Analysis of the Palmitoylation and Membrane Targeting Domain of Neuromodulin (GAP-43) by Site-Specific Mutagenesis†

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ABSTRACT: Neuromodulin (GAP-43) is a neurospecific calmodulin binding protein which is implicated in neuronal growth and regeneration. It is concentrated in neuronal growth cones and associates with membranes through the palmitoylation of the N-terminal peptide MLCCMRRTK at Cys-3 and Cys-4. In the present study, we have identified critical amino acid residues required for palmitoylation and membrane association of neuromodulin *in vivo*. Several neuromodulin constructs with point mutations were tested for membrane association and palmitoylation. Wild-type neuromodulin expressed in COS-7 cells incorporated [^{3}H]palmitic acid, whereas a mutant in which both Cys-3 and Cys-4 were substituted with glycine was not palmitoylated *in vivo*. Mutant proteins in which either Cys-3 or Cys-4 was substituted with leucine incorporated 75% and 25% of [^{3}H]palmitic acid, respectively, compared to wild-type neuromodulin. The relative distribution of mutant neuromodulins expressed in COS-7 cells was quantitated by immunoblot analysis of the membrane and cytosolic fractions. There was a general correlation between membrane association of mutant neuromodulins and the extent to which they were palmitoylated *in vivo*. Additional point mutations in the acylation domain of neuromodulin indicated that a short hydrophobic amino acid sequence N-terminal to Cys-4 may be required for optimal palmitoylation and membrane association. We conclude that Cys-4 is critical for the palmitoylation and membrane association of neuromodulin.

The neural-specific phosphoprotein neuromodulin (also known as GAP-43, P-57, B-50, and F-1) is a major component of neuronal growth cone membranes (Skene, 1989). The protein has been implicated in several important neuronal functions including axonal growth and regeneration (Skene & Willard, 1981; Benowitz & Lewis, 1983; Doster et al., 1991), regulation of free calmodulin levels in neurons (Andreasen et al., 1983; Liu & Storm, 1990), long-term potentiation in hippocampus (Akers & Routtenberg, 1987), neurotransmitter release (Dekker et al., 1989), and G-protein regulation (Strittmatter et al., 1990). Immunofluorescence staining of neuromodulin in cultured neurons showed that the protein is concentrated in the axons and axonal growth cones (Meiri et al., 1988; Goslin et al., 1990). In developing rat brains and adult bovine brains, neuromodulin is tightly bound to membranes and is resistant to extractions under various conditions including high salt and high pH (Cimler et al., 1985; Skene et al., 1986; Skene & Virag, 1989).

Neuromodulin is palmitoylated *in vivo*, and this modification may provide a hydrophobic moiety for anchoring the protein to membranes (Skene & Virag, 1989). Site-directed mutagenesis of both Cys-3 and Cys-4 of the N-terminal sequence MLCCMRRTK of neuromodulin abolished its association with membranes (Zuber et al., 1989; Liu et al., 1991). The N-terminal 10 amino acid sequence of neuromodulin was able to direct chloramphenicol acetyltransferase (CAT) to membranes (Zuber et al., 1989). Therefore, palmitoylation of the N-terminal domain of neuromodulin is necessary, and may be sufficient for membrane association of neuromodulin.

Although a number of palmitoylated proteins have been identified, no apparent consensus amino acid sequence has been defined for palmitoylation [for reviews, see Sefton and Buss (1987), Towler et al. (1988), Schmidt (1989), and James and Olson (1990)]. Whereas most of the palmitoylated proteins, including the vesicular stomatitis virus glycoprotein (Schmidt & Schlesinger, 1979), acetylcholine receptor (Olson et al., 1984), β_2-adrenergic receptor (O'Dowd et al., 1989), and CD4 (Crise & Rose, 1992), are transmembrane proteins in which palmitoylation does not appear to have a significant effect on membrane association, neuromodulin is a peripheral membrane protein requiring palmitoylation for its membrane attachment. Little is known about the domain structures determining palmitoylation of peripheral membrane proteins such as neuromodulin. An earlier report suggested that both cysteines of neuromodulin were required for its membrane targeting because substitution of either Cys-3 or Cys-4 with threonine abolished membrane binding of the protein (Zuber et al., 1989). The data reported in this study suggest that palmitoylation of a single cysteine in neuromodulin is sufficient for membrane association and that the membrane attachment of neuromodulin may be exclusively determined by its palmitoylation.

