targeting in PC12 cells

Our dialanine-scanning analysis demonstrated that the SVLV targeting of TacA is limited only by mutation of the residues that interfered with internalization (leucine residues), indicating that the motif's role in SVLV targeting is not easily distinguishable from its previously defined role in internalization. We therefore sought to address the specificity of the VAChT dileucine-containing motif as an SVTM. In order to address this, we examined additional Tac chimeras of several membrane proteins that contain other cytosolic internalization motifs. First, a chimera between Tac and the N-terminus of transferrin receptor (TacTfR), which contains a tyrosine internalization motif, was analyzed for its subcellular distribution. TacTfR was internalized from the plasma membrane but did not traffic to SVLVs as analyzed by density gradient (data not shown), supporting that the specificity of the VAChT dileucine-containing motif as an SVTM extends beyond internalization. However, dialanine-scanning mutagenesis of the

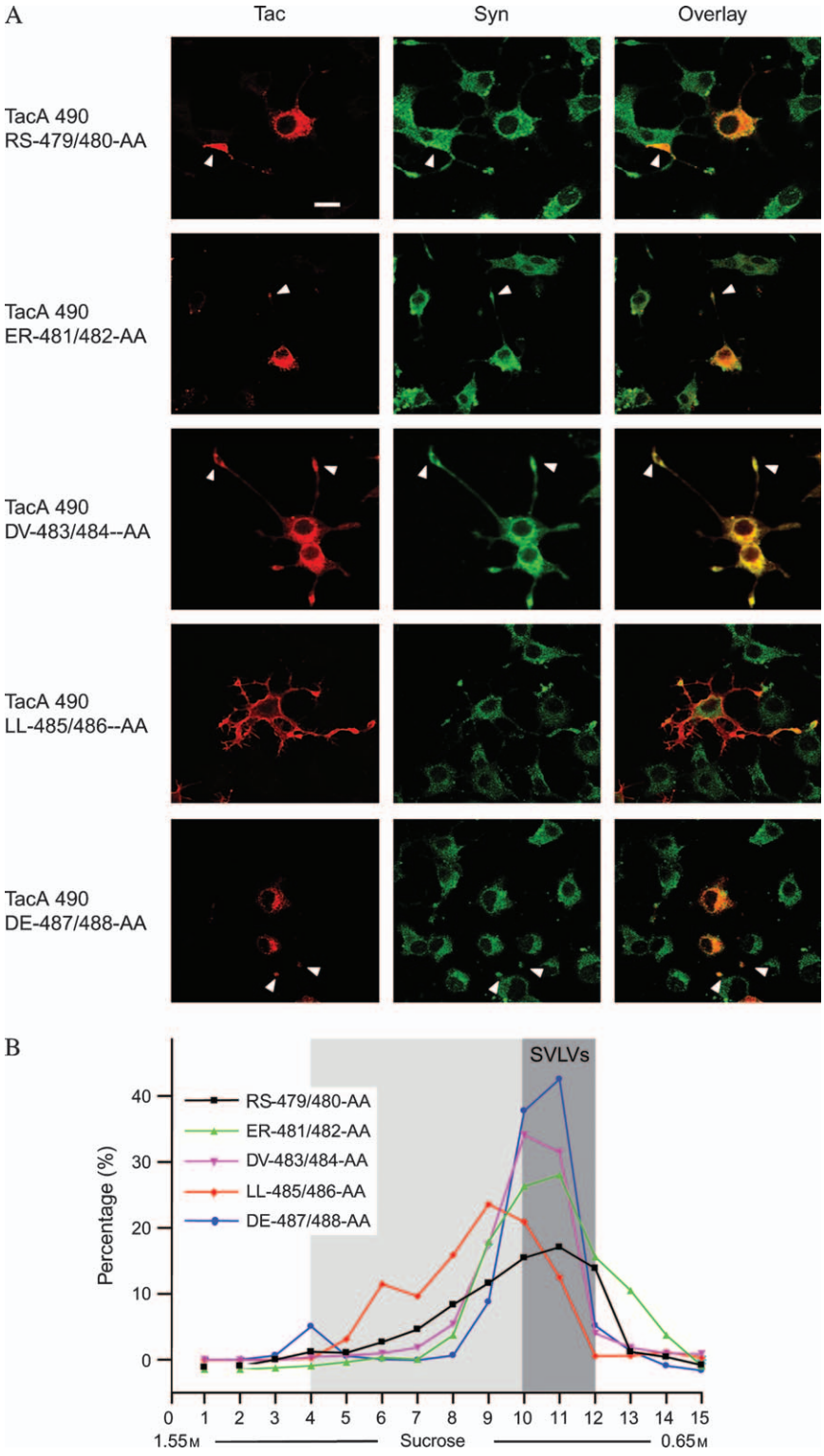

Figure 5: Dileucine-containing motif of VAChT C-terminus serves as an SVTM. A) The SVLV targeting analysis of dialanine mutants as detected through immunofluorescent staining. Overexpressed TacA490 mutants in differentiated PC12 cells were visualized (red) and compared to staining patterns of synaptophysin (green). Only TacA490 LL-485/486-AA showed disrupted SVLV targeting, localizing preferentially to the cell surface. Arrows indicate the tips of the processes for colocalization of TacA variants and synaptophysin. Bar = 20 μm. B) The SVLV targeting analysis of dialanine mutants through density gradient analysis. Postnuclear supernatants of transiently transfected PC12 cells were fractionated with sucrose gradients. Western blots for TacA490 mutant proteins were semiquantified and calculated as a fraction of total signal. Gray shading marks SVLV corresponding fractions.

VAChT dileucine-containing motif suggested that none of the specific residues within this motif, other than the leucine residues, are required for the chimera's SV targeting (Figure 5). Therefore, it was unclear to what extent the exact motif in VAChT was specific as an SVTM and to what extent the dileucine motif could be generalized as an SVTM. Thus, we tested the potential SV targeting of another classical dileucine motif-containing protein, GLUT4. In PC12 cells, GLUT4, regulated in part through a C-terminal dileucine internalization motif, R(XXX)LL, traffics to

