Stimulation of Cellular Sphingomyelin Import by the Chemokine Connective Tissue-activating Peptide III*

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The selective import of phospholipids into cells could be mediated by proteins secreted from the cells into the extracellular compartment. We observed that the supernatants obtained from suspensions of thrombin-activated platelets stimulated the exchange of pyrene (py)-labeled sphingomyelin between lipid vesicles in vitro. The proteins with sphingomyelin transfer activity were purified and identified as the chemokine connective tissue-activating peptide III (CTAP-III) and platelet basic protein. Isolated CTAP-III stimulated the exchange of py-sphingomyelin between lipid vesicles but did not affect the translocations of py-labeled phosphatidylcholine and phosphatidylethanolamine. CTAP-III rapidly increased the transfer of py-sphingomyelin from low density lipoproteins into peripheral blood lymphocytes, other immune cells, and fibroblasts. In the presence of heparin, CTAP-III was unable to insert sphingomyelin into the peripheral blood lymphocytes. The activation energy of the py-sphingomyelin transfer suggested that the translocation proceeded entirely in a hydrophobic environment. [3H]Sphingomyelin transferred to the cells by CTAP-III was hydrolyzed to [3H]ceramide and [3H]sphingosine after activation with tumor necrosis factor α. The generation of the [3H]sphingolipid messengers was catalyzed by acid sphingomyelinase. Our results identify CTAP-III as the first mediator of the selective (endocytosis-independent) cellular import of sphingomyelin allowing the paracrine modulation of the sphingolipid signaling.

The central role of phospholipids in intracellular signaling processes is well established. Degradation of membrane phospholipids yields water-soluble (e.g. eicosanoids, inositol trisphosphate) and lipophilic messenger molecules (such as diacylglycerol and ceramide), which initiate several signal transduction chains. Phospholipids are also essential for the biogenesis of caveolae, lateral domains of the cell membrane consisting of sphingolipids, cholesterol, and specific proteins (reviewed in Ref. 1). Specific phospholipids are also required for membrane fusion processes, in particular phosphatidylethanolamine (2). The intramembrane and intrablayer distribution of the phospholipids can be rapidly changed in response to a particular physiological situation. During apoptosis, phosphatidylserine is transferred to the outer leaflet of cell membranes, where it serves as a recognition signal for the clearance of apoptotic cells (3, 4). In view of their prominent roles in cellular functions, it is evident that the concentration and localization of the individual phospholipids within the membrane bilayer need to be carefully regulated.

This is accomplished by enzymes involved in the remodeling and synthesis of the phospholipids (acyltransferases, sphingomyelin synthase, etc.) and by proteins transferring phospholipids between the different intracellular membrane systems (interbilayer transfer). Furthermore, a group of proteins catalyzes the intrabilayer movements of phospholipids between the leaflets of the cellular membrane bilayer (5). Early work on human blood cells suggested that cells may also acquire phospholipids from extracellular sources (6). Among the phospholipid donors, low density lipoproteins (LDL)1 and other lipoprotein particles are considered to be of particular relevance. The lipoproteins are major phospholipid carriers within the plasma compartment. Their phospholipid contents are only slightly lower than their cholesterol concentrations. By means of the endocytosis of the LDL particles, among the multiple components of the lipoproteins, cells also take up phospholipids.

Recent data point to the existence of a selective, endocytosis-independent phospholipid uptake pathway. Through this import, the phospholipid composition of the cells can be rapidly changed. This was shown to be relevant for the assembly of protein complexes on the platelet surface necessary for the synthesis of thrombin (7). The uptake of phosphatidylinositol may modulate intracellular signal transduction processes (8). The data thus indicated that specific proteins mediate the selective phospholipid import into the cells. Proteins catalyzing the transfer of phospholipids between the plasma lipoproteins (9) are apparently not involved in the cellular phospholipid uptake.

