Palmitoylated Peptides from the Cysteine-rich Domain of SNAP-23 Cause Membrane Fusion Depending on Peptide Length, Position of Cysteines, and Extent of Palmitoylation*

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Synaptosome-associated proteins SNAP-23/25, members of a family of proteins essential for exocytosis, have a highly conserved central cysteine-rich domain that plays an important role in membrane targeting. More than one cysteine in this domain is modified by palmitic acid through a thioester linkage. In an effort to address the biological significance of acylation of this domain, we have generated synthetic peptides corresponding to the cysteine-rich region of SNAP-23 and covalently modified the cysteines with palmitic acid. The interaction of acylated and nonacylated peptides with lipid vesicles and natural membranes has been investigated. Our results indicate that palmitoylation is essential for membrane association. The palmitoylated peptides were able to fuse both model and natural membranes. The extent of fusion depended on the length of the peptides and the number and positions of covalently linked palmitic acids. Peptide-mediated fusion was suppressed by lysolipid and involved both outer and inner leaflets of the lipid bilayer, which is characteristic of natural membrane fusion. Our results suggest an important role for the cysteine-rich palmitoylated domain of SNAP-23 in promoting membrane fusion in cells.

The mixing of specific membrane compartments by the process of fusion is a highly regulated event in eukaryotic cells. The fusion process is exquisitely controlled in a manner that does not disturb the structural and functional identity of organelles (1, 2). Several proteins that are involved at different steps in the transport process, from the formation of a transport vesicle until its fusion with the target membrane, have been identified (3–7). The SNARE¹ proteins play a crucial role in the process of intracellular membrane fusion. For fusion to occur, transport vesicles with distinct v-SNAREs have to pair with cognate t-SNAREs at the appropriate target membrane. Syntaxin and SNAP-23/25 are members of the t-SNARE family of proteins, whereas synaptobrevin/vesicle-associated membrane protein are v-SNARE proteins (3–7).

Syntaxin, SNAP-25, and vesicle-associated membrane proteins mediate fusion through the formation of helical bundles that span opposing membranes. Hydrophobic C-terminal domains anchor syntaxin and vesicle-associated membrane proteins to the lipid bilayer of the plasma and vesicle membranes, respectively. The crystal structure of the SNARE complex has identified the regions of SNAP-25 that are involved in binary and ternary interactions (8, 9). SNAP-25 and its non-neuronal homolog, syndet/SNAP-23 (10, 11) possess cysteine residues clustered in a relatively unstructured segment between two helical domains. The cysteine residues in this segment are palmitoylated (12-14). Mutants of syndet and SNAP-25 lacking cysteines, and therefore not palmitoylated, are localized predominantly in the cytoplasm of transfected cells (15, 16). Based on mutagenesis of SNAP-25, a minimal plasma membranetargeting domain composed of residues 85-120 has been identified. This segment has the ability to target green fluorescent protein to plasma membranes in transfected cells (17). There have been reports which suggest that cysteines and consequently palmitoylation, are not essential for targeting of SNAP-25 to membranes, as membrane association is facilitated by syntaxin 1A (18). However, recent reports suggest that membrane targeting of SNAP-25 is independent of interaction with syntaxin 1A and the cysteine-rich domain is necessary for function in intact cells (19).

To understand the role of the proposed membrane targeting segment of the SNAP family of proteins in membrane assembly of the parent protein and its possible role in the fusion process, we have studied the interaction of synthetic peptides spanning the cysteine-rich region of SNAP-23, with and without covalently linked palmitic acids, with model and natural membranes. A schematic sketch showing the various domains of SNAP-23, the primary structure of the segment with the cysteine cluster in SNAP proteins from different species, and the synthetic peptides investigated in the present study are presented in Fig. 1, A and B. We observed that palmitoylation is essential for the peptides to cause membrane fusion. Our results also provide insights into dependence of membrane fusion on peptide length, position of cysteines, and extent of palmitoylation.

EXPERIMENTAL PROCEDURES

Materials—Fmoc amino acids used in peptide syntheses were purchased from Advanced Chemtech (Louisville, KY). All phospholipids including those labeled with fluorescent tags were purchased from Avanti Polar Lipids (Birmingham, AL). Calcein was from Sigma. All other chemicals were of the highest grade commercially available.