a unique vesicle population (31–35). Examination of the steady-state localization of TacGLUT revealed that the chimera, similarly to the full-length protein, segregated to fractions slightly heavier than synaptophysin-containing fractions in density gradients (Figure 6B). In order to ensure that TacGluT did not comigrate with SVLVs, velocity gradients fractionation was performed to separate SVLV light fractions with neighboring fractions. Exclusion from SVLVs was confirmed as TacGLUT was found only in the bottom fraction of the gradient (Figure 6C). Although both GLUT4 and VAChT chimera contain similar dileucine motifs, [R(TPS)LL versus E(RDV)LL], their differential targeting suggests that the environment or nature of the residues of the dileucine motif may influence the specificity of this motif for SVLV targeting. Finally, we examined a closely related dileucine motif, E(XXX)[I/L]L, found in the similar environment of the C-terminus of VMAT2. Vesicular monoamine transporter 2 was previously shown to have preferential targeting to LDCVs in PC12 cells, which is thought to be regulated by an acidic patch at the distal end of the C-terminus (36). The presence of this additional signaling motif also allowed us to examine how neighboring domains influence the dileucine-mediated targeting event. We therefore generated a chimera, TacM, in which the C-terminus of VMAT2 was fused to Tac protein (Figure 6A). In both transiently transfected and stable cell lines, as determined by sucrose and velocity gradients, TacM was found to traffic preferentially to LDCV fractions, but also to SVLV fractions (Figure 6D; velocity gradient not shown), suggesting the C-terminus of VMAT2 is not sufficient for its preferential LDCV targeting. To determine if the distal sequence of the acidic patch influences the function of C-terminus in targeting as indicated previously, we next constructed TacMs in which the acidic patch was deleted (Figure 6A). In contrast to the two-peak distribution seen in the density gradient for TacM, the steady-state distribution of TacMs showed a single peak that comigrated with synaptophysin-containing fractions (Figure 6 E). The trafficking of TacMs to SVLVs strongly suggests that the VMAT2 dileucine motif, when not influenced by neighboring signals, is also able to direct SVLV targeting, indicating that this motif might be generalized as an SVTM for several SV proteins.