MATERIALS AND METHODS

Plasmid Construction. The cDNA encoding the full-length murine neuromodulin (Cimler et al., 1987) was subcloned into the expression vector pCDM8 (a generous gift from Dr. Brian Seed, Massachusetts General Hospital). Site-directed mutagenesis of cysteines-3 and -4 to glycine residues was carried out as previously described (Chapman et al., 1991). Mutagenesis of the N-terminal amino acid sequence of neuromodulin was accomplished by polymerase chain reaction (PCR) to produce a 525 bp product encoding the 5′-neuromodulin sequence. The PCR product was digested with *Hin*dIII and *Nae*I to generate a 300 bp fragment, and the fragment with the mutation was ligated to pCDM8-NM at *Hin*dIII and *Nae*I sites (Liu et al., 1991). The PCR primers used were as follows: upstream: T2, 5′-GGGGAAGCT-

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TACCATGACGTGCTGTATGAGAAGAACCA-3′; T3, 5′-GGGGAAGCTTACCATGCTGACCTGTCTGAGA-AGAACCA-3′; L3, 5′-GGGGAAGCTTACCATGCTGCT-CTGTATGAGAAGAACCA-3′; L4, 5′-GGGGAAGCT-TACCATGCTGTGCTTAATGAGAAGAACCA-3′; R5, 5′-GGGGAAGCTTACCATGCTGTGCTGTAAGAGAA-GAACCA-3′; 3Q, 5′-GGGGAAGCTTACCATGCTGT-GCTGTATGCAACAAACCCAACAG-3′; downstream primer, 5′-TGCTTTCTGCAGTCTCCGTTGGA-3′. All mutations were confirmed by direct DNA sequencing.

Cell Culture and Transfections. COS-7 cells were grown in DMEM supplemented with 10% fetal calf serum and maintained in a humidified 35 °C incubator with 5% CO_2. Transient expression of wild-type and mutant neuromodulin proteins was accomplished using the calcium phosphate method (Chen & Okayama, 1987) or the lipofection method (Felgner et al., 1988; Liu et al., 1991). For lipofection transfections, COS-7 cells were plated onto 60-mm dishes at near-confluency and transfected with 1 μg of plasmid DNA and 25 μL of Lipofectin (BRL) in 1.5 mL of 20 mM Hepes, pH 7.4, 150 mM NaCl. After 5-h incubation, 3 mL of media was added. The plates were replaced with fresh media after 12 h. After 48 h, cells were collected and homogenized in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.5 mM PMSF on ice. Total particulate and cytosolic fractions were obtained by centrifugation at 20000*g* or 100000*g* for 30 min.

Immunoblot, Immunoprecipitation, and in Vivo Labeling. For immunoblot analysis of transfected cells, equal quantities of particulate and cytosolic proteins were separated by SDS–PAGE. The proteins were transferred onto nitrocellulose membrane and blotted with a rabbit anti-neuromodulin antibody as previously described (Liu et al., 1991). For immunoprecipitation (IP) of neuromodulin, transfected cells were labeled with 50 μCi/mL [^{35}S]methionine (^{35}S-Express, NEN) in methionine- and cysteine-free media (Sigma) or with 0.7–1 mCi/mL [^{3}H]palmitic acid (NEN) for 1–1.5 h. The cells were directly lysed with 0.1% SDS in 20 mM Na_2HPO_4, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 1% NP-40 (IP buffer). Insoluble materials were discarded after centrifugation at 15000*g* for 5 min. One microgram monoclonal anti-GAP-43 91E12 (Boehringer Mannheim; Skene & Virag, 1989; Goslin et al., 1990) and 15 μL of protein G-plus–agarose (Oncogene Science) were added to each sample and incubated overnight at 4 °C. The immunocomplex was pelleted by centrifugation and washed twice with IP buffer. The protein G–agarose immunocomplex was wash once more with 0.5 M NaCl in IP buffer and then with 10 mM Tris-HCl ((pH 7.5)/1 mM EDTA before the labeled proteins were heated at 60 °C for 5 min in 20 mM Tris-HCl, pH 6.8, 2% SDS, 1 mM EDTA, 1 mM DTT, 15% glycerol, and 0.01% bromphenol blue and separated by SDS–PAGE. Gels were treated with Amplify (Amersham) and exposed at –80 °C to Kodak X-OMAT film for 1–8 weeks.

Immunohistochemical Staining of COS-7 Cells Expressing Neuromodulin and Neuromodulin Mutant Proteins. Transfected COS-7 cells were fixed at 48 h after transfection with 3% paraformaldehyde in PBS for 15 min at room temperature, and permeabilized with –20 °C ethanol for 5 min. Nonspecific binding sites were blocked with 4% BSA in PBS for at least 30 min. Cells were stained with a monoclonal antibody against neuromodulin (Boehringer Mannheim, 2 μg/mL) and FITC-conjugated goat anti-mouse antibodies (Boehringer Mannheim, 1:1000), as previously described (Liu et al., 1991). For staining of Golgi apparatus, cells were incubated with 4 μg/mL TRITC-conjugated wheat germ agglutinin (WGA, Sigma) in solution with the FITC-conjugated secondary antibodies.