We demonstrate in the present study that stimulated platelets release proteins that specifically exchange sphingomyelin between phospholipid vesicles in vitro. Among these proteins,

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1 The abbreviations used are: LDL, low density lipoproteins; LDLR, LDL (apoE/apoB) receptor; PBP, platelet basic protein; CTAP-III, connective tissue-activating peptide III; NAP-2, neutrophil-activating peptide 2; IL-8, interleukin-8; βTG, β-thromboglobulin antigen; PBL, peripheral blood lymphocytes; A-SMase, acid sphingomyelinase; N-SMase, neutral sphingomyelinase; py, pyrene; PACE, polyaacrylamide gel electrophoresis; TNFα, tumor necrosis factor α; TNF-R55, 55-kDa TNF receptor.
connective tissue-activating peptide III (CTAP-III), a cytokine belonging to the CXC-subfamily of chemokines, exerted the strongest stimulation of the cellular sphingomyelin uptake. CTAP-III, which is massively secreted by the activated platelets (10, 11), represents a N-terminally extended precursor of the chemokine neutrophil-activating peptide 2 (NAP-2). The latter protein is generated from CTAP-III through the cleavage of a N-terminal peptide, which is mediated by proteases associated with monocytes and neutrophil granulocytes (12, 13). CTAP-III, which is devoid of the stimulatory functions on neutrophils characteristic for NAP-2 (14), has been reported to act as a weak growth factor for fibroblasts (15). Thus, the physiological role of CTAP-III has not been clearly evident so far. The results of our study identify CTAP-III as a transcellular mediator of the cellular sphingomyelin import.

MATERIALS AND METHODS

Reagents and Antibodies—Platelet basic protein (PBP) and CTAP-III were isolated from released supernatants of thrombin-activated platelets by sequential immunoaffinity chromatography, cation exchange chromatography, and reversed phase chromatography (13, 16). NAP-2 was generated from CTAP-III by limited digestion with chymotrypsin and purified by reversed phase chromatography (17). Recombinant CTAP-III and IL-8 bearing a His tag at the N terminus were produced in Escherichia coli according to previously described methods (18), the His tag being removed from IL-8 to yield the 72-amino acid form of the chemokine. All chemokine preparations exceeded 98% purity according to overexpressed silver-stained SDS-PAGE and automated N-terminal sequence analysis. The peptide corresponding to the 15 N-terminal amino acids of CTAP-III was synthesized using solid phase methods and Fmoc-(9-fluorenyl)methoxycarbonyl chemistry and purified on a Poros R2/H column (PerSeptive Biosystems, Wiesbaden, Germany). The monoclonal antibody reactive to all variably truncated isoforms of β-TG Ag (monoclonal antibody C-24) was isolated using anti-CD15 antibodies conjugated to microbeads. Skin ceramide moiety, was kindly donated by Dr. Thierry Levade (University of Toulouse, France).

Platelet basic protein (PBP) and CTAP-III, which is devoid of the stimulatory functions on neutrophils (12, 13), represents an N-terminally extended precursor of the platelet basic protein (PBP) bearing a His tag at the N terminus (18). The proteins of the fractions from both columns were analyzed by SDS-PAGE.

In Vitro Phospholipid Exchange—For the preparation of phospholipid donor vesicles, 250 μg of egg phosphatidylcholine were dissolved together with 45 μg of phosphatidic acid, and 7.5 μg of either pyrophosphatidylcholine, py-phosphatidylcholine, or py-phosphatidylethanolamine in ethanol. The mixture was dispersed by very slow injection into 300 μl of Tris buffer. For the production of acceptor vesicles, 10 mg of egg phosphatidylcholine in ethanol was dispersed in 1 ml of Tris buffer. 50 μl of acceptor vesicle solution was mixed with 10 μl of donor vesicles and 40 μl of Tris buffer. To this suspension, 200 μl of either Tris buffer or column fractions was added. After 0 and 30 min, 100 μl of the suspensions was loaded onto a small anion exchange column (DEAE-Sepharose C6-LB), and the column was eluted with 1.5 ml of Tris buffer. The eluted acceptor vesicles were solubilized with 2% Triton X-100 at 37 °C, and their pyrene monomer fluorescence was measured.