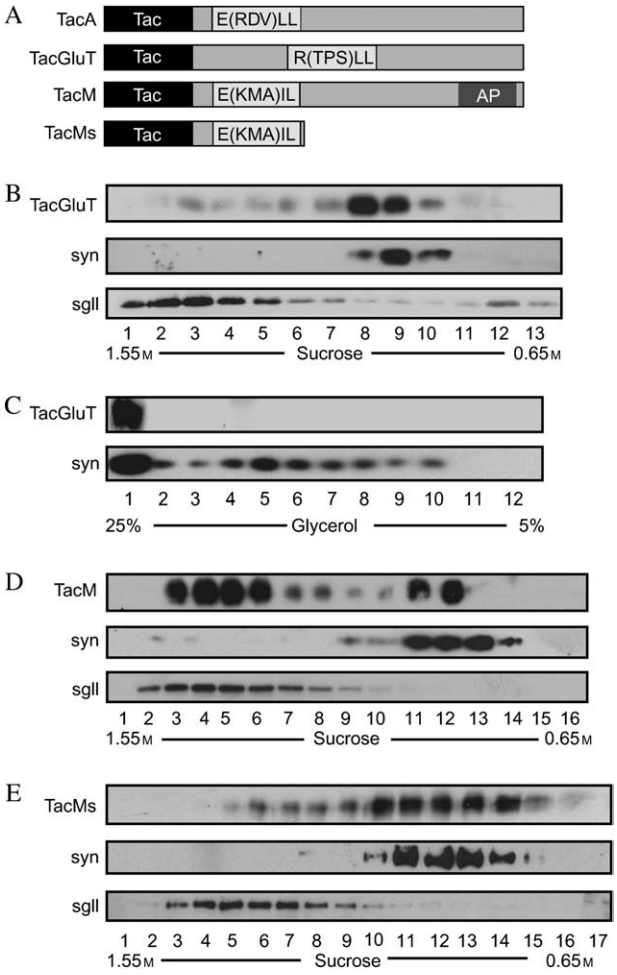

Figure 6: Vesicular targeting of membrane proteins that contain dileucine-containing motifs. A) Schematic diagram of constructs for TacA, TacGluT, TacM and TacMs chimera. Dileucine motifs indicated in gray boxes. TacGluT and TacM contain the cytoplasmic C-terminal tail for GLUT4 and VMAT2, respectively. AP = acidic patch. TacMs contains the distal deletion mutation of TacM. B) TacGluT does not target to SVLVs in PC12 cells. Postnuclear supernatants of PC12 cells transiently expressing TacGluT were fractionated through sucrose gradients. TacGluT does not comigrate with SVLVs-containing fractions. C) TacGluT is excluded from SVLV-containing fractions in glycerol gradients. D) TacM localizes to both LDCVs and SVLVs in PC12 cells in sucrose gradients. E) TacMs localizes to SVLVs but not LDCVs in PC12 cells in sucrose gradients. TacMs comigrates with SVLV fractions.

Discussion

In this study, by using chimeras between an unrelated plasma membrane-localized protein, Tac, and the C-terminus of VAChT, we have shown that the C-terminus of VAChT is necessary and sufficient for SVLV targeting in PC12 cells. Consistently, deletion analysis of this region demonstrates that a 10-aa dileucine-containing motif is sufficient for this SVLV targeting. Further examination of this motif has suggested that it contains dual signals indistinguishable for internalization and SV targeting. Through the use of additional control chimeras, we have shown that this signal is specific as an SVTM and that it may be generalized for multiple SV proteins.

Our approach to investigate the SVLV trafficking signals of VAChT through the use of Tac chimeric proteins allowed us to determine the sufficiency of potential targeting signals within VAChT. This system permitted independent analysis of the ability of VAChT C-terminal cytosolic regions to traffic a nonrelated protein, Tac, to SVLVs. Previous studies of VAChT trafficking have used

mutagenesis of full-length VAChT or chimera between VAChT and VMAT2. Because of the high sequence similarity between these two transporters [~65% among transmembrane regions (12)], studies of this nature cannot exclude the potential role of the transmembrane domains or the luminal loop of the transporters in vesicular targeting. In addition to determining sufficiency, the Tac chimeric approach also allowed us to focus on internalization and sorting steps from the plasma membrane, which mimic SV recycling and biogenesis at neuronal terminals. Consistent with VNT chimeric studies (16), our results using Tac chimera in PC12 cells imply that neither the N-terminal domain nor the transmembrane domain regions of VAChT are required for SVLV trafficking at the terminal. However, our data does not address whether these regions are important in axonal targeting, an important additional sorting step for SV proteins in neurons (7). Our preliminary studies using TacA chimera in primary neurons show that the steady-state distribution of the protein is not restricted to axons but extends to all neuronal processes (data not shown). This may suggest that in neurons the N-terminus or transmembrane regions of VAChT may be required for the proper axonal targeting of the protein while the C-terminus may be sufficient to direct SV recycling at the terminal.