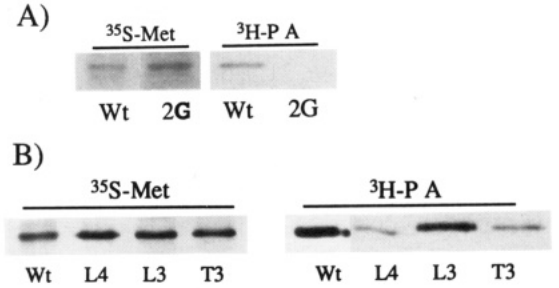

FIGURE 1: Incorporation of [^{3}H]palmitic acid into wild-type and mutant neuromodulins *in vivo*. COS-7 cells were transfected by the lipofection method and serum-starved for 1 h before addition of [^{3}H]-palmitic acid (A, 0.5 mCi/mL; B, 1 mCi/mL; NEN) or [^{35}S]-methionine (A, 50 μCi/mL; B, 100 μCi/mL ^{35}S-Express; NEN) and labeled for 1–1.5 h. Cells were lysed directly with 0.1% SDS in immunoprecipitation buffer. Neuromodulin was immunoprecipitated with monoclonal anti-GAP-43 (Boehringer Mannheim) and separated by SDS–PAGE for fluorography. The gels were exposed to film at –80 °C for 7–8 weeks (^{3}H) or 3–5 days (^{35}S). Wt, wild-type neuromodulin; 2G, Gly-3, Gly-4 neuromodulin; L4, Leu-4 neuromodulin; L3, Leu-3 neuromodulin; T3, Thr-3 neuromodulin.

Table I: Incorporation of [^{3}H]Palmitic Acid into Neuromodulin and Neuromodulin Mutants in Vivo

neuromodulin	description	% incorpn of [^{3}H]palmitic acid[a]
Wt	wild type	100
L3	Cys-3 to Leu-3	75
T3	Cys-3 to Thr-3	25
L4	Cys-4 to Leu-4	25
2G	Cys-3, -4 to Gly-3, -4	0.00

[a] Relative incorporation of [^{3}H]palmitic acid into neuromodulin mutants was quantitated from the data in Figure 1 by laser densitometry with wild-type neuromodulin taken as 100%.

RESULTS

In Vivo Palmitoylation of Neuromodulin. To determine if neuromodulin is palmitoylated at Cys-3 and Cys-4 *in vivo*, we metabolically labeled COS-7 cells transiently expressing wild-type neuromodulin or mutants lacking Cys-3 or Cys-4 with [^{3}H]palmitic acid. Neuromodulin was immunoprecipitated and analyzed for incorporation of [^{3}H]palmitate by fluorography. Although wild-type neuromodulin was labeled with [^{3}H]palmitic acid, a mutant in which both Cys-3 and Cys-4 were mutated to glycine (2G)[1] was not labeled (Figure 1A). To define the role of each individual cysteine for palmitoylation, we made single-cysteine mutants L3 and L4, in which either Cys-3 or Cys-4 was substituted with leucine residues. We also constructed a Cys-3 to Thr-3 mutant (T3), the same mutation that was used in the study by Zuber et al. (1989). The T3, L3, and L4 mutant proteins all incorporated [^{3}H]palmitic acid *in vivo*, indicating that either Cys-3 or Cys-4 can be independently palmitoylated (Figure 1B). The relative amounts of palmitoylation, however, varied considerably (Table I). Compared to wild-type neuromodulin, L3 and L4 incorporated 75% and 25% of [^{3}H]palmitic acid, respectively.

[1] Abbreviations: T2, neuromodulin in which Leu-2 is substituted with Thr; T3, neuromodulin in which Cys-3 is substituted with Thr; L3, neuromodulin in which Cys-3 is substituted with Leu; L4, neuromodulin in which Cys-4 is substituted with Leu; R5, neuromodulin in which Met-5 is substituted with Arg; 3Q, neuromodulin in which Arg-6, Arg-7, and Lys-9 are substituted with Gln residues; 2G, neuromodulin in which Cys-3 and Cys-4 are both substituted with Gly residues.