Peptide Synthesis and On-resin Acylation—Peptides were synthesized manually using solid phase procedures and Fmoc chemistry. Cysteine sulfhydryl groups were blocked with acetamidomethyl group. The N termini of peptides were acetylated in a mixture of acetic anhy-

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¹ The abbreviations used are: SNARE, soluble NSF attachment protein receptor; NSF, N-ethylmaleimide-sensitive factor; SNAP-23/ SNAP-25, synaptosome-associated proteins of 23 and 25 kDa; PC, 1-palmitoyl-2-oleoylphosphatidylcholine; PE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; SM, sphingomyelin; CL, cholesterol; NBD-PE, N-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; Rho-PE, N-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; MES, 2-(N-morpholino)ethanesulfonic acid; LUV, large unilamellar vesicles; SUV, small unilamellar vesicles; Fmoc, N-(9-fluorenyl)methoxycarbonyl; LPC, lysophosphatidylcholine.

A



в

Sequences of synthetic peptides palmitoylated at cysteine

KF <u>C</u> GL <u>CVCPC</u> NRTKNFESGKAYKTTWG	27P4 (27C4)
KFSGLSV <u>C</u> P <u>C</u> NRTKNFESGKAYKTTWG	27P2 (27C2)
KF <u>C</u> GLSVTP <u>C</u> NRTKNFE <u>C</u> GKAYK <u>C</u> TWG	Sc27P4 (Sc27C4)
KF <u>C</u> GL <u>CVC</u> P <u>C</u> NRTGYEKKFSTATKNWG	Sp27P4 (Sp27C4)
WKF <u>C</u> GL <u>C</u> V <u>C</u> P <u>C</u> NRT	14P4 (14C4)
WKFSGLSV <u>C</u> P <u>C</u> NRT	14P2 (14C2)

FIG. 1. Schematic sketch of SNAP-23, sequences of the region containing the cysteine cluster from various mammalian species and peptides synthesized. *A*, sketch showing the highly conserved coiled-coil domains of SNAP-23 and the cysteine cluster representing the palmitoylation sites. Sequences of human SNAP-23 and the corresponding regions of SNAP proteins from various species within the palmitoylation domain and the adjacent residues are indicated. The conserved cysteine residues in the SNAP proteins are *underlined*. *B*, sequence of the peptides synthesized. Cysteine residues that have been palmitoylated are *underlined*. Corresponding nonpalmitoylated peptides are denoted as 27C4, 27C2, Sc27C4, Sp27C4, 14C4, and 14C2, respectively, and indicated in parentheses.

dride/triethylamine/dimethyl formamide (0.1:0:0.02:1, v/v). Palmitoylation was carried out as described before (20). Briefly, cysteine sulfhydryl groups of resin-bound peptides were deprotected using 5 eq of mercuric acetate (per cysteine) in dimethyl formamide for 1 h, followed by treatment with 50 eq of β -mercaptoethanol for another hour. The procedure was repeated three times to ensure complete removal of acetamidomethyl. On-resin palmitoylation of free -SH groups was carried out using palmitic acid activated with N-hydroxybenzoatriazole and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate. Peptides were cleaved from the resin purified by reverse phase high performance liquid chromatography/reverse phase fast performance liquid chromatography and characterized by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Expected mass was observed for the nonpalmitoylated and palmitoylated peptides. Stock solutions were made in trifluoroethanol or dimethyl sulfoxide. Peptide concentration was determined by monitoring absorbance at 280 nm.

Preparation of Lipid Vesicles—Lipid vesicles with compositions PC, PC:PE (1:1), PC:PE:SM:CL (1:1:1:1.5), and PC with 30 mol % cholesterol were prepared by the extrusion method or by sonication. Lipid stocks were diluted in chloroform to appropriate molar concentrations and dried to form a thin film. The lipid films were left under vacuum overnight to remove residual solvent and subsequently hydrated in 5 mM HEPES, pH 7.4, containing 150 mM NaCl. SUV were obtained by sonication of the lipid suspension. LUV were obtained by extrusion through polycarbonate filters of 100 nm pore size (21). For circular dichroism studies, peptide was added to lipid solution in chloroform and dried to form a mixed film. The film was hydrated and SUV were prepared by sonication.