Our Tac chimeric analysis also first shows that the dileucine-containing sequence (residues 479–488) within the VAChT C-terminus is sufficient as an SVTM in PC12 cells. Consistent with this, a similar region (residues 481–490) was previously indicated to be required in the targeting of full-length VAChT in SN56 cells (22). On the other hand, our chimeric study was unable to confirm another report that a nonclassical tyrosine motif (Y524–Y527) at the distal end of the C-terminus of VAChT serves as an alternative internalization motif or an SVLV trafficking role of a classical tyrosine motif (21). Our systematic analysis of deletion mutants of these two tyrosine motifs failed to show any significant decrease in their internalization at 30 min or any alteration of their steady-state SVLV trafficking (Figures 2 and 4). It is plausible to suggest that these previously identified motifs, while not required for SVLV targeting in our system, may play a role in regulating the efficiency of VAChT targeting. More sensitive approaches may help to address how they influence the dominant dileucine-containing motif.

Further examination of the signaling properties of the dileucine motif demonstrated that this signal is specific as an SVTM. In agreement with our dialanine-scanning results and analysis of additional Tac chimeras, the specific residues within the VAChT dileucine-containing motif are not essential for its SVLV targeting. However, TacGLUT containing a similar dileucine motif did not traffic to SVLVs, suggesting that the overall environment of the motif, as well as factors such as proximity of the motif to the transmembrane domain, or accessibility of cytosolic machinery to the motif may determine the specificity of the dileucine-containing motif as an SVTM. Furthermore, our data suggest that the dileucine-containing motif can be influenced by adjacent regions of the protein, such as the acidic patch in VMAT2. One possible explanation is that this additional signaling motif may mask the SVTM and act as a dominant earlier sorting signal at the *trans* Golgi network (TGN). The other possibility is that this motif may promote the Golgi–endosomal trafficking during retrieval of VMAT2 and thus enhance its overall targeting to secretory granules at the TGN. When the acidic patch is deleted, the closely related dileucine motif of VMAT2 was able to direct targeting to SVLVs, suggesting that this signal may be generalized to several SV proteins. Interestingly, the notion that the VMAT2 C-terminus may contain signals for targeting to both LDCVs and SVLVs suggests some regulation of secretory vesicle protein trafficking between the two types of vesicles in neurons.

The potential for this type of dileucine motif to be a more general SVLV signal is further supported by several previous findings. In synaptotagmin 1, the cytoplasmic motif, E(VDA)ML, accounts for at least a portion of its SVLV trafficking in PC12 cells (8). Furthermore, the dileucine motif of tyrosinase, E(KQP)LL, is responsible for its targeting to SVLVs when exogenously expressed in PC12 cells (8). Interestingly, dileucine-like motifs have been identified in several other vesicular transport proteins such as VGLUT1, E(KCG)FV (37). This recent report has suggested that this dileucine-like motif is critical for the internalization and trafficking of the transporter. This sequence is upstream of a polyproline motif that is responsible for interaction of VGLUT1 with endophilin 1, which may regulate this dileucine-like dependent trafficking (37,38).

The mechanisms of the dileucine-mediated trafficking of synaptotagmin and tyrosinase are mediated by a fairly well-characterized mechanism for SV biogenesis in PC12 cells (39), ADP ribosylation factor GTPase–AP-3-directed endosomal budding (8). However, AP-3 binding has not been able to be detected for VAChT (21). Thus, VAChT targeting may be regulated through alternative cytosolic machinery. Accordingly, we have identified a novel functional interaction between the C-terminus of VAChT and SNX5, a lipid-binding protein involved in the endosomal trafficking of membrane proteins (Colgan et al., unpublished data). This suggests that VAChT may also traffic through an endosomal intermediate. The dual properties of the dileucine-containing motif may regulate trafficking through an endosomal intermediate by serving first as an internalization signal at the plasma membrane and subsequently acting as an SVTM at an endosomal compartment. This sequential targeting model may rely on the chronological function of multiple regulatory sorting machineries or may take advantage of similar but subcellularly compartmentalized machinery that govern different targeting events. Similar mechanisms are thought to occur in nonneuronal cell lines, where this type of dileucine motif

can be recognized both at the plasma membrane as well as endosomal and lysosomal compartments (29). Alternatively, the dileucine motif signal may mediate direct and selective internalization from the plasma membrane that has been suggested as a major pathway for recycling SVs in the nerve terminal (40). Nonetheless, the dual role of the dileucine-containing motif in internalization and SV targeting provides a molecular mechanism underlying the efficient recruitment of essential SV components during its biogenesis and recycling.