T3 was labeled about 3-fold less than L3, suggesting that palmitoylation of Cys-4 is sensitive to the amino acid present at position 3, with a preference for a more hydrophobic amino acid. Data comparing L3 and L4 mutants suggest that native neuromodulin may be palmitoylated preferentially at Cys-4. Alternatively, the palmitoylation of Cys-3 may be highly dependent on the presence of Cys-4, and possibly enhanced by its prior acylation. These data indicate that cysteine palmitoylation can be highly dependent on adjacent amino acid residues.

Palmitoylation of Neuromodulin Determines Its Membrane Association. Since the wild-type, L3, L4, and T3 mutant neuromodulins showed differential palmitoylation, we examined the distribution of these proteins between the membrane and cytosolic fractions from transfected COS-7 cells by immunoblot analysis (Figure 2). The percentage of each mutant protein in membranes relative to the cytosol was quantitated by laser densitometry (Table II). In adult bovine brain, neuromodulin is found in the membrane and cytosolic fractions, and this distribution is thought to reflect pools of palmitoylated and nonpalmitoylated neuromodulin. In adult brain, approximately 60% of neuromodulin is associated with the membrane fraction (Cimler et al., 1985), and immunoblot analysis of neuromodulin expressed in COS-7 cells gave a similar distribution (Table II). The 2G mutant, which showed no palmitoylation, was only detected in the cytosolic fraction. Fifty percent of the L3 mutant and 16% of the L4 mutant were associated with the membrane (Table II). There was a strong correlation between the extent of palmitoylation of the protein and membrane targeting. Nineteen percent of the single-cysteine mutant T3, which was palmitoylated significantly less than wild-type or L3 neuromodulin, was membrane-associated. These data demonstrate that palmitoylation of a single Cys residue in the membrane binding domain of neuromodulin is sufficient to target the protein to membranes.

Immunofluorescence Staining of Neuromodulins Expressed in COS-7 Cells. The subcellular distribution of neuromodulin mutants expressed in COS-7 cells was also examined by immunofluorescence staining. Wild-type neuromodulin (Wt) was concentrated in the plasma membranes as well as the Golgi apparatus, adjacent to the nucleus (Figure 3). The Golgi localization of neuromodulin was confirmed by double immunofluorescence staining with wheat germ agglutinin (WGA) which strongly stains the Golgi apparatus (Figure 3). In cultured primary neurons, neuromodulin has also been found to accumulate in the Golgi apparatus (Goslin et al., 1990; Liu and Storm, unpublished observation). As expected, the 2G mutant showed a diffuse pattern of staining, particularly in the perinuclear region, characteristic of a cytosolic protein. There was no detectable staining in plasma or intracellular membranes (Figure 3).

L3 neuromodulin, which was palmitoylated almost as extensively as wild-type neuromodulin, showed a distribution in COS-7 cells that was very similar to wild-type neuromodulin with patchy labeling in the plasma membrane and accumulation in the Golgi apparatus. In contrast, L4 and T3, which were poorer substrates for palmitoylation, showed minimal staining in the plasma membrane and resembled the 2G mutant in their distribution (Figure 3).

Neuromodulin and Neuromodulin Mutants Containing at Least One Acyl Group Strongly Associate with Membranes. Several of the neuromodulin cysteine mutants were palmitoylated to a much lesser extent than wild-type neuromodulin (e.g., T3 and L4), and showed significantly lower membrane/cytosolic ratios. However, one would expect that the addition of a single palmitic acid residue to neuromodulin would promote strong membrane association. From hydrophobic contributions, a single palmitic acid residue would shift the aqueous to membrane-phase partition coefficient of neuromodulin by approximately 10^6-fold in favor of membrane association (Tanford, 1962). In order to show that the membrane-bound forms of the neuromodulin mutants were all strongly associated with membranes, we extracted the membrane-bound proteins with buffers that normally remove peripheral membrane proteins (Figure 4). The absolute amount of each protein expressed in COS-7 cells varied because of different transfection frequencies, and therefore equal staining for each protein was not seen on the Western blot