Fusion Assay in the Presence of Peptides—Resonance energy transfer between NBD-PE (energy donor) and Rho-PE (energy acceptor) was used to monitor membrane fusion (22). Two populations of liposomes were prepared, one labeled with 2 mol % each of NBD-PE and Rho-PE



Peptide/ lipid

FIG. 2. Fusogenic activity of the palmitoylated peptides. A, fusion of SUV and LUV by 27P4 at a peptide/lipid ratio 0.01. Lipid composition of vesicles were: 1, PC:PE:SM:CL (1:1:1:1.5); 2, PC:PE (1:1), 3, PC; 4, PC:CL (30 mol % CL). B, percent fusion of SUV and LUV of lipid composition PC:PE:SM:CL (1:1:1:1.5) in the presence of various peptides with increasing peptide/lipid ratios. SV-208 is a fusion peptide derived from Sendai virus F1 protein. The sequence of the peptide is IKLTQHYFGLLTAFGSNGTIG.

and the other unlabeled. The labeled and unlabeled vesicles were mixed at a ratio of 1:9 to a final concentration of 200 μ M in 1.2 ml in 5 mM HEPES containing 150 mM NaCl. Peptides were added and after 5 min, fluorescence was recorded with excitation at 465 nm and emission set at 530 nm. The slit widths of the excitation and emission monochromators were 5 nm. Percent fusion was calculated using the equation (F $F_o/(F_t - F_o) \times 100$, where $F_o =$ fluorescence intensity before adding peptide, F = fluorescence intensity after adding peptide, and $F_t =$ fluorescence intensity after complete dilution by detergent (Triton X-100). Fusion assays were done in the presence of 10 mM dithionite or lysophosphatidylcholine (LPC) (23) as follows. The labeled vesicles were incubated on ice for 1 h in the presence of 10 mM dithionite. Excess dithionite was removed by passing through Sephadex G-50 spin columns. To check the effect of LPC, 10 $\mu g/ml$ LPC was added to the vesicles before recording the RET changes induced by the peptides. Three independent experiments were carried out.

Binding of Peptides to Lipid Vesicles—Peptides (5 μ M) were titrated with increasing amounts of lipid vesicles. The tryptophan fluorescence was monitored with the excitation monochromator set at 280 nm, and the emission spectrum was recorded from 320 to 400 nm with 5-nm slit widths. All fluorescence measurements reported in this study were done in a F-4500 Hitachi fluorescence spectrometer. The accessibility of tryptophan to the aqueous quencher iodide in the presence of lipid vesicles was determined as follows. KI was added in increasing amounts from a 4 M stock containing 1 mM Na₂S₂O₃. The data were analyzed according to the Stern-Volmer equation, $F_o/F = 1 + K_{\rm SV}[Q]$, where F_o and F represent the fluorescence intensities in the absence and presence of quencher [Q], respectively and $K_{\rm SV}$ is the Stern-Volmer quenching constant. Normalized accessibility factor was calculated from the ratios of $K_{\rm SV}$ at a lipid:peptide ratio of 50:1 and $K_{\rm SV}$ for peptide in the absence of lipid.

Circular Dichroism (CD) Spectroscopy—Spectra were recorded in a JASCO J-715 spectropolarimeter in water and also in SUV composed of PC:PE:SM:CL (1:1:1:1.5). Scanning was done in the wavelength range

A

27P



FIG. 3. Characteristics of peptide-mediated model membrane fusion. A, effect of the addition of LPC (10 μ g/ml), an indicator for the existence of a hemifusion intermediate on the fusogenic property of peptides. Percent fusion induced by the palmitoylated peptides in the presence and absence of LPC at peptide/lipid ratio 0.005 is shown. The liposomes were composed of PC:PE:SM:CL (1:1:1:1.5). B, extent of inner leaflet mixing upon bleaching of the outer leaflet NBD fluorescence by 10 mM dithionite is compared with the leaflet mixing in untreated liposomes with composition as indicated in A. The peptide/lipid ratio was 0.005. *Inset*, the initial NBD fluorescence intensity with and without bleaching by dithionite. 60% reduction in initial NBD fluorescence indicates complete bleaching of outer leaflet.

of 195 to 250 nm. Path length was 1 mm. Data are represented as mean residue ellipticity.

Content Leakage Assay—Lipid films formed after drying in vacuum were hydrated in 70 mM calcein solution made in HEPES buffer, pH 7.4. SUV were prepared by sonication. Untrapped calcein was separated by gel filtration on a Sephadex G-75 column in 5 mM HEPES containing 150 mM NaCl and 5 mM EDTA, pH 7.4. Release of calcein on addition of peptides was monitored with excitation at 490 nm and emission at 520 nm. Percent release was calculated from the equation $(F - F_o)/(F_t - F_o) \times 100$, where F_o and F are fluorescence intensities in the absence and presence of peptides and F_t is the fluorescence intensity after adding Triton X-100.

Preparation of Pancreatic Zymogen Granules and Plasma Membranes-Granules and membranes were isolated as described by Nadin et al. (24). The precautions mentioned by Edwardson (25) were taken. Granules from a single pancreas were suspended in 500 μ l of 5 mM MES containing 280 mM sucrose, pH 6.0, 1 mM phenylmethylsulfonyl fluoride, 1 µg of pepstatin, and 1 mM EDTA was also included. Protein was assayed by the Bradford method (26). Pancreatic membranes were prepared by method of Meldolesi et al. (27). EDTA and pepstatin were included in the buffers during processing. Membranes were resuspended in 5 mM MES containing 280 mM sucrose, pH 6.5, and stored in aliquots at -20 °C at a protein concentration of 1 mg/ml. KCl-treated membranes were prepared by incubating the membranes in 0.5 M KCl for 30 min at 4 °C (25). Membranes were recovered by centrifugation at $70,000 \times g$ for 30 min. The pellets were resuspended in 0.5 M KCl and the recovery step was repeated. The membrane pellet was washed in sucrose/MES buffer and resuspended at a protein concentration of 1 mg/ml in the same buffer.



FIG. 4. Peptide association with model membranes monitored by changes in tryptophan fluorescence. A, relative increase in the tryptophan fluorescence of peptides upon titration with SUV with lipid compositions PC (\oplus), PC:CL (ϕ), PC:PE (\blacktriangle), PC:PE:SM:CL (\blacksquare). Lipid ratios were as described in the legend to Fig. 2A. Nonacylated peptides did not show any increase in tryptophan fluorescence, on titration with lipid vesicles. B, accessibility of tryptophan to aqueous quencher (I⁻). The normalized accessibility factors (*naf*) were calculated as described under "Experimental Procedures" from $K_{\rm SV}$ of the peptides in the presence of liposomes composed of PC (\square), PC:CL (\blacksquare), PC:PE (\blacksquare), and PC:PE:SM:CL (\blacksquare).

Fusion between Zymogen Granules and Pancreatic Plasma Membranes-Membrane fusion was assayed by the measurement of relief of self-quench of fluorescent probe R18 (28). 1 µl of octadecylrhodamine (R18) from a 20 mM stock solution in ethanol was added to 300 μ l of granule suspension and incubated at 37 °C for 5 min. Granules were collected by centrifugation at 900 \times g for 10 min and resuspended in the original volume. In the fusion assay, 5 μ l of the granules (~10 μ g of protein) were resuspended in 600 µl of MES buffer, pH 6.5, containing 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 μ g/ml pepstatin, and 280 mM sucrose. Fluorescence was recorded with excitation at 560 nm and emission at 590 nm. After equilibration for 3 min, the granules were challenged with target membranes (~20 μg of protein). In the case of KCl-treated membranes, $\sim 30 \ \mu g$ of protein was used. Other components like peptides, Ca2+, and EGTA were added to the cuvette in accordance with the experimental requirement. Percent fusion was calculated considering the fusion induced by 1 mM Ca^{2+} as 100%

Preparation of Resealed Ghosts—Red blood cells were isolated from rat blood and resealed ghost membranes were prepared using the method described by Mobley *et al.* (29). Phospholipid concentration of the resealed ghosts was estimated by the Fiske-Subbarow method (30). The ghost membranes were resuspended in incubation buffer (10 mM sodium phosphate, pH 7.4, containing 120 mM KCl, 30 mM NaCl, and 1 mM EDTA). Fresh preparations were used in all the experiments.

Lipid Mixing between Red Cell Membranes—Lipid mixing was analyzed by measuring dequenching of R18 (29), by a procedure similar to the zymogen granule plasma membrane fusion assay. Resealed ghost membranes were incubated with R18 at a final concentration of 1 mol of R18/50 mol of red cell lipid for 30 min at room temperature. Unbound

Sc27P



FIG. 5. Secondary structure of peptides. Circular dichroism spectra of the peptides recorded in water (\blacklozenge) and lipid (\bigcirc) vesicles composed of PC:PE:SM:CL (1:1:1:1.5).

probe was removed by repelleting the ghosts at 22,000 × g for 20 min and washed in the incubation buffer. For the assay 50 μ M phospholipid containing labeled and unlabeled ghosts at a molar lipid ratio of 1:4 were taken in incubation buffer. Fluorescence was recorded for 3 min before the addition of peptides. Peptides were added and fluorescence changes were monitored as a function of time. Fluorescence intensity of intact R18-labeled red cell ghosts was 20–30% that of labeled ghosts treated with 0.1% Triton X-100. Percent fusion was calculated using the formula: 100 × (($F - F_o$)/($F_{max} - F_o$)), where F, F_o , and F_{max} are the fluorescence intensites before addition of peptide, after addition of peptide, and after complete dilution of the probe by addition of 1% Triton X-100.

RESULTS

Syntheses of Peptides-Peptides corresponding to 14 or 27 residues of the membrane-targeting domain of human SNAP-23 were synthesized by solid phase methods. Four of the five cysteines present in this domain are conserved in human SNAP-25. In 27P4, the nonconserved cysteine was replaced with phenylalanine, as observed in the sequence of human SNAP-25 (Fig. 1). To generate peptides with two palmitic acids, two cysteines were substituted with serines (27P2 and 14P2). In Sc27P4, the cysteines at positions 6 and 8 that are present in 27P4 were exchanged with the amino acids at positions 18 and 24, to spread out the cysteines and examine whether their clustering was important for activity. The amino acids after the cysteine cluster in 27P4 were also scrambled (Sp27P4) with a view to examine the role of this segment in membrane fusion. Palmitoylation was carried out with peptides attached to the resin after removal of the cysteine thiol protecting group. Cleavage from the resin yielded the palmitoylated peptides. In the 14-residue peptides, tryptophan was introduced at the N terminus to facilitate quantitation as well as to monitor interaction with lipids by fluorescence spectroscopy.

Fusion of Liposomes—The ability of peptides to fuse lipid vesicles of varying composition was examined by a fluorescence-dequenching assay (22) that monitors lipid mixing. In this assay, NBD-PE and Rho-PE are present in "donor" liposomes and fluorescence of NBD is quenched as a result of energy transfer. When donor liposomes fuse with unlabeled "acceptor" liposomes, the average distance between the fluorophores increases, which results in an increase of NBD fluorescence. The ability of 27P4 to fuse SUV and LUV of varying lipid composition is shown in Fig. 2A. Considerable fusion is observed with vesicles composed of PC:PE:SM:CL. LUV fused more effectively than SUV. Because maximum fusion was observed with PC:PE:SM:CL vesicles, the fusogenic ability of other peptides related to 27P4 was examined on SUV and LUV





FIG. 6. Membrane permeabilization measured by the release of entrapped calcein. *A*, percentage of calcein released by the palmitoylated peptides with an increasing peptide/lipid ratio from liposomes with the composition PC:PE:SM:CL (1:1:1:1.5). *B*, percentage of calcein release induced by peptide 27P4 at a peptide/lipid ratio 0.04 from liposomes. *1*, PC:CL; *2*, PC; *3*, PC:PE; *4*, PC:PE:SM:CL. The ratios of the various lipids are as described in the legend to Fig. 2A. Calcein release was measured 5 min after addition of peptide. Beyond this time no further release was observed.

with this composition. The data shown in Fig. 2*B* indicate that 27P4 causes maximum fusion. 27P2 and 14P4 show similar extents of fusion, which is less than 27P4 but comparable with Sc27P4. The shorter peptide with two palmitic acids causes fusion to a considerably lower extent. The peptide Sp27P4 is marginally less effective in causing fusion at high peptide concentrations as compared with 27P4. However, the extent of fusion caused by Sp27P4 is more than the other peptides. The four palmitic acids clustered at the N terminus appear to significantly influence the fusogenic properties of the peptides. A peptide corresponding to a segment of the Sendai virus F1 protein (IKLTQHYFGLLTAFGSNGTIG) that has been demonstrated to induce fusion of lipid vesicles (31) is less efficient than 27P4 and Sp27P4 in causing fusion.

Effect of Lysolipid on Membrane Fusion and Involvement of Inner and Outer Leaflets—Hemifusion is an intermediate state where the outer membrane leaflets mix to form a stalk structure (32–38). Biological membrane fusion is presumed to proceed through this state. Lysolipids stabilize positive curvature upon integration in the outer leaflet because of their inverted cone geometry (38). Because hemifusion is associated with curvature of the outer leaflet, lysolipids are inhibitory to fusion before membrane merger (37). We investigated whether peptide-mediated fusion involved a hemifusion intermediate, and the ability of the peptides to convert the hemifusion state to complete fusion wherein the inner leaflet lipids of the fusing membranes are also involved. The data presented in Fig. 3A indicate reduction of fusion in the presence of LPC for all the





Time (sec)

five peptides that cause fusion. This suggests hemifusion as a possible step in the fusion process. To examine whether peptide-mediated fusion involved both membrane leaflets, liposomes where the fluorescence of NBD present in outer leaflet was quenched by dithionite treatment, were used in the fusion assay (39). A considerable amount of resonance energy transfer was observed even after the quenching of outer leaflet fluorescence by dithionite (Fig. 3B). This shows that the peptides induced lipid mixing in the inner leaflet along with the outer leaflet. The efficiency of the inner leaflet mixing by these peptides appears to be a little lower than the outer leaflet mixing.

Environment of Trp in the Presence of Lipid Vesicles—Because the fluorescence of Trp is sensitive to environment (40), the fluorescence of Trp in synthetic peptides (described in Fig. 1) in the presence of lipid vesicles was monitored. All the peptides have a single Trp residue. It was observed that the nonpalmitoylated peptides when excited at 280 nm showed an emission maximum at 350 nm in buffer. Addition of lipid vesicles did not result in change in the fluorescence properties suggesting that the peptides do not bind to lipid vesicles. This observation is also consistent with the inability of these peptides to fuse lipid vesicles. Changes in fluorescence were observed when lipid vesicles were added to the palmitoylated peptides (Fig. 4A). A linear increase in F/F_o was observed for 14P2 with various lipids. For 27P2, Sp27P4, and Sc27P4, the F/F_o plateaued at a lipid/peptide ratio of 20, whereas in 14P4 and 27P4, the plateau was observed at a ratio of 10. The data indicate that the tetrapalmitoylated peptides with clustered cysteines bind strongly to lipid vesicles irrespective of the nature of the head group. The accessibility of Trp to iodide was then examined. The data in Fig. 4B indicate that while Trp is solvent exposed in the nonpalmitoylated peptides, it is shielded for the aqueous quencher in the palmitoylated 27-residue peptides as compared with the 14-residue peptides. Despite difference in the fusogenic properties, the Trp location seems to be similar in 27P2, 27P4 and Sc27P4 and Sp27P4.

Conformation of Peptides—The conformations of the peptides were examined in buffer and lipid vesicles (Fig. 5). The palmitoylated peptides were not ordered in buffer and in the presence of lipid vesicles showed minima at \sim 208 and 222 nm, which is characteristic of helical conformation (41). The nonpalmitoylated peptides did not show ordered structure in buffer and lipid vesicles. These differences could arise as a result of the palmitoylated peptides binding to lipid vesicles and lipidinduced conformational changes in 27P2 and 27P4.

Leakage of Vesicle Contents—Whether the fusion process is accompanied by leakage of vesicle contents was next examined.

The data presented in Fig. 6A indicate that a maximum release of 40% was observed with 27P4. No further release was observed beyond a peptide/lipid ratio of 0.03. Release of calcein as a function of lipid composition was examined for 27P4 (Fig. 6B). Unlike fusion, substantial release of calcein was also observed with PC vesicles. Calcein release was less pronounced in the presence of other palmitoylated peptides, which were less effective in causing fusion. However, concentrations of the peptides at which there was discernible calcein release were higher than the concentrations at which they induced leaflet mixing.

Fusion of Natural Membranes—The ability of the peptides to fuse membrane vesicles with plasma membranes in vitro was investigated using zymogen granules and pancreatic plasma membranes. Their fusogenicity has also been compared with that of Ca²⁺, which has been shown to stimulate fusion between zymogen granules and plasma membranes in vitro (25, 28). The data shown in Fig. 7A indicate that 27-residue peptides have the ability to fuse the granules to the plasma membrane. Ca^{2+} was more effective than the peptides in inducing fusion. The extent of fusion caused by 27P4 and Sp27P4 was similar. The shorter palmitoylated peptides as well as SV208 peptide did not cause fusion. When the extent of fusion was normalized with respect to Ca²⁺, 27P4 showed 50% fusion. Fusion by 27P4 was similar in the absence and presence of EGTA, indicating that peptide-mediated fusion was Ca²⁺-independent. The dependence of fusion on peptide concentration is shown in Fig. 7B. 27P4 and Sp27P4 showed similar extents of fusion at various peptide concentrations used in the experiment.

The relation between Ca²⁺ and peptide-mediated fusion was investigated (Fig. 8). Fusion mediated by Ca²⁺ was observed to be independent from peptide-mediated fusion. As shown in Fig. 8A, when fusion was initiated by peptide, addition of Ca²⁻ resulted in an increase the membrane mixing to an extent equal to that caused by Ca^{2+} alone. But when fusion was initiated by Ca²⁺, no further increase in fusion was observed with the addition of peptides. A possible explanation for this observation could be that the Ca²⁺-induced lipid mixing is the maximum attainable for this system under the conditions of the study and therefore addition of peptides could not result in a further increase in the fusion. From the results shown in Fig. 8B, it was evident that Ca^{2+} -mediated fusion was inhibited by EGTA, whereas addition of EGTA did not affect peptide-mediated fusion. Ca²⁺ was unable to increase lipid mixing in the presence of EGTA. Thus, peptide- and Ca²⁺-mediated fusion are independent events. To examine whether peripherally bound proteins are involved in the fusion process, the effect of KCl treatment on fusion was investigated. The data shown in Fig. 9 indicate that salt wash abrogates Ca²⁺-mediated fusion but not fusion caused by peptides. This further confirms that Ca^{2+} - and peptide-induced fusion are independent events.

The fusogenic ability of synthetic peptides on other membrane systems like red blood cell ghost membranes was investigated. The data shown in Fig. 10 indicate that the palmitoylated 27-residue peptides also cause fusion of red blood cell ghost membranes. In this system, the SV208 peptide also causes fusion, although to a lower extent.

DISCUSSION

The fusion events in eukaryotic cells are orchestrated events, which are finely tuned by interaction with various proteins of the SNARE family (1-7). SNAP-23/25, a crucial player in the fusion process (8, 9) is a fairly hydrophilic protein but membrane-associated. The protein does not have equivalents of "fusion peptide segments" that exist in viral fusion proteins (42). However, it has multiple cysteine residues, which are



FIG. 8. Effects of EGTA and Ca^{2+} on peptide-induced fusion between zymogen granules and plasma membrane. A, effect of peptides on Ca^{2+} -induced fusion and effect of Ca^{2+} on peptide-induced fusion for 27P4 and 27P2. B, effect of addition of Ca^{2+} on fusion initiated by 27P2 and 27P4 in the presence of 10 mM EGTA. In both A and B, the Ca^{2+} and peptide concentrations are 1 mM and 1 μ M, respectively. Control indicates the basal level of fusion between the zymogen granules and plasma membranes. The experimental conditions for recording R18 fluorescence were same as those mentioned in the legend of Fig. 7. A second dip in the fluorescence intensity seen here arises from the addition of peptides/ Ca^{2+} during the process of recording. Arrows indicate the time at which the various components were added.

proximal to each other in a central domain. Continuous stretches of cysteines are present in cysteine string proteins, which are known to be involved in exocytosis (43). Most of these cysteines are palmitoylated. In cysteine string proteins, the cysteines and hence palmitoylation could be essential for membrane targeting (43, 44). The role of cysteines and palmitoylation in the SNAP family proteins with respect to membrane attachment and fusion is yet to be clearly elucidated. We have investigated the membrane binding and the ability of peptides corresponding to the cysteine-containing SNAP segment to cause fusion of natural and model membranes.

Our studies indicate that palmitoylation is necessary for binding to lipid vesicles. In the shorter 14-residue peptide, four covalently linked palmitic acids are essential for fusion. The 27-residue peptide where the four cysteines are clustered (27P4) caused maximum fusion. When the amino acids after the cysteine cluster were scrambled, there was a slight decrease in the extent of fusion of model membranes but not natural membranes. The peptide Sc27P4 with relatively evenly spaced out cysteines was less effective in causing fusion and the efficiency was comparable with that of 27P2 where only two palmitic acids are present at the N terminus. Hence, it appears that four palmitic acids linked to a cluster of cysteines is an important determinant for perturbation of the lipid layer so as

A



FIG. 9. Requirement of peripheral proteins on the plasma membrane for peptide-induced and Ca^{2+} -induced fusion. *A*, fusion induced by the peptides, 27P2, 27P4, and Ca^{2+} prior to treatment of the plasma membrane with KCl. The concentrations of the peptides were 1 μ M and that of Ca^{2+} and EGTA were 1 mM. *B*, fusion after treatment of plasma membranes with 0.5 N KCl. The amount of Ca^{2+} , EGTA, and the peptides used was the same as in *A*. *Control* indicates the basal level of fusion between the zymogen granules and plasma membrane. Experimental conditions for R18 fluorescence measurements were the same as indicated in the legend to Fig. 7.

to promote vesicle fusion. All the acylated fusogenic peptides in this study fused vesicles that had the lipid composition PC:PE: SM:CL with a higher efficiency compared with lipid vesicles of other compositions. This observation could be relevant as there are reports that SNAREs might be clustered in sphingolipid cholesterol rafts in polarized apical sorting (45, 46). This lipid composition is also very close to natural synaptic vesicle membrane composition and the model membranes composed of these four lipids have been shown to exhibit optimized fusogenic capabilities (47).

The fusion process appears to proceed via a transient hemifusion state as evident from sensitivity to lysolipid. Fusion also involves both bilayer leaflets as indicated by bleaching of NBD in the outer leaflet by dithionite. However, unlike fusion caused by peptide mimics of SNARE (48) and vesicular stomatitis virus-G protein transmembrane segments (23), Ca^{2+} does not



FIG. 10. Fusion between red blood cell ghost membranes induced by peptides. A, peptides (2 μ M) were added to a suspension of R18-labeled and unlabeled ghost membranes. Time-dependent changes in R18 fluorescence are shown. 1, 27P4; 2, 27P2; 3, SV208; 4, 27C4, 5, 14P4; 6, 14C4, 7, control. Fusion (%) was calculated as described under "Experimental Procedures." B, peptide concentration-dependent fusion between red blood cell ghost membranes.

potentiate the fusogenic property of 27P4. Even in the case of fusion of zymogen granules to plasma membranes, the process appears to be independent of Ca^{2+} and also independent of peripheral membrane proteins.

Although proteins play crucial roles in the regulation of biomembrane fusion, the process requires coming together and coalescence of lipid bilayers. The role of lipids in membrane fusion has been addressed (49–51). Defects in membrane lipid packing are necessary for interaction between the hydrophilic regions of the bilayers. The 27- or 14-residue peptides are not intrinsically hydrophobic and are quite different from viral fusion peptides (42). They do not associate with lipid vesicles in the absence of palmitoylation. Even on palmitoylation, the peptide chain does not appear to span the lipid bilayer. Despite these differences, the fusion process seems to follow the steps proposed in the Stalk hypothesis (32–38), involving binding, formation of a hemifusion intermediate, and complete fusion.

A direct role for SNARE proteins in membrane fusion has emerged from studies where isolated SNARE proteins have been reconstituted in model membranes. Formation of SNARE complexes has been shown to be essential for this fusion (52– 54). Recently, the SNARE protein domains other than those involved in ternary complex formation have been implicated to be important for fusion (48). Palmitoylation of SNAP-25 in neurites and PC 12 cells is dynamically regulated and the degree of palmitoylation influences membrane binding (13). Our results indicate that palmitoylated and nonpalmitoylated peptides exhibit distinctive differences in membrane-binding properties and ability to promote fusion of membranes. It is therefore possible that the palmitoylation status of the cysteine cluster of SNAP proteins could play a crucial role in regulating membrane fusion in cells.

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