Materials and Methods

Chemicals and antibodies

General chemicals used in this report were purchased from Sigma (St Louis, MO, USA) unless otherwise noted. The following antibodies were used in immunofluorescent staining: biotin-conjugated mouse anti-CD25 (Affinity BioReagents, Golden, CO, USA), polyclonal synaptophysin and secretogranin II (SYSY, Goettingen, Germany), secondary Cy3-conjugated goat anti-mouse and Alexa 488-conjugated goat anti-rabbit (Jackson Immunoresearch Lab, West Grove, PA, USA). The following antibodies were used for Western [illegible] polyclonal IL-2R alpha (Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal anti-IL2 receptor (Covance, Princeton, NJ, USA), polyclonal anti-synaptophysin and anti-secretogranin II (SYSY), secondary horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit (Pierce, Rockford, IL, USA). Antibodies used for ELISA internalization assay are as follows: biotin-conjugated mouse anti-CD25 (Affinity BioReagents), polyclonal goat anti-mouse IgG (BD Pharmingen, San Diego, CA, USA).

Plasmid construction and mutagenesis

Chimeric protein TacA was generated by polymerase chain reaction (PCR) amplification of the C-terminus of VAChT (aa 465–530) with the introduction of *Xba*1 and *Xho*1 restriction sites flanking the region. Polymerase chain reaction product was digested and subcloned into Tac/pcDNA3.1 as described (16). C-terminus deletion mutant chimeras were constructed similarly, with PCR amplification introducing an *Xba*1 site at the 5′ region and a stop codon and *Xho*1 site at the 3′ region. Subsequent digestion was followed by subcloning into Tac/pcDNA3.1 using *Xba*1 and *Xho*1. 8ATacDLM was constructed using an extended 3′ primer encoding an *Xba*1 site followed by eight alanines and the consensus motif for aa 479–483. Polymerase chain reaction amplification introducing a stop codon and a *Not*1 site downstream of the dileucine region was followed by digestion and subcloning into Tac/pcDNA3.1 using *Xba*1 and *Not*1 restriction enzymes. Dialanine-scanning point mutations were generated by using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) as directed using TacA490/pcDNA3.1 as a backbone. TacGLUT was generated by PCR, with oligos that isolated the C-terminus of human GLUT4 and introduced Xba1 and Not1 restriction sites flanking the C-terminus. Polymerase chain reaction product was digested and subcloned into Tac/pcDNA3.1 using Xba1 and Not1. TacM constructs were generated similarly to TacA with the exception that the C-terminus of VMAT2 was amplified by PCR and used for subcloning. TacMs was generated by introducing a stop codon after the dileucine-containing region by QuickChange site-directed mutagenesis using TacM/pcDNA 3.1 as a backbone. All constructs were confirmed by sequencing.

Cell culture and transfection

All cells were maintained in 5% CO_2 at 37°C in medium containing penicillin and streptomycin unless otherwise noted. PC12 cells were maintained in DMEM (Invitrogen, San Diego, CA, USA) with 10% equine serum (Hyclone, Logan, UT, USA), 5% cosmic calf serum (Hyclone) and 2 mM L-glutamine (Invitrogen). For immunofluorescent analysis, cells were seeded on poly-D-lysine and Matrigel-coated glass coverslips 24 h before transfection. Cells on coverslips were then transfected using either LipofectAMINE 2000 (Invitrogen) or Superfect (Qaigen, Hilden, Germany) reagent according to the manufacturer's instructions. Twelve hours later, cells were differentiated using 100 nM nerve growth factor for 24 h before fixation with 4% paraformaldehyde. For biochemical assays, transfections were done using LipofectAMINE 2000. Transfected cells were incubated at 37°C for 36–48 h before harvest.

Stable lines of wild-type Tac, TacA, TacA490 dialanine-scanning mutants and TacM were generated by further selection with G418 (500 mg/mL). Positive transformants were screened by immunofluorescence staining and Western blot analysis. At least, three stable lines were tested for consistent subcellular localization of the heterogeneously expressed membrane proteins. All stable cell line-derived results were also confirmed in transiently expressing independent PC12 cell lines.