Loading of Lipoproteins with Labeled Phospholipids—LDL and high density lipoprotein labeled were labeled with fluorescent and radioactive phospholipids by incubation of fresh human plasma with py-phospholipids, [14C]- and [3H]phospholipids (present in lipid vesicles), and subsequent isolation of lipoprotein fractions by ultracentrifugation as described (21). The specific activities ranged between 1.0 and 9.4 × 10^4 cpm/nmol of phospholipid. Py-phospholipids were present at 10–25 ng of pyrene/μg of LDL protein.

Incubation of Cells with Labeled Lipid Donors—In general, cells were suspended with lipoproteins or vesicles (labeled with [14C]-, [3H]- and [125I]-labeled lipotides) in the suspension buffer at 37 °C. In the case of py-phospholipid-containing donors, the fluorescence was monitored directly after the suspensions were added under on-line conditions. Incorporation of py-phospholipid into the cells was followed by the increase in monomer intensity after addition of the cells to the donors. In all experiments shown under “Results,” the increase in monomer intensity was accompanied by a decrease of the excimer to monomer ratio of py-fluorescence intensity. These changes in the py-fluorescence monomer and excimer intensities specifically indicate the selective (endocytosis-independent) phospholipid transfer and the monomer and excimer fluorescence of the suspensions were determined at emission wavelengths of 380 nm and 480 nm, respectively, with excitation at 340 nm (excitation and emission slits of 5 and 10 nm). Following incubation with radioactively labeled lipoproteins, the cells were separated from the donors by centrifugation and washed once, and the cell associated radioactivity was determined.

Lipid Separations—After incubation with the [14C]phospho-sphingomyelin-labeled lipoproteins, the cell suspensions were separated into aqueous and organic phases by the procedure of Bligh and Dyer (22). The upper phase was analyzed for its amount of [14C] reflecting the quantity of [14C]phosphocholine liberated from [14C]sphingomyelin. Concomitantly, the quantity of cell-associated [14C]sphingomyelin was estimated separating the phospholipids of the lower phase by one-dimensional thin layer chromatography using the solvent CHCl3/CH3OH/NH3/H2O (45/37/12/8, v/v). In the case of incubation with [3H]sphingomyelin-LDL, following the Bligh and Dyer separation procedure, the lower phase was subjected to one-dimensional thin layer chromatography using the solvent CHCl3/CH3OH/NH3/H2O (60/35/5/8, v/v) to separate the sphingophospholipids ceramide, sphingosine, and sphingomyelin. The area corresponding to standards of these sphingolipids was scraped off, and the radioactivity was determined in a scintillation counter.

Miscellaneous—Protein concentrations were measured according to the Bradford method by means of a kit using α-globulin as a standard (Bio-Rad, Munich). The concentrations of β-TG Ag and related proteins in platelet releasates were determined according to a previously described sandwich-enzyme-linked immunosorbent assay system (23). The mean values under “Results” are given ± S.D.

RESULTS

Platelet Basic Protein and Related Proteins Stimulate Sphingomyelin Transfer in Vitro—The extracellular media recovered
from suspensions of thrombin-activated platelets were shown to contain proteinaceous factors that enhance the transfer of ethanolamine phospholipids between lipid vesicles in vitro (7). In the present study, we analyzed whether the media would influence the exchange of the choline phospholipids phosphatidylcholine and sphingomyelin. Supernatants obtained from suspensions of thrombin-activated platelets were added to a mixture consisting of donor vesicles, containing py-labeled phospholipids, and acceptor vesicles. Extracellular media from suspensions of untreated platelets did not affect the transfer of the py-labeled choline phospholipids between the two types of vesicles (Table I). The supernatants recovered from thrombin-activated platelets stimulated the exchange of py-sphingomyelin by 3.4-fold compared with the one obtained from untreated platelets. The exchange of py-phosphatidylcholine was unaffected. The acceleration of py-sphingomyelin transfer was enhanced after dialysis of the supernatants from activated platelets, suggesting that low molecular weight components partially suppress the sphingomyelin transfer (Table I). Briefly boiling the supernatant and precipitation of its proteins with ammonium sulfate strongly reduced the exchange of py-sphingomyelin (not shown). Transfer of py-sphingomyelin from lipid vesicles to the PBL was also stimulated by the presence of the extracellular media gained from thrombin-activated platelet suspensions (Table I). Again, the transfer of py-phosphatidylcholine was not affected. The results suggested that platelet releasates contained proteins mediating the sphingomyelin exchange.

To isolate and identify these proteins, supernatants from thrombin-stimulated platelet suspensions were fractionated by gel filtration on a Sephadex column. The fractions eluted from the column were tested for their capacity to affect the py-sphingomyelin exchange in vitro. The sphingomyelin exchange activity was mostly present in a broad peak between fractions 28 and 31 (Fig. 1A). The fractions were pooled and the proteins separated by gel electrophoresis. Several bands were observed. A prominent band was noted in the 8-kDa range (Fig. 1A, right-hand side). To further purify the proteins catalyzing sphingomyelin exchange, the combined fractions 28–31 were applied onto an anion exchange column. The proteins were eluted from this latter column with a linear NaCl gradient. The highest sphingomyelin transfer activity was found in fraction 5.

![Figure 1](http://www.jbc.org/)

**FIG. 1.** Isolation of platelet releasate proteins mediating sphingomyelin exchange. A, separation of platelet-secreted proteins by Sephadex chromatography. The supernatants obtained from suspensions of thrombin-stimulated platelets were separated by passage through a Sephadex G-75SF column. The fractions eluted were added to a mixture of donor vesicles (containing py-sphingomyelin) and acceptor vesicles. The py contents of the acceptor vesicles were determined as described under "Materials and Methods." The values given refer to control suspensions without platelet releasates. The separation of the proteins of the pooled fractions 28–31 by SDS-PAGE (15% gel) is shown on the right. B, further purification of the proteins with sphingomyelin exchange activity. The pooled fractions 28–31 eluted from the Sephadex column were passaged over an anion exchange Resource Q column. A continuous NaCl gradient was used to elute the proteins. Subsequently, the py-sphingomyelin exchange activity was determined by means of the in vitro system of donor and acceptor vesicles. Fraction 5, showing the strongest stimulation of py-sphingomyelin transfer, was subjected to gel electrophoresis (right). The arrow indicates a single band with an apparent molecular mass of 8 kDa.

| Vesicle-vesicle transfer | Vesicle-cell transfer |
|--------------------------|-----------------------|
| **% of pyrene in donor vesicles** | **ng of pyrene/10^6 PBL** |
| Control | 2.1 ± 0.6 | 0.6 ± 0.2 |
| Supernatant from untreated platelets | 1.9 ± 0.3 | 0.5 ± 0.1 |
| Supernatant from thrombin-activated platelets | 6.5 ± 1.1 | 2.9 ± 0.6 |
| Supernatant from thrombin-activated platelets + dialysis | 8.6 ± 1.8 | 0.4 ± 0.2 |
| Supernatant from thrombin-activated platelets | 1.8 ± 0.7 | 1.8 ± 2.4 |

**TABLE I**

Releases of activated platelets stimulate sphingomyelin exchange

Platelets were activated with thrombin (0.5 unit/ml), and the platelet free supernatants were recovered and added to a mixture of donor vesicles (enriched with either py-sphingomyelin (py-SM) or py-phosphatidylcholine (py-PC)) and acceptor vesicles. After a 30-min incubation, the acceptor vesicles were isolated and their pyrene contents were determined. In other experiments, the transfer of the py-labeled choline phospholipids between lipid vesicles (3.8 μg of egg phosphatidylcholine and 0.09 μg of py-phospholipid) and PBLs was assessed within an 8-min incubation period in the presence of the supernatants obtained from platelet suspensions. Mean values are shown from four to six experiments.
centration dependence of the stimulation indicated a steep increase between 0 and 4.4 μM. At the latter concentration, at which an apparent saturation was reached, the transfer of py-sphingomyelin was enhanced by 6.5-fold. The exchange of py-labeled phosphatidylcholine and of py-phosphatidylethanolamine were unaffected (Fig. 2A). Addition of NAP-2 (4.4 μM) stimulated the py-sphingomyelin transfer by 2.9-fold (Fig. 2B). Equimolar concentrations of PBP were nearly equally effective as native and recombinant CTAP-III. A synthetic 15-amino acid peptide encompassing the residues that are cleaved off after 10 min, 2–3% of the PBL and skin fibroblasts (Fig. 3B). Accordingly, CTAP-III selectively stimulated the exchange of sphingomyelin.

Enhancement of Cellular Sphingomyelin Import by CTAP-III—We next evaluated the effect of CTAP-III on the selective cellular uptake of sphingomyelin. LDL particles, the lipoprotein fraction with the highest amount of sphingomyelin in human plasma, were employed as donors for the sphingolipid. Blood cells and fibroblasts, which could be targets for the proteins secreted by the activated platelets under in vivo conditions, were analyzed for their capacity to incorporate sphingomyelin in a CTAP-III-dependent way. The specific transfer of sphingomyelin was assessed by following the changes in the py-fluorescence characteristics under on-line conditions (see "Materials and Methods"). CTAP-III (3.3 μM) rapidly stimulated the translocation of LDL derived py-sphingomyelin into the PBL and skin fibroblasts (Fig. 3A). After 10 min, 2–3% of the sphingomyelin initially present in the donor vesicles had been transferred to the cells in the absence of the chemokine. With CTAP-III, the amount taken up by the cells was increased by 4- to 5-fold. The concentration dependence of the stimulation of sphingomyelin uptake by CTAP-III showed a sharp increase between 0 and 3.3 μM, an apparent saturation being observed at higher concentrations (Fig. 3B). At 3.3 μM, CTAP-III did not affect the transfer of py-phosphatidylcholine and py-phosphatidylethanolamine from the LDL particles into the PBL within a 10-min interval (data not shown).

The enhancement of the py-sphingomyelin incorporation by CTAP-III was comparable in LDL receptor-positive (LDLR+/+) and LDL receptor-negative (LDLR−/−) fibroblasts, as well as in Jurkat cells (Fig. 3C). In the presence of the anti-β-TG Ag antibody, the increase in the sphingomyelin import into the LDLR+/+ fibroblasts and Jurkat cells as induced by CTAP-III was diminished by 93% and 86%, respectively. The
The effect of the indicated proteins (all at 2–3 μM) on the transfer of py-sphingomyelin from LDL (1 μg of protein/ml) or lipid vesicles (3.8 μg of egg phosphatidylcholine and 0.08 μg of py-sphingomyelin) to PBL (10^6) was analyzed within an 8-min incubation period. The anti-β-TG antibody was added at 0.1 mg/ml. Means are shown from three to five experiments.

| Protein | LDL | CTAP-III | CTAP-III + anti-β-TG | NAP-2 | IL-8 | Lysozyme | Lipid vesicles | CTAP-III |
|---------|-----|----------|---------------------|-------|-----|----------|--------------|---------|
|         |     | py-sphingomyelin uptake | fold elevation against control |       |     |          |              |         |
| PBP     | 5.9 ± 1.3 | 6.4 ± 0.6 | 1.1 ± 0.4 | 4.4 ± 0.8 | 0.6 ± 0.4 | 1.0 ± 0.1 | 6.8 ± 1.3 |
| CTAP-III|       |           |            |       |     |          |              |         |
| NAP-2   |       |           |            |       |     |          |              |         |
| IL-8    |       |           |            |       |     |          |              |         |
| Lysozyme|       |           |            |       |     |          |              |         |
| Lipid vesicles |       |           |            |       |     |          |              |         |
| CTAP-III|       |           |            |       |     |          |              |         |

The temperature dependence for the selective uptake process mediated by CTAP-III was completely prevented by the anti-β-TG Ag antibody. NAP-2 enhanced the py-sphingomyelin uptake by 4-fold, whereas IL-8 was ineffective (Table II). IL-8 differed from the β-TG Ag proteins in the electrostatic properties of the C-terminal, positively charged, and neutral amino acids prevailing in the β-TG Ag proteins, whereas more residues with negative charges were present in IL-8. The basic, water-soluble lysozyme did not promote the uptake of py-sphingomyelin (Table II). This excludes the notion that the presence of the positive charges per se was sufficient for the phospholipid transfer function of CTAP-III (and related peptides). When lipid vesicles were employed as phospholipid donors instead of the lipoproteins, the transfer of py-sphingo myelin into the cells was accelerated by 7-fold (Table II). Thus, CTAP-III promoted the cellular uptake of py-sphingomyelin.

Basic amino acids, in particular lysine, may play a role for the interaction of CTAP-III with plasma membrane glycosaminoglycans, thereby physically approaching CTAP-III to the cells. The CTAP-III-mediated translocation of py-sphingomyelin from LDL to the PBL was reduced by 94% in the presence of heparin (Fig. 4A). Interactions of CTAP-III with cell surface glycosaminoglycans might thus be implicated in the phospholipid transfer activated by the chemokine. The temperature dependence for the selective uptake process mediated by CTAP-III was determined. PBL were equilibrated at four different temperatures between 4 °C and 37 °C. Arrhenius plots where generated from the temperature dependence of LDL derived py-sphingomyelin transfer in the presence of CTAP-III (Fig. 4B). The activation energies calculated after linearization of the curve amounted to 7.4 kcal/mol. This indicates that water was excluded from the CTAP-III promoted transfer of sphingomyelin (see "Discussion").

**CTAP-III Promotes Cellular Sphingomyelin Import**

**TABLE II**

**Cellular py-sphingomyelin uptake is differentially stimulated by chemokines**

| Exogenous Sphingomyelin | Jurkat cells | Fibroblasts |
|-------------------------|-------------|-------------|
| [3H]Phosphatidylcholine | 2.5 ± 0.6   | 2.6 ± 0.7   |
| [14C]Sphingomyelin      | 1.0 ± 0.3   | 1.3 ± 0.4   |
| [3H]Phosphatidylcholine | 1.1 ± 0.4/2.4 ± 0.3 |
| [14C]Sphingomyelin      |             |             |

In cells that had been incubated with LDL supplemented with [14C]phosphatidylcholine, the cell-associated radioactivity was unchanged by CTAP-III (Table III). In further experiments, the LDL particles were double labeled with [3H]phosphatidylcholine plus [14C]sphingomyelin, and the lipoproteins were incubated with the Jurkat cells. After the end of the incubation, the cellular amounts of 3H and 14C were determined. Although the quantity of cell-associated 3H was unaffected by the presence of CTAP-III, the cellular amount of 14C was augmented. Accordingly, the chemokine specifically increased the transfer of labeled sphingomyelin into the cells, the CTAP-III-stimulated import being independent of the endocytosis of the particles.

To analyze whether the sphingomyelin delivered to the cells...
under the control of CTAP-III was available for the production of sphingolipid messenger molecules, the formation of the sphingomyelin hydrolysis product phosphocholine was measured (Fig. 5A). Following incubation of Jurkat cells with [14C]sphingomyelin-LDL in the presence of CTAP-III and subsequent cell activation with TNFα, a 4.2-fold-elevated formation of [14C]phosphocholine was noted. The TNFα-dependent generation of [14C]phosphocholine was reduced by 95% by the anti-β-