Ca^{2+}-Myristoyl Switch and Membrane Binding of Chemically Acylated Neurocalcins†

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ABSTRACT: Neurocalcin is a member of a novel family of neuronal calcium sensors that belongs to the superfamily of EF-hand Ca^{2+}-binding proteins. Neurocalcin is myristoylated on its N-terminus in vivo and can associate with biological membranes in a calcium and myristoyl-dependent manner. This process known as "Ca^{2+}-myristoyl switch" has been best described for the photoreceptor specific protein, recoverin, as well as for several other neuronal calcium sensors. Here, we used reversed micelles to chemically acylate nonmyristoylated neurocalcin at its N-terminus with fatty acids of different lengths (from C12 to C16). This approach allowed us to prepare neurocalcin derivatives in which a single fatty acid is selectively linked to the N-terminal glycine of the polypeptide chain through an amide bond. The membrane binding properties of the monoacylated neurocalcins were then examined by cosedimentation with phospholipid vesicles and direct binding to lipid monolayers by surface plasmon resonance spectroscopy (Biacore). Our results show that neurocalcins monoacylated with lauric, myristic, or palmitic acid were able to associate with membrane in a calcium-dependent manner. This indicates that the Ca^{2+}-myristoyl switch can function with different lipid moieties and is not strictly restricted to myristate. The ability to modify at will the fatty acid linked to the N-terminal glycine should be useful to analyze the contribution of the fatty acid moiety to the biological function of this family of neuronal calcium sensors.

Many eukaryotic and viral proteins are modified by the covalent attachment of a myristic acid to their N-termini (*1*−*4*). N-Terminal myristoylation is carried out by the myristoyl-CoA:protein *N*-myristoyltransferase (NMT)[1] and occurs during or soon after translation of the polypeptide (*5*, *6*). NMT catalyzes the transfer of the fatty acid from acylated coenzyme A to the polypeptide N-terminus to form an amide bond, which is essentially irreversible. NMT has a high specificity for 14:0 CoA, although it can also use 12:0, 14:1n-9, and 14:2n-6 CoA. In most tissues, proteins are acylated with only the 14:0 myristic acid. However, in photoreceptor cells, for yet unclear reasons, proteins are heterogeneously acylated with 12:0, 14:1n-9, and 14:2n-6 in addition to 14:0 (*7*).

In numerous cases, N-terminal myristoylation has been shown to be critical for the function of the corresponding proteins. Acylation can affect the protein structure, the enzymatic activity, or the ability of the acylated protein to interact with specific partners. In general, myristoylation will also facilitate binding of proteins to membrane. Indeed, many known myristoylated proteins are involved in signal transduction, such as protein kinases, heterotrimeric G proteins, ADP-ribosylation factors, and calcium-binding proteins. These latter proteins constitute a subgroup of EF-hand Ca^{2+}-binding proteins that are mainly expressed in the brain and photoreceptors (*8*). This subfamily of so-called neuronal calcium sensors (NCS) contains at present more than 40 members including recoverin, S-modulin, visinin, frequenin, GCAP (guanylyl cyclase activating protein), GCIP (guanylyl cyclase inhibitory protein), and multiple isoforms of visinin-like proteins, including VILIP, hippocalcin, and neurocalcin. Highly conserved orthologs of frequenin and VILIP/neurocalcin have been found in *Aplysia*, *Caenorabhditis elegans*, *Drosophila*, and yeast (*Schizzosaccharomyces pombe* and *Saccharomyces cerevisae*). Recoverin and S-modulin are photoreceptor specific proteins that are involved in the regulation of light adaptation: in their Ca^{2+}-bound form, they block the phosphorylation of photoexcited rhodopsin thus prolonging the photoresponse. GCAP and GCIP are

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[1] Abbreviations: NMT, *N*-myristoyltransferase; NCS, neuronal calcium sensors; RnaseA, ribonuclease A; AOT, sulfosuccinic acid bis-[2-ethylhexyl] ester; DTT, dithiothreitol; EGTA, ethylene glycol-bis-(b-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, (*N*-{2-hydroxyethyl}piperazine-*N*′-{2-ethanesulfonic acid}); ESI-MS, electrospray ionisation mass spectrometry; HPA, hydrophobic association; MADLI-TOF MS, matrix-assisted laser desorption time-of-flight mass spectrometry; Q TOF MS, quadrupole time-of-flight mass spectrometry; RP-HPLC, reversed-phase-high performance liquid chromatography; SDS−PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SPR, surface plasmon resonance.

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Ca^{2+}-dependent activator and inhibitor, respectively, of the photoreceptor specific guanylyl cyclases. Frequenin was originally shown to facilitate neurotransmission in *Drosophila*. More recently the *Saccharomyces cerevisiae* frequenin homologue has been identified as a subunit of the phosphatidyl inositol 4-OH kinase (*9*). The precise function of the visinin-like proteins is not yet clearly established although some reports indicate that they could modulate cAMP signaling and/or calmodulin-dependent signaling pathways (*10*−*13*).

These NCS proteins typically contain between 180 and 210 amino acids, with 45% sequence identity (*8*). They exhibit a consensus signal for N-terminal myristoylation and four EF-hand Ca^{2+}-binding motifs, although in all cases the first EF hand is unable to bind calcium. Several of these proteins have been shown to be myristoylated either in vivo and/or in vitro. In several instances it was demonstrated that the covalently attached myristoyl group at the N-terminus allows the NCS proteins to interact with biological membranes in a Ca^{2+} dependent manner in a process called "Ca^{2+}-myristoyl switch". The molecular mechanisms of the Ca^{2+}-myristoyl switch have been analyzed in depth in the case of recoverin, a photoreceptor specific protein (*14*−*18*). X-ray and NMR structures of both Ca^{2+}-bound and Ca^{2+}-free myr-recoverin have revealed that in the Ca^{2+} free state, the myristoyl group is sequestered in a deep hydrophobic pocket where it is clamped by residues from the three EF-hands. Upon Ca^{2+} binding, the myristoyl group is extruded and therefore free to bind to membranes and/or protein targets. Ca^{2+} binding is accompanied by a 45° rotation of the N-terminal domain, which exposes hydrophobic residues that might contribute additional free energy to interact with lipids. Crystal structure of recombinant bovine neurocalcin (*19*) and the NMR structure of GCAP-2 (*20*) strongly suggests that all NCS proteins share a similar three-dimensional structure.