Immunofluorescence and confocal microscopy

Immunofluorescent staining was performed as previously described (41). In brief, cells seeded on glass coverslips were fixed with 4% paraformaldehyde in PBS, pH 7.4. After fixation, cells were permeabilized and blocked for 30 min in blocking buffer (BB, 2% BSA, 1% fish skin gelatin and 0.02% saponin in PBS). Cells were then incubated with primary antibody in BB for 1 h at room temperature. Coverslips were washed and incubated with the appropriate Alexa- or Cy3-conjugated secondary antibody for 1 h at room temperature. Confocal images were acquired with a Fluoview 500 laser scanning confocal imaging system (Olympus, Tokyo, Japan) configured with a fluorescence microscope fitted with Plan Apo × 60 and × 100 oil objectives (Olympus). Confocal images were collected sequentially at 1024 × 1024 resolution to minimize bleed through of fluorescence between channels.

Fractionation analysis

PC12 cells stably expressing, or transiently transfected with plasmid constructs, were harvested in Buffer A (150 mM NaCl, 10 mM HEPES pH 7.4, 1 mM EGTA, 0.1 mM $MgCl_2$) with protease inhibitors. Cells were cracked by eight passes through a cell cracker (clearance: 0.02 μm). Postnuclear supernatants were then loaded onto prepared density or velocity gradients and spun in a Beckman SW41 rotor. For sucrose density fractionation, sucrose gradients were prepared using a gradient mixer to form continuous gradients with sucrose concentrations from 0.65 M to 1.55 M and spun in SW41 rotor at 30 000 × ***g*** for 8 h at 4°C. (Beckman Instruments, Palo Alto, CA, USA). Glycerol velocity fractionation was done through a glycerol gradient of 5–25% glycerol (v/v) and spun in SW41 rotor at 37 000 × ***g*** for 1 h at 4°C. Fractions from density gradients were collected from the bottom and velocity gradients collected from the top to minimize contamination. All gradients were numbered from heavy to light.

Western blot analysis

Proteins were detected in gradient fractions by immunoblotting as previously described (42). Equal amounts of gradient from each fraction were denatured in 3× SDS sample buffer (New England Biolab, Beverly, MA, USA) and separated by electrophoresis through 10% SDS–PAGE. After electrophoresis, proteins were transferred to nitrocellulose (BA-85, Schleicher-Schuell Bioscience, Keene, NH, USA) and TacA chimeric proteins, synaptophysin, secretogranin II or other proteins were visualized by immunoblotting with appropriate antibodies in combination with enhanced chemiluminescence (Super-Signal West Pico, Pierce). Protein immunoreactive signals were scanned and the intensity of bands was semiquantified using the NIH IMAGING PROGRAM.

Internalization assay

Internalization assay was done as previously described (16). Briefly, PC12 cells transfected by electroporation with cDNA plasmids were harvested 36 h later and resuspended with serum-free medium [DMEM, 20 mM HEPES–KOH (pH 7.2), 0.2% BSA]. The cell suspension was incubated

with biotinylated anti-CD-25 (Tac) monoclonal antibody at 4°C for 2 h to label Tac chimeric proteins localized to the cell surface. After washing of unbound antibody, internalization was allowed for 30 min by 37°C incubation. Internalization was stopped and the sample equally divided. In one fraction, cell surface-labeled proteins were quenched through avidin incubation. Cells in both fractions were then solubilized and loaded in triplicate aliquots onto 96-well ELISA plate precoated with goat anti-mouse IgG and incubated overnight at 4°C. Plates were washed of unbound biotin signal and developed using 3,3′,5,5′ tetramethylbenzidine liquid substrate system for 30 min. End point absorbance measurements were taken at 450 nm using a BioRAD plate reader. Percent internalized was calculated as follows: (Ca−Va)/(Ct−Vt), where Ca = internalized (avidin quenched) transfected cells; Va = internalized (avidin quenched) vector transfected cells; Ct = total (unquenched) transfected cells; Vt = total (unquenched) vector transfected cells. Standard curves were run with each experiment to ensure inclusion within the linear range of detection. Mutants were normalized to control TacA490 constructs to determine percent change in internalization and to normalize across experiments.

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