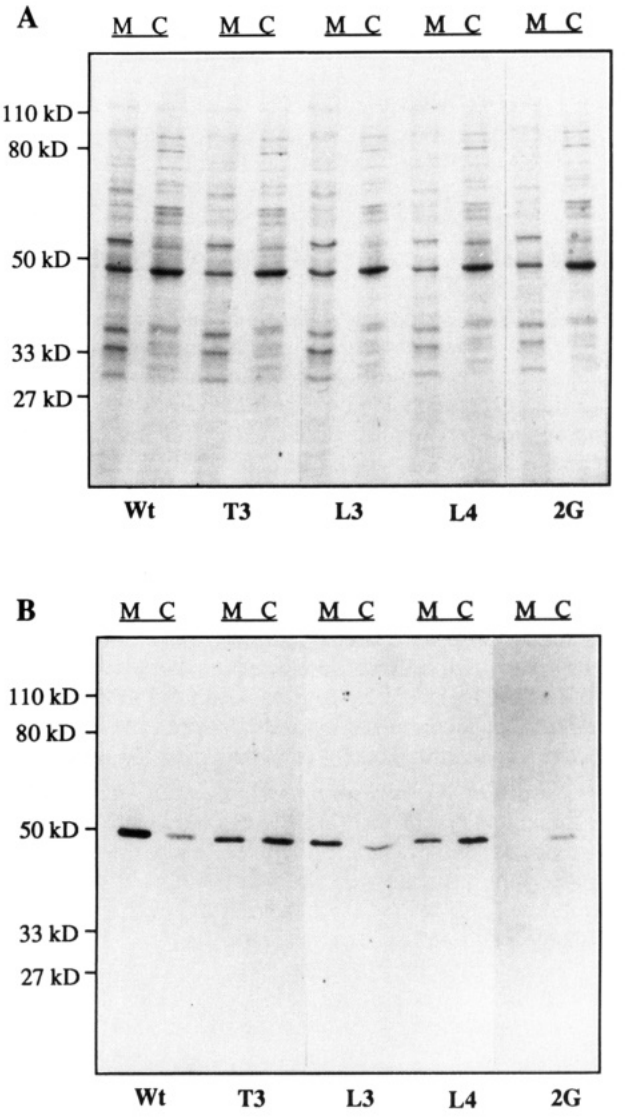

FIGURE 2: Distribution of neuromodulin and mutant neuromodulins between the membrane and cytosolic fractions. Neuromodulins were transiently expressed in COS-7 cells as described under Materials and Methods, and the membrane and cytosolic fractions were analyzed for neuromodulins by immunoblot. Cells were collected and homogenized in 10 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 0.5 mM PMSF on ice. Total particulates were pelleted by centrifugation at 100000*g* for 30 min and washed once with homogenization buffer. Two micrograms of total protein from membrane (M) and cytosolic (C) fractions was separated by SDS–PAGE and transferred onto nitrocellulose paper for immunoblot analysis as described under Materials and Methods. (A) Coomassie blue staining of the SDS gel; (B) immunoblot of the same gel. Wt, wild-type neuromodulin; 2G, Gly-3, Gly-4 neuromodulin; L4, Leu-4 neuromodulin; L3, Leu-3 neuromodulin; T3, Thr-3 neuromodulin.

Table II: Ratio of Neuromodulin and Neuromodulin Mutants in Membrane and Cytosolic Fractions Determined by Immunoblot Analysis

neuromodulin	amino acid sequence	% of neuromodulin in membrane[a]
Wt (wild type)	MLCCMRRTKQ...	62
3Q	MLCCMQQTKQ...	60
R5	MLCCRRRTKQ...	59
T2	MTCCMRRTKQ...	58
L3	MLLCMRRTKQ...	50
T3	MLTCMRRTKQ...	19
L4	MLCLMRRTKQ...	16
2G	MLGGMRRTKQ...	ND[b]

[a] The percentage of neuromodulin in the membranes relative to the cytosol was quantitated from the immunoblot analysis as described in Figures 2 and 5, and normalized against the total proteins of membrane and cytosolic fractions. [b] ND, not detected.

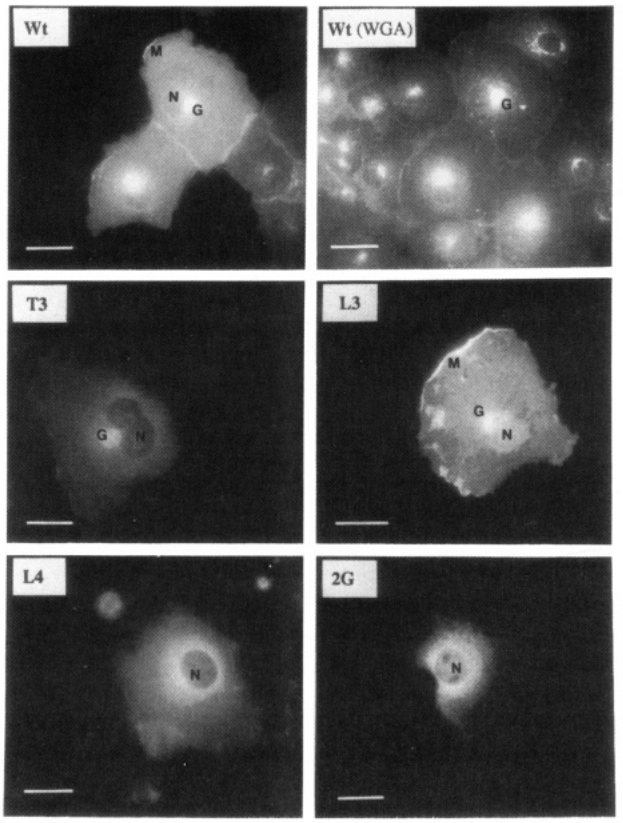

FIGURE 3: Immunofluorescence staining of neuromodulin and neuromodulin mutants transiently expressed in COS-7 cells. Cells expressing wild-type (Wt), T3, L3, L4, and 2G neuromodulins were fixed and permeabilized with –20 °C ethanol and incubated with 2 μg/mL monoclonal anti-GAP-43 91E12 (Boehringer Mannheim) overnight at 4 °C, followed by incubation with FITC-conjugated goat anti-mouse antibodies (Boehringer Mannheim, 1:1000) or TRITC-conjugated WGA (24 μg/mL, Sigma). Wt, wild-type neuromodulin; 2G, Gly-3, Gly-4 neuromodulin; L4, Leu-4 neuromodulin; L3, Leu-3 neuromodulin; T3, Thr-3 neuromodulin. N, nucleus; G, Golgi apparatus; M, plasma membrane. Bar = 20 μm.

reported in Figure 4. This experiment was repeated 3 times, and the data from the Western blots were quantitated by laser densitometry and reported in Table III. Wt, T3, L3, and L4 neuromodulins showed very similar responses to washes with 100 mM $NaHCO_3$, pH 11, buffer or with 1 M NaCl solutions, indicating that monopalmitoylated neuromodulins, like the wild-type protein, were strongly associated with membranes (Table III). Like neuromodulin in rat and bovine brains (Andreasen et al., 1983; Cimler et al., 1985; Skene & Virag, 1989), the mutant proteins could only be extracted with detergent-containing solutions (data not shown). As expected

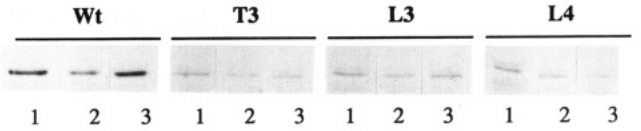

FIGURE 4: Neuromodulin and neuromodulin mutants with only one cysteine residue are strongly associated with membranes. Membranes prepared from COS-7 cells expressing the wild-type neuromodulin and T3, L3, and L4 mutant neuromodulins were analyzed by immunoblot analysis following extraction with (1) 1 M NaCl in 10 mM Tris-HCl/1 mM EDTA, (2) 100 mM $NaHCO_3$, pH 11, for 30 min on ice, or (3) 10 mM Tris-HCl (pH 7.5)/1 mM EDTA for 15 min at room temperature. The membranes were pelleted by centrifugation at 20000*g* for 15 min at 4 °C and analyzed by immunoblot using a rabbit antibody against neuromodulin. Wt, wild-type neuromodulin; L4, Leu-4 neuromodulin; L3, Leu-3 neuromodulin; T3, Thr-3 neuromodulin. Note the expression efficiency was higher in Wt-transfected cells.

Table III: Percentage of Neuromodulin Remaining in the Membranes Washed with Different Solutions

neuromodulin	conditions	% neuromodulin in membrane[a]
Wt	high salt	>95
	low salt	90 ± 5
	pH 11	72 ± 8
T3	high salt	92 ± 5
	low salt	86 ± 5
	pH 11	78 ± 10
L3	high salt	>95
	low salt	90 ± 3
	pH 11	80 ± 8
L4	high salt	>95
	low salt	82 ± 5
	pH 11	80 ± 8

[a] The amount of neuromodulin remaining in the membranes was quantitated by immunoblot analysis, and neuromodulin present in the membranes before extractions was used as reference (100%). High salt, 1 M NaCl, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.5; low salt, 1 mM EDTA/10 mM Tris-HCl, pH 7.5; pH 11, 100 mM $NaHCO_3$, pH 11.

from thermodynamic considerations, incorporation of a single palmitic acid into neuromodulin is sufficient for membrane association. The differences in the membrane/cytosol ratio for the various mutant neuromodulins containing single cysteine residues reflected their suitability as substrates for palmitoylation rather than their absolute affinity for the membrane.

Amino Acid Sequence Motif for Palmitoylation of Neuromodulin. Comparison of a number of palmitoylated proteins, including neuromodulin, reveals that their acylation domains contain hydrophobic residues (e.g., leucine or isoleucine) neighboring the cysteines and positively charged amino acids in the palmitoylation domains (Table III). To evaluate the importance of these general features for palmitoylation and membrane association, we prepared several additional neuromodulin mutants in which specific amino acid residues near Cys-4 were modified. These mutant proteins were expressed in COS-7 cells, and their distribution between the membrane and cytosolic fractions was determined by immunoblot analysis (Figure 5). The three basic amino acids at Arg-6, Arg-7, and Lys-9 were not crucial determinants for membrane association, since conversion of these residues to glutamine did not have a large effect on the membrane/cytosol ratio (Table II). The hydrophobic residues at Met-5 and Leu-2 were also not crucial determinants for membrane association since the mutants R5 (Met-5 to Arg-5) and T2 (Leu-2 to Thr-2) had membrane to cytosol ratios of 7.0. These data illustrate that there is no clear consensus sequence or general sequence descriptor for palmitoylation other than the

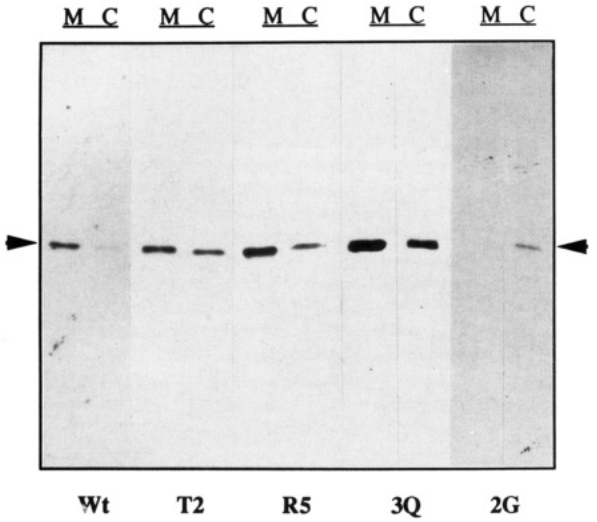

FIGURE 5: Immunoblot analysis of the distribution by neuromodulin and mutant neuromodulins between the membrane and cytosolic fractions. COS-7 cells were transfected by the lipofection method as described under Materials and Methods. Cells were collected and homogenized in 10 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 0.5 mM PMSF on ice. Total particulates were pelleted by centrifugation at 100000*g* for 30 min and washed once with homogenization buffer. Two micrograms of total protein from membrane (M) and cytosolic fractions (C) was separated by SDS–PAGE for immunoblot analysis using a rabbit anti-neuromodulin antibody. Wt, wild-type neuromodulin; 2G, Gly-3, Gly-4 neuromodulin; T2, Thr-2 neuromodulin; R5, Arg-5 neuromodulin; 3Q, Gln-6, Gln-7, and Gln-9 neuromodulin.

presence of one or more Cys residues with neighboring hydrophobic residues.

DISCUSSION

It has been proposed that palmitoylation may affect the lateral diffusion of VSV G protein (Scullion et al., 1987) and palmitoylation of β_2-adrenergic receptors is critical in coupling of the receptor to adenylyl cyclases (O'Dowd et al., 1989). Although the functional importance of neuromodulin palmitoylation has not been clearly defined, palmitoylation clearly localizes the protein to membranes and may be important for neuron growth cone localization (Liu et al., 1991). We have proposed that neuromodulin may function to bind and localize calmodulin at the membrane with release of calmodulin in response to protein kinase C phosphorylation (Alexander et al., 1987; Liu & Storm, 1990; Apel et al., 1990). In addition, neuromodulin may interact with the membrane cytoskeleton and control membrane dynamics. If these models for neuromodulin are operative, then palmitoylation of the protein should be important for localizing it at the membrane surface for interactions with the cytoskeleton and calmodulin. It has also been suggested that neuromodulin may stimulate G-protein activities and palmitoylation of neuromodulin inhibited this activity (Sudo et al., 1992). Consequently, acylation and deacylation of neuromodulin may be a key to control of its functions in neurons.

Neuromodulin was originally purified from bovine brain after extraction of the protein from membrane preparations with nonionic detergents (Andreasen et al., 1983). An examination of the amino acid sequence of neuromodulin, however, revealed no stretches of hydrophobic amino acid sequences that could account for membrane binding (Wakim et al., 1987). The observations that neuromodulin may be palmitoylated at Cys-3 and/or Cys-4 (Skene & Virag, 1989) and the report that its N-terminal 10 amino acid sequence can target fusion proteins to membranes (Zuber et al., 1989) strongly suggested that palmitoylation of these cysteines may be the principal source for membrane association. In fact, a mutant neuromodulin with both Cys-3 and Cys-4 mutated to Gly did not associate with the membranes, suggesting that one or both of these cysteine residues are necessary for palmitoylation and membrane binding.

An earlier study reported that mutations of neuromodulin at either Cys-3 or Cys-4 to threonine residues resulted in loss of membrane association (Zuber et al., 1989). These data suggested that both cysteine residues may be required for membrane binding and palmitoylation of the protein. Our data indicate that either cysteine residue can be palmitoylated *in vivo* and that palmitoylation of a single residue is sufficient for strong membrane association. An examination of neuromodulin mutants indicated that there is a general correlation between membrane targeting and the extent of palmitoylation. While either Cys-3 or Cys-4 can be palmitoylated independently of the other, quantitation of the incorporation of [^{3}H]-palmitic acid into neuromodulin *in vivo* demonstrated that the presence of Cys-4 is of major importance for palmitoylation of the protein. The greater labeling of Leu-3 versus Leu-4 mutant neuromodulins suggested that Cys-4 may be the preferred site for palmitoylation of the native protein or, alternatively, that the presence of Cys-4 may be crucial for palmitoylation of Cys-3. Differential palmitoylation at two cysteines has also been reported for CD4, in which cysteines-394 and -397 are both acylated, but Cys-394 is the major site for palmitoylation (Crise & Rose, 1992). However, since the incorporation of [^{3}H]palmitic acid into the mutant neuromodulins does not measure the steady-state stoichiometry of palmitoylation, it is also possible that the differential labeling of L3 and L4 may simply reflect differences in their rate of acylation and de-acylation. The Thr-3 mutant showed a much lower membrane targeting than Leu-3 because the threonine substitution lowered palmitoylation at Cys-4, not because palmitoylation of both cysteines is required for membrane association. In addition, the single-cysteine mutants still bound very strongly to membranes. These data suggest that the wild-type protein can be attached to membranes via mono-palmitoylation at either Cys-4 or Cys-3.

Table IV: Amino Acid Sequences of Palmitoylation Sites of Proteins

neuromodulin	NH_2-MLCCMRRTK-COOH	Cimler et al., 1987
band III	NH_2-QIICLAVL-COOH	Okubo et al., 1991
CD 4	NH_2-FFCVRCRHRR-COOH	Crise & Rose, 1992
K(A)-ras	NH_2-PGCVKIKK-COOH	Hancock et al., 1989
H. M2 AChR	NH_2-LLMCHYKNI-COOH	O'Dowd et al., 1989
B. rhodopsin	NH_2-TLCCGKNPL-COOH	Ovchinnikov et al., 1988
SFV-E1	NH_2-VVTCIGLLRR-COOH	Schmidt et al., 1988
VSV-G	NH_2-IHLCIKLK-COOH	Mack & Kruppa, 1988
transferrin receptor	NH_2-PKRCSGSICYG-COOH	Jing & Trowbridge, 1987
H. β_2-adrenergic receptor	NH_2-LLCCLRRSS-COOH	O'Dowd et al., 1989

Unlike myristoylated and isoprenylated proteins, in which the recognition domains for acylation are confined within a four to seven amino acid sequence (Hancock et al., 1989; Schmidt, 1989; Casey et al., 1989; James & Olson, 1990), a consensus sequence for palmitoylation has not yet been defined. Acyl-transferase activity has been localized to the Golgi apparatus, ER membranes, and growth cone membranes in neurons (Rizzolo & Kornfeld, 1988; Gutierrez & Magee, 1991; Patterson & Skene, 1989), there may be multiple forms of palmitoyltransferase with different specificities present in any given cell. It is interesting that most of the palmitoylated proteins contain positively charged and hydrophobic amino acid residues near the acylated cysteine residues (Table IV). Our data indicate that the presence of Arg-6, Arg-7, and Lys-9 in neuromodulin is not crucial for membrane targeting. However, the presence of hydrophobic amino acids adjacent to Cys-4 may be important since the mutant L3 was a better palmitoylation substrate than T3. The presence of hydrophobic residues appears to be more important for palmitoylation of neuromodulin than the presence of positively charged amino acids, although both motifs are seen in most palmitoylation substrate domains (Table IV).

In summary, mutagenesis of specific amino acids in the palmitoylation domain of neuromodulin and examination of the sequence of other palmitoylated proteins failed to identify a clear consensus sequence or a well-defined general descriptor for palmitoylation. Palmitoylation of either cysteine residue in neuromodulin is sufficient for membrane association, and there is a direct correlation between membrane targeting and the extent of palmitoylation. Either Cys-3 or Cys-4 of neuromodulin can be palmitoylated *in vivo*, and this palmitoylation is both necessary and sufficient for the attachment of the protein to membranes.

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