In the present study, we have investigated whether the Ca^{2+}-myristoyl switch is strictly restricted to C14 or could function with other fatty acids, acting more generally as a Ca^{2+}-acyl switch. For this purpose, nonmyristoylated neurocalcin had to be chemically acylated in a controlled manner. Conjugating fatty acids that are poorly soluble in water, to proteins that are poorly soluble in organic solvents, is still a challenging task. Protein acylation can be undertaken using either classical chemical methods (*21*−*25*) or reversed micelles as microreactors (*26*−*28*). In micellar media, derivatization of proteins by water-insoluble reagents can be limited to a small number of sites. On the other hand, carrying out this reaction in water is difficult and leads to a much more extensively modified protein. Reversed micelles were used to acylate enzymes (*26*−*28*), antibodies, and Fab fragments (*29*, *30*). Reversed micelles acting as microreactors have been used for N-terminal acylation of ribonuclease A (RnaseA). Monoacylated RnasesA were produced in 10-mg quantities and used for both in vivo and in vitro membrane interaction studies (*31*, *32*).

The same methodology was adapted here to nonmyristoylated neurocalcin expressed in *Escherichia coli*. The proteins were characterized by reversed-phase−high-performance liquid chromatography (RP-HPLC) and electrospray ionization mass spectrometry (ESI-MS). The peptide bearing the acyl group was identified by enzymatic hydrolysis of the protein and by matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS). The precise location of the fatty acid was determined by analyzing the fragmentation of this peptide by tandem mass spectrometry. All fatty-acylated derivatives were found to be specifically monoacylated at the N-terminus. Analysis of the membrane binding properties of the different derivatives indicated that they all exhibit a Ca^{2+}-acyl switch.

MATERIALS AND METHODS

Unmyristoylated and myristoylated bovine neurocalcin δ were obtained as recombinant proteins from *E. coli*. Expression and purification of both proteins were performed as previously described (*33*). About 10−20 mg of recombinant myristoylated or unmyristoylated neurocalcin δ were obtained from 1 L of culture. The neurocalcin content in purified preparations was determined from the absorbance at 280 nm using a molar extinction coefficient of 20 mM^{-1} cm^{-1}. Protein purity was checked by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). Proteins were stored in 20 mM, HEPES[1]-Na buffer, pH 7.4 containing 150 mM NaCl and 1 mM dithiotreitol (DTT). Fatty acid chlorides (lauryl, myristoyl, palmitoyl), AOT (sulfosuccinic acid bis [2-ethylhexyl] ester) (used as received), brain lipids (brain extract typeVII), detergents octylglucoside, and Triton X-114 were from Sigma (St. Louis, MO). Triton X-114 was precondensed as follows. A total of 20 g of detergent were mixed with 980 mL of 20 mM HEPES-Na and 150 mM NaCl, pH 7.4. This solution was placed at 0 °C for 2−4 h. The solution was incubated overnight at 30 °C (to allow detergent condensation). The upper phase (aqueous detergent depleted phase) was removed and replaced by the same volume of fresh 20 mM HEPES-Na, 150 mM NaCl, pH 7.4 buffer. The solution was mixed and the condensation was repeated twice more. After the third condensation, the upper aqueous phase was discarded and the concentration of Triton X-114 in the final lower phase was determined by comparison of its absorption at 275 nm with that of the commercial detergent solution. The precondensed Triton X-114 was stored in a darkness bottle at room temperature.

Bio-Beads SM-2 adsorbent (20−50 mesh) were from Bio-Rad (Hercules, CA, US). 2,2,4-Trimethyl pentane, from Aldrich (St Quentin-Fallavier, France), was dried on 3 Å molecular sieve. Trifluoroacetic acid (protein sequencing grade) was from Fluka (St Quentin-Fallavier, France). Acetone and acetonitrile, both HPLC grade, were supplied by Riedel de Haen (Seelze, Germany). Deionized water was obtained from a MilliQ apparatus (Millipore, Milford, MA). All other reagents were of analytical grade.

Preparation and Purification of Acylated Neurocalcin δ. Unmyristoylated neurocalcin was acylated in reversed micelles following the methodology previously established for Rnase A. Optimal conditions determined for hydrophobic modification of neurocalcin δ using AOT reversed micelles are as follows: [water]/[AOT] = 12, [acyl chloride]/[neurocalcin δ] = 2, borate buffer 50 mM pH 10.1. Prior to acylation, the unmyristoylated neurocalcin solution was desalted using sephadex G25 column equilibrated with water, the fractions containing the proteins were pooled and freeze-dried. Three milligrams of protein was injected in 7.5 mL of 0.1 M AOT in 2,2,4-trimethyl pentane. Fatty acid chloride

dissolved in 0.3 mL of 2,2,4 trimethyl pentane was then added. After reacting for 2 h, the protein fraction was precipitated by cold acetone (−20 °C). After a 20-min centrifugation at 2500*g* and −20 °C, the protein precipitate was collected, and was washed four times as follows: 7.5 mL of cold acetone (−20 °C) was added, the solution was centrifuged for 10 min at 2500*g* and −20 °C, and the supernatant-containing isooctane and AOT were discarded. AOT presence in the different fractions was checked by thin-layer chromatography. After washing, 1 mL of acetone was added to the precipitate followed by 1 h of evaporation at room temperature under vacuum. The proteins were dissolved in 1.2 mL of 20 mM HEPES-Na, pH 7.4, containing 150 mM NaCl, 2 mM $CaCl_2$, 1 mM DTT, and 1% Triton X-114.

The above solution contained two major components when analyzed by RP-HPLC. The acylated protein was separated from the nonacylated counterpart by using a Triton X-114 temperature-induced phase separation procedure at 30 °C. Precondensed Triton X-114 prepared as described above was used throughout the procedure. The protein solution in 20 mM HEPES-Na, 150 mM NaCl, 2 mM $CaCl_2$, 1 mM DTT, 1% Triton X-114, pH 7.4, buffer was mixed and incubated on ice for 5−10 min. Two-tenths of a milliliter was layered on 0.3 mL of a sucrose cushion (6% w/v sucrose in 10 mM HEPES-Na, 150 mM NaCl, 0.06% Triton X-114, pH 7.4) into the first microcentrifuge tube. After 3 min incubation at 30 °C, the sample was centrifuged at 3000*g* for 3 min. The upper aqueous phase (about 0.2 mL) was transferred into a second tube and Triton X-114 was added in order to obtain a final concentration of 0.5%. It was mixed and incubated for 5 min on ice before being layered on the same sucrose cushion (first tube) followed by 3 min incubation at 30 °C. The sample was centrifuged at 3000*g* for 3 min. The upper aqueous phase (about 0.2 mL) was transferred into a third tube, and Triton X-114 was added in order to obtain a final concentration of 2%. It was mixed and incubated for 5 min on ice followed by 3 min incubation at 30 °C and centrifugation at 3000*g* for 3 min. The final upper depleted aqueous phase was transferred into a clean tube. The sucrose cushion (first tube) was removed from the detergent-rich phase, the solution was completed with 20 mM HEPES-Na, 150 mM NaCl, 2 mM EGTA, 1 mM DTT, pH 7.4, buffer to 1 mL of total volume.

The acylated protein was recovered in about 1.5% Triton X-114. The detergent was then removed by using Bio-Beads as follows: 80 mg/mL of Bio-Beads were added each hour during 4 h while the solution was gently stirred using a rocking shaker at room temperature. After sedimentation of Bio-Beads (320 mg total), less than 0.04% of Triton X-114 (as determined by absorption at 275 nm) were present in the recovered protein solution.

The purity of the acylated proteins was checked by RP-HPLC analysis. The overall yield of pure modified neurocalcin was on average about 20% of the starting material.

Mass Spectrometry Characterization of Acylated Neurocalcin δ. The extent of chemical modification was determined by electrospray ionization mass spectroscopy (ESI-MS), using a Finnigan SSQ710 (San José, CA) mass spectrometer equipped with an electrospray inlet system. Protein solutions in methanol, water, and acetic acid (50/50/0.5) were injected at 0.5 μL min^{-1}. The instrument was used in the positive mode. Spectra were recorded in an *m*/*z* range of 1000−2000.

Assignment of chemical modification was deduced from mass spectrometry of the peptides resulting from protein hydrolysis. About 100 pmol of protein were submitted to digestion using Asp-*N* endoproteinase from a *Pseudomonas fragi* mutant (Boehringer Mannheim, Germany). One part by weight enzyme with 200 parts by weight protein in 50 mM Tris buffer, pH 8.0, were incubated during 15 h at 37 °C. The resulting peptide mixture was desalted using C18-micro-column (Ziptip-C18, Millipore, Bedford, MA) for all proteins except for C16-neuro mixture which was desalted using a C4-Ziptip. The peptides in methanol, water, acetic acid, (50/50/0.5) solution were analyzed after cocrystallization with a matrix of dihydroxybenzoic acid (saturated solution in 0.1% trifluoroacetic acid) by using a MALDI-TOF mass spectrometer Voyager-STR biospectrometry workstation (Perseptive biosystems, Framingham, MA) in the positive and reflector modes. The acylated peptides were identified by comparing experimental masses with the theoretical masses of the nonmodified peptides released from Asp-*N* digestion of the protein. The location of the acyl moiety was determined by analyzing the fragmentation of the peptide bearing the acyl group using a hybrid quadrupole-time-of-flight electrospray mass spectrometer Q TOF 2 (Micromass, Manchester, U.K.). After desalting, the hydrolysate solution was deposited, via a gel loader, in a golden glass needle of the nanospray (Protana, Odense, DK).

Vesicle Binding Studies. Vesicles were prepared from a dried lipid film obtained from 100 mg of brain lipids in 5 mL of chloroform solution by removing the organic solvent under vacuum. The lipid film was hydrated by 2 mL of 10 mM HEPES-Na, pH 7.4, containing 20 mM KCl and 180 mM sucrose. Liposomes were obtained from ultrasonic irradiation (four cycles of 3 min at 100 W separated by 3 min periods), the suspension was centrifuged 20 min at 5000*g* in order to eliminate titan particles. The supernatant was subjected to four cycles of freeze−thawing, the liposomes were centrifuged for 30 min at 100000*g*, the pellet was resuspended in 10 mM HEPES-Na buffer, pH 7.4, containing 20 mM KCl and 180 mM sucrose. The hydrodynamic mean diameters of the aggregates determined by quasielastic light scattering (Nanosizer N4MD, Coultronics) ranged from 180 to 250 nm.

For binding assays, 15 μg of acylated neurocalcin were incubated with liposomes (about 500 μg of lipids) in 300 μL of 20 mM HEPES-Na, pH 7.5, 100 mM KCl, containing either 2 mM EGTA[1] or 1 mM $CaCl_2$. After 30 min incubation at 22 °C, the mixtures were centrifuged for 1 h at 100000*g* in an airfuge (Beckman) at 22 °C. Both supernatant and pellet fractions were denaturated in 100 μL of sample buffer and identical volumes were electrophoresed on a 12.5% SDS gel, which was stained with Coomassie Blue.

Surface Plasmon Resonance Measurements. Binding kinetics were determined by surface plasmon resonance (SPR) using a BIAcore X (Biacore AB, Uppsala, Sweden). In SPR technology, the complex formation between a flowing analyte (here, the acylated protein) on top of an immobilized ligand (an hybrid alkane-thiol/phospholipid bilayer) is observed. The hydrophobic chip consisted of 1-octadecanethiol self-assembled monolayer on a gold surface designed to facilitate

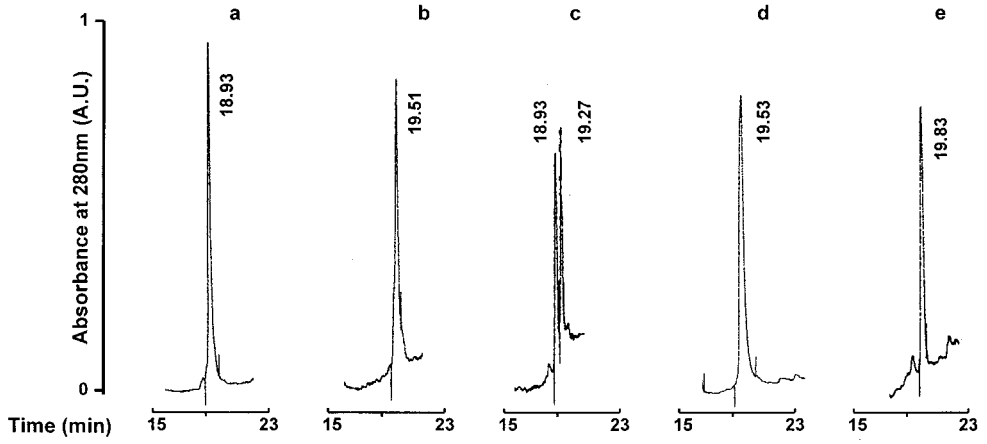

FIGURE 1: RP-HPLC profiles of neurocalcin (a), myr-neurocalcin (b), and C12-neurocalcin (c), C14-neurocalcin (d), C16-neurocalcin (e) obtained after Triton X-114 purification procedure.

Table 1: RP-HPLC and ESI-MS Parameters of Neurocalcins[a]

sample	RP-HPLC retention time (min)	molar mass (g mol^{-1} ± SD)	expected mass variation (g mol^{-1})	number of fatty acid
neurocalcin	18.93	22 110 ± 2	0	0
Myr-neurocalcin	19.51	22 325 ± 2	210	1
C12-neurocalcin	19.27	22 300 ± 2	182	1
C14-neurocalcin	19.53	22 326 ± 2	210	1
C16-neurocalcin	19.83	22 354 ± 2	238	1

[a] Retention time of proteins determined on a C-18 grafted silica column are indicated in column 1. Molecular masses found for each sample are given column 2. Column 3 indicates the mass variation expected for one acyl moiety linked. Column 4 gives the number of acyl moieties per protein molecule as deduced from the difference of molecular mass of the sample compared to neurocalcin.

liposome mediated hydrophobic adsorption. The hydrophobic chip was obtained by adsorption of 1-octadecanethiol [1 mM in ethanol, water (4/1) solution] onto bare gold and characterized as previously described (*34*). Before vesicles fusion, the surface of the sensor chip was cleaned by a 2 min injection of 40 mM octyl-β-D-glucoside at a flow rate of 20 μL min^{-1}. Conditions of vesicles preparation for 5 mg of brain lipids, were adapted as follows: the lipid film was hydrated by 5 mL of buffer (without sucrose) and ultrasonic irradiation reduced (three cycles of 0.5 min at 40 W separated by 3 min periods). Coating of the chip was obtained by injection the vesicle suspension (80 μL at a flow rate of 2 μL min^{-1}) over the surface. The lipid layer was washed 2 min with NaOH (40 mM, 20 μL min^{-1}) which stabilized the coverage (*34*−*36*). The optical thickness of the lipid layer formed, determined by spr spectroscopy [homemade surface plasmon resonance apparatus in Kretshmann set up (*37*)] was 22 ± 4 Å. This is in accordance with the formation of a lipid monolayer (*34*, *37*) and corresponds to 1700 ± 100 RU measured on Biacore apparatus. Proteins samples in buffer (0.02−5 μM) were centrifuged 15 min at 100000*g* and injected at flow rates ranging from 5 to 20 μL min^{-1}. Reversion to buffer flow was done 6 min after injection. A 2 min NaOH (40 mM, 20 μL min^{-1}) wash between two successive protein injections was required for a complete regeneration of the phospholipid monolayer. All solutions in HEPES-Na buffer were filtered and thoroughly degassed. Temperature was maintained at 25 °C.

Analyses of association and dissociation curves were carried out by using BIAeval 3.1 global analysis software. Association phase was taken from 20 to 320 s after injection and dissociation phase was considered in the time interval 80−240 s after buffer injection. Equilibrium constants (K_D) were determined from Scatchard plots as done for recoverin (*36*) and artificially acylated Rnases (*38*). Concentration of free analyte, under continuous flow, is equal to the bulk protein concentration (*C*), concentration of complex (protein bound) can be measured directly as the steady-state response (R_{eq}). The steady-state level (in RU) is related to the free protein concentration by the equation $R_{eq} = R_{max} - K_D R_{eq}/C$; where R_{max} is the maximum protein binding capacity.

RESULTS

Chemical Acylation of Neurocalcin. Neurocalcin produced in *E. coli* was used as starting material for protein acylation, while myristoylated neurocalcin produced by coexpressing neurocalcin and the yeast *N*-myristoyl transferase, was used as a positive control. After acylation with lauric, myristic and palmitic acid chlorides, RP-HPLC of the protein mixture always yielded two major fractions in a 50/50 ratio: the starting material (identified by its retention time) and the putative acylated derivative. The retention time of the acylated product increased with the fatty acid chain length (Table 1). All these compounds showed the same electrophoretic mobility on SDS−PAGE. The acylated proteins were purified by using a Triton X-114 temperature-induced phase separation at 30 °C. After elimination of the detergent using Bio-Beads, the purity of neurocalcin derivatives was checked by RP-HPLC as shown in Figure 1. The purification of myristoyl and palmitoyl derivatives was efficient, as attested by the presence of less than 10% of unmodified neurocalcin. However, the lauryl derivative could not be efficiently separated from the unmodified neurocalcin by the Triton X-114 procedure; nevertheless, the mixture containing neurocalcin and the lauryl derivative (50/50) was considered for subsequent experiments. As shown in Figure 1, the retention time of the derivatives increased with the length of the acyl chain of the fatty acid, and as expected, the chemically obtained C14-neurocalcin retention time was identical to the myr-neurocalcin one.

An ESI-MS analysis of each derivative was performed in order to determine the number of fatty acids covalently bound

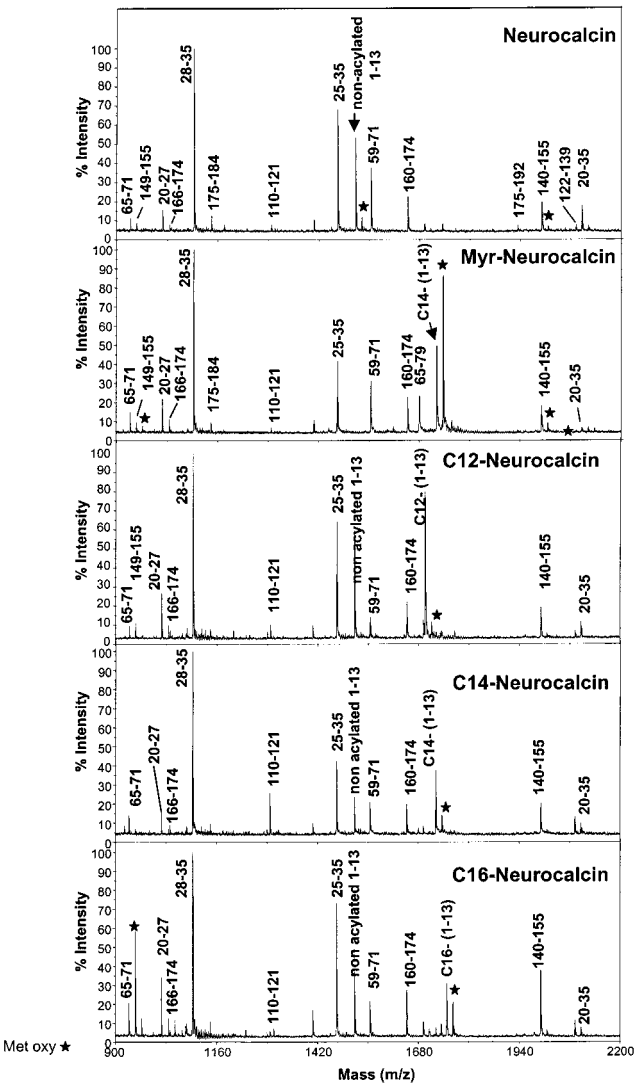

FIGURE 2: MALDI-TOF mass spectra of peptides resulting from neurocalcins after digestion by Asp *N* endoproteinase (*Pseudomonas fragi*). Each fragment was identified as explained in material and methods section (Swiss Prot). The (1−13) peptide bearing the acyl group is named Cx-(1−13), where x is the acyl chain carbon number.

per neurocalcin polypeptide (Table 1). A mass variation of 182, 210, or 238 was, respectively, expected for the linkage of a single lauric, myristic, or palmitic fatty acid moiety per protein molecule. The mass data for all samples are consistent with the presence of a single fatty acid residue per protein molecule.

To determine the exact location of the linked acyl chain, we compared the peptide masses maps of neurocalcin, myr-neurocalcin and those of chemically acylated derivatives after proteolytic cleavage with the Asp-*N* endoproteinase from a *Pseudomonas fragi* mutant. MALDI-TOF spectras obtained for neurocalcin, myr-neuro, C12-neuro, C14-neuro and C16-neuro are shown in Figure 2. After digestion of neurocalcins, a large part of the expected proteolytic peptides could be identified by MALDI-TOF analysis. All acylated peptides (resulting from myr-neuro, C12-neuro, C14-neuro, or C16-neuro) showed a mass increase for the N-terminal peptide GKQNSKLRPEVMQ that corresponds precisely to the mass of the acyl group considered. No additional acyl group could be detected in all other peptides identified, indicating that in all cases, acylation was selective for the N-terminal peptide. To determine precisely the site of modification within the N-terminal peptide, the hydrolysates were directly submitted to MS/MS electrospray, ions corresponding to the N-terminal peptide were selected and fragmented (Figure 3). Analysis of the b (N-terminal end) and Y″ (C-terminal end) fragments revealed that the acyl group was borne by the N-terminal dipeptide GK. No modification was observed on the other part of the N-terminal peptide GKQNSKLRPEVMQ as confirmed by the occurrence of Y″-12 fragments in C12-neuro and C14-neuro. Internal fragments such as KQ, KQN in myr-neuro and in C12 and C16-neuro or KQNSKLRPEVM in C16-neuro show that they did not bear the acyl group. Therefore in all cases, the chemically attached fatty acid is linked via an amide bond to the N-terminal G.

All of these results are in agreement with a selective N-terminal chemical monoacylation of neurocalcin. The possibility of modifying at will the length of the N-terminal fatty acid allowed us to study its role in the Ca^{2+}- and acyl-dependent binding of neurocalcin to membranes.

Membrane Binding of Neurocalcin Derivatives. Bovine brain lipid vesicles were incubated with the proteins in the presence or absence of Ca^{2+} and pelleted by high-speed centrifugation (100000*g*). Both supernatant and pelleted protein content were analyzed by SDS−polyacrylamide gel electrophoresis (Figure 4). Nonmyristoylated neurocalcin did not bind to vesicles and remained in the supernatant in the presence or absence of Ca^{2+}. Myr-neurocalcin was recovered in the lipid pellet when incubated in the presence of Ca^{2+}, whereas it remained in the supernatant in the presence of EGTA. The chemically acylated C14 and C16-neurocalcin derivatives behaved similarly. They were associated with the vesicles pellet when the sedimentation was carried out in the presence of Ca^{2+} while they were recovered in the supernatant fraction when sedimented in the presence of EGTA. The mixture (unmodified neurocalcin/C12-neurocalcin) resulting from lauric acid modification was assayed in the same conditions. In that case, protein was evidenced both in the pellet and in the supernatant in the presence of Ca^{2+}. In the presence of EGTA, all proteins were recovered in the supernatant (data not shown). This result suggests that the C12-neurocalcin derivative present in the mixture can effectively bind vesicles in the presence of Ca^{2+}. Control experiments showed that chemically modified neurocalcins centrifuged at high speed in the presence of Ca^{2+}, but without lipids, remained soluble (data not shown). All of these data establish that chemically modified neurocalcins bind to lipid vesiscles in a Ca^{2+}-dependent manner.

The kinetics of binding of acylated neurocalcins to a brain lipid hybrid layer were investigated by using the BIAcore technique. For this purpose, a monolayer of brain lipids was formed at the surface of an 1-octadecanethiol layer adsorbed on the bare gold surface of a sensor chip, the quality of which was controlled before each experiment by inspection of the surface plasmon resonance spectra (see Materials and Methods). Formation of the brain lipid monolayer yielded a reproducible and stable signal of 1700−1800 RU (data not shown). As shown in Figure 5A, myr-neurocalcin bound efficiently to the lipid monolayer in the presence of Ca^{2+} whereas only a residual binding was observed in the absence of Ca^{2+}. As a control, we checked that myr-neurocalcin did

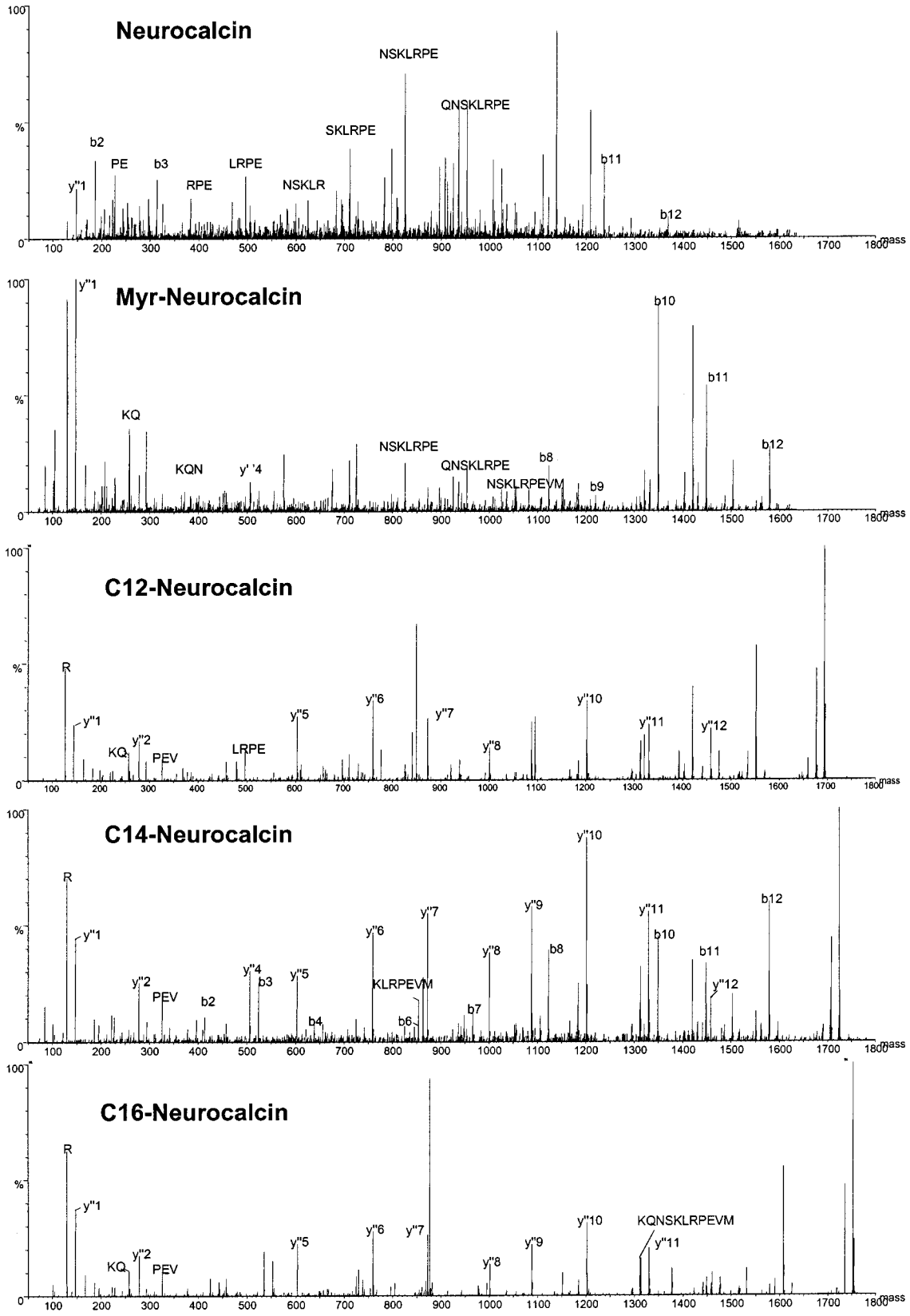

FIGURE 3: Tandem mass spectra resulting from fragmentation of the (1−13) N-terminal peptides. The b fragments (obtained from the N-terminal end) and Y″ fragments (obtained from the C-terminal end) were identified by comparison with the calculated masses of the theoretical fragments. The other fragments (internal ones) are indicated by their sequence.

not bind to the bare alkane-thiol layer (i.e., without brain lipid). In the same conditions, unmyristoylated-neurocalcin did not bind to the lipid coverage neither in the presence nor in the absence of Ca^{2+}. Therefore, the Ca^{2+} -myristoyl switch of neurocalcin can be monitored in real time by spr spectroscopy using this hybrid layer configuration.

The affinity of myr-neurocalcin was determined by recording sensorgrams at increasing protein concentrations

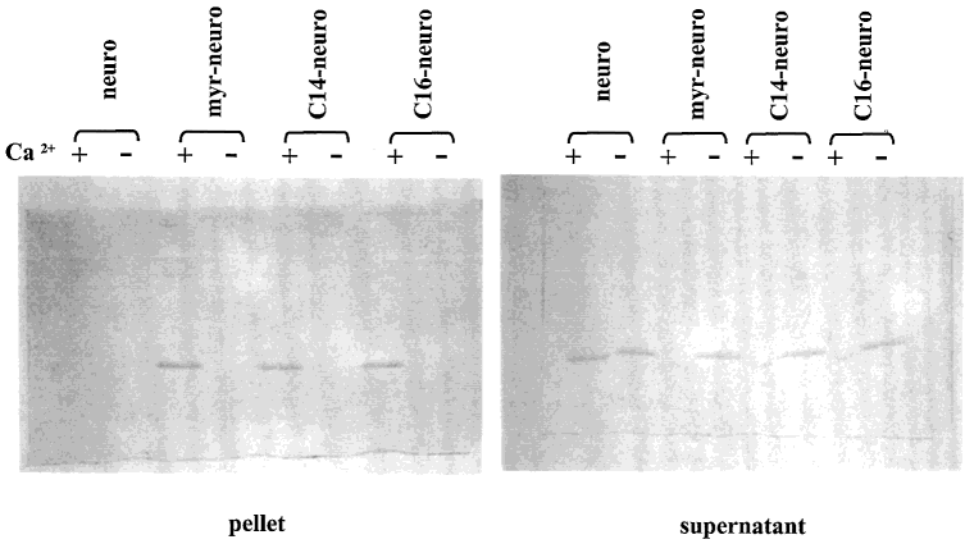

FIGURE 4: Binding of acylated neurocalcins to bovine brain liposomes. Proteins were incubated with liposomes either in the presence of EGTA ($-Ca^{2+}$) or in the presence of $CaCl_2$ ($+Ca^{2+}$). After high-speed centrifugation, equal volumes of pellet and supernatant were electrophoresed on a 12.5% SDS gel which was stained with Coomassie Blue.

from 0.02 to 2 μM in the presence 2 mM Ca^{2+} (Figure 5A). The kinetic data were fitted with the 'separated association and dissociation phase' method. The values of resonance units at equilibrium (R_{eq}) obtained were used for determination of equilibrium constants, Scatchard plots derived from three independent set of experiments give a K_D value of 0.35 $\pm$ 0.05 μM.

Similarly, the binding properties of C14 and C16-neurocalcins toward the phospholipid supported layer were examined (Figure 5, panels B and C). In absence of Ca^{2+}, the chemically modified C14 and C16-neurocalcins did not bind significantly to the lipid monolayer: at the highest protein concentration maximum tested, less than 250 RU could be detected. In presence of Ca^{2+}, C14 and C16-neurocalcins bound efficiently to the phospholipid coated chips. Importantly, for a given protein concentration (i.e., 0.5 μM) C14-neurocalcin (900 RU) gave a steady state response highly similar to myr-neurocalcin (850 RU) while C16-neurocalcin (2000 RU) was about twice higher. Scatchard plots derived from data analysis of those experiments gave a K_D value of 0.39 $\pm$ 0.05 μM for C14-neurocalcins and 0.28 $\pm$ 0.05 μM for C16-neurocalcins. These results indicate that the binding of C16-neurocalcin is facilitated compared to the C14/myr-neurocalcins although the increase in affinity of C16-neurocalcin appeared less than expected for the presence of two additional methylene groups.

DISCUSSION

In this study, we show that neurocalcin can be selectively chemically acylated by different fatty acid chlorides on its N-terminus. The reaction was carried out in size-tunable nanoreactors; reversed micelles; the protein soluble in the aqueous core is protected by a molecular surfactant barrier while the lipid is introduced in the organic phase. Under optimized conditions, neurocalcin was monoacylated by lauric, myristic, and palmitic acid as demonstrated by ESI-MS. Further characterization of these derivatives by MALDI-TOF and MS/MS mass spectrometry indicated that the single fatty acid is linked to the N-terminal glycine of the polypeptide chain through an amide bond. Preferential acylation of the N-terminal glycine could be interpreted as a result of two facts: (i) a good accessibility due the flexibility of the terminal part of the polypeptide chain in the case of unmyristoylated neurocalcin (*19*) which favors interaction with the surfactant shell of the micelle, and (ii) p*K* values of the amino groups indicate that the N-terminal-amino group should react faster than ϵ-amino groups with fatty acid chlorides as previously observed with RnaseA (*31*, *32*). The chemically acylated C12, C14, and C16-neurocalcins were purified using a Triton X-114 temperature-induced phase separation procedure and their membrane association properties were examined. All three acylated-neurocalcin exhibited a calcium-dependent membrane association as demonstrated by cosedimentation with phospholipid vesicles and/or direct binding to lipid monolayers in Biacore experiments.

Our results indicate therefore that the calcium-acyl switch can function with C12 or C16 fatty acids in addition to the standard C14. The calcium-myristoyl switch model states that the myristoyl group is sequestered within a hydrophobic pocket in the Ca^{2+}-free protein and that binding of calcium triggers a conformational change that results in the release of the fatty acid that can then insert into the membrane. The present data suggest that the hydrophobic cavity in the Ca^{2+}-free neurocalcin can accommodate these various fatty acids despite their difference in chain length. Moreover, the calcium-induced conformational change is apparently not impaired by the C12 or C16 modification. Earlier studies have shown that myristoylation affect the Ca^{2+}-binding of neurocalcin (*33*). The unmyristoylated neurocalcin binds Ca^{2+} with a higher affinity than the myristoylated protein but without apparent cooperativity. It was proposed that the energy of calcium binding was used in part for the solvation of the fatty acid group. It will be of interest to compare the Ca^{2+} -binding properties of the C12 and C16 modified neurocalcins with those of the myristoylated protein in order to test this hypothesis.

We have used surface plasmon spectroscopy to characterize the apparent affinity of acylated neurocalcins toward an hybrid bilayer membrane. In the presence of Ca^{2+}, the affinity of C14/myr-neurocalcin for brain lipids was rather high with

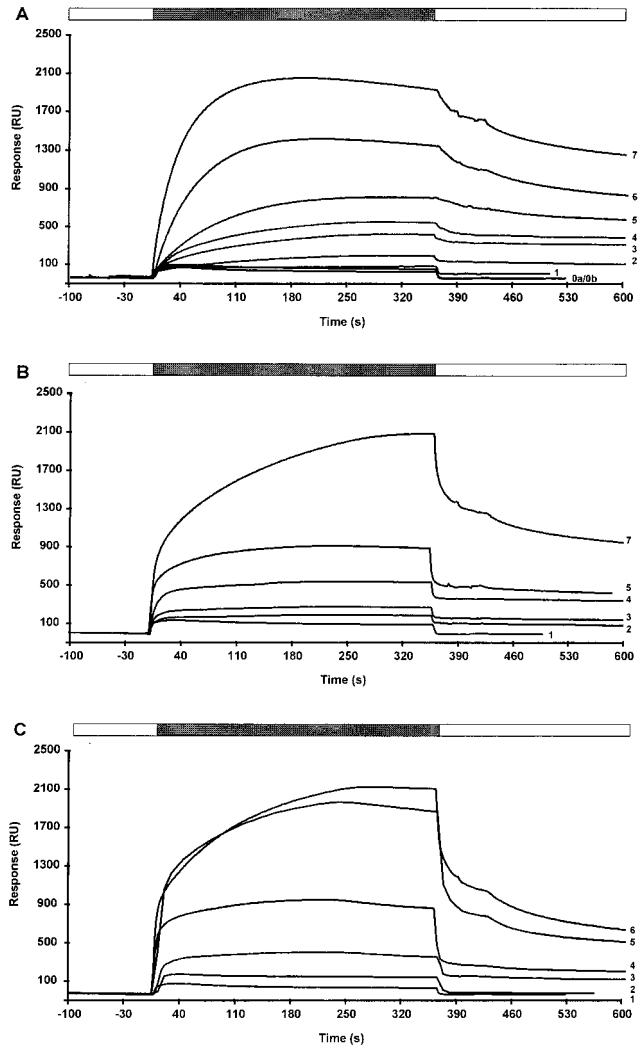

FIGURE 5: Affinity of acylated neurocalcins toward a bovine brain phospholipid monolayer. (A) Overlay plots of sensorgrams obtained either in absence of Ca^{2+} for neurocalcin (0.5 μM, trace 0a) and myr-neurocalcin (0.5 μM, trace 1), or in the presence of 2 mM Ca^{2+} for neurocalcin (0.5 μM, trace 0b) and for myr-neurocalcin with concentrations ranging from 0.05 to 2 μM. (B) Overlay plots of sensorgrams obtained either in absence of Ca^{2+} for C14-neurocalcin (0.5 μM, trace 1); or in the presence of 2 mM Ca^{2+} with protein concentrations ranging from 0.05 to 2 μM. (C) Overlay plots of sensorgrams obtained either in absence of Ca^{2+} for C16-neurocalcin (0.5 μM, trace 1) or in the presence of 2 mM Ca^{2+} with protein concentrations ranging from 0.05 to 1 μM. Protein injections are represented by the horizontal filled bar while open bars represent flow with running buffer. Protein concentrations are indicated on the corresponding sensorgrams (2, 0.05 μM; 3, 0.1 μM; 4, 0.2 μM; 5, 0.5 μM; 6, 1 μM; 7, 2 μM). A flow rate of 5 μL/min was applied for protein injection.

a K_D of 0.35−0.39 μM. This affinity is about 100-fold higher than the affinity of myr-recoverin for ROS membranes [K_D = 27 μM (*38*)] but lower than that of myr-Src for acidic phospholipids [K_D = 0.1 μM (*39*)]. More importantly, we showed that the binding of the C16-derivatized neurocalcin (K_D = 0.28 μM) is facilitated compared to the C14/myr-neurocalcins. However, the increment in the apparent affinity of C16-neurocalcin compared to C14/myr-derivative is less than one would have expected. Indeed, previous studies have shown that the binding energy of acylated peptides (*40*) and/or acylated proteins (*41*) to phospholipid vesicles increases linearly with the number of carbons in the acyl chain with a slope of 0.8 kcal mol^{-1}/CH_2 group. The two additional carbons in the C16 acyl chain should in theory increase the apparent association constant of C16-neurocalcin by at least 1 order of magnitude over that of the C14 one. The small increase of membrane affinity of the C16 acylated protein compared to the C14 one might indicate that the C16 acyl chain interact more strongly than the myristic chain with the hydrophobic pocket of neurocalcin. A more stable interaction of the C16 acyl chain with neurocalcin would indeed reduced the overall association of the acylated protein with membrane, as previously noted by Peitzsch and McLaughlin (*40*).

Monoacylated proteins appear as ideally suited to act as signaling switches in cellular regulatory processes. Because the acylation per se provide only weak membrane association energy, the attachment of acylated proteins to the membrane requires additional interactions between the polypeptides and the phospholipids. Small chemical and/or structural changes of the polypeptide can therefore dramatically affect the partitioning of the acylated protein between the cytosolic and the membrane compartment. In some cases, additional lipid modifications are found to assist membrane association. The most frequent modification is the addition of palmitoyl groups by means of a thioester linkage to cysteine residues. Some G-protein α-subunits and proteine tyrosine kinases are thus doubly myristoylated and palmitoylated (*42*). In other cases, electrostatic interactions between basic residues of the polypeptide and acidic lipids enhance the membrane binding of some myristoylated proteins such as the myristoylated alanine-rich kinase C substrate, Src, the ADP-ribosylation factor and human immunodeficiency virus-1 matrix proteins (*43*). It is likely that in the case of neurocalcin (or recoverin), calcium binding to the protein has two independent structural consequences, that nevertheless cooperate intimately for triggering membrane binding: (1) extrusion of the myristoyl group from the hydrophobic cleft that results from the Ca^{2+}-induced displacement of several residues that serve to clamp the fatty acid in the calcium free state, as best illustrated by the structural studies of recoverin; (2) acquisition of a conformation that favors the association of the protein with membrane either through electrostatic interactions or by exposing hydrophobic patches.

In conclusion, the methodological approach described here should provide new avenues to study the structural and functional roles of protein myristoylation. For example, several photoreceptor proteins have been found to be heterogeneously N-terminal fatty acylated (*7*). Acylation with 12:0, 14:1n-9 or 14:2n-6 fatty acids are supposed to weaken the membrane affinity as compared to 14:0 myristic acid. As a consequence, the lower membrane affinity may diminish the strength of interaction of these acylated proteins with their membrane bound targets as suggested by functional studies of heterogeneously acylated $G_{t\alpha}$ and recoverin. Chemical acylation with different fatty acids as described here might be applied to clarify these issues. Besides, it has been suggested that, in some instances, the myristic group of acylated NCS proteins could directly interact with the target enzyme(s). It could be possible now to incorporate myristic acid analogues containing photoactivatable groups in their acyl chain into NCS proteins in order to probe for such potential direct contacts between the fatty acid group and the target proteins.

Recently, Bader et al. have also described a chemical approach to modified Ras proteins with lipopeptides (*44*). They synthesized different lipidated peptides that were covalently coupled to the C-terminus of bacterially expressed truncated Ras proteins lacking their natural lipid modification. The in vitro lipidated proteins were shown to be able to bind to artificial membranes and to interact with the downstream effector Raf. An oncogenic Ras variant derivatized with the lipopeptides was able to induce cell transformation when microinjected into PC12 cells. The ability to modify at will by chemical approaches the lipids attached to proteins should help to understand the role of these critical modifications in protein structure, in protein compartimentalization, and in cell signaling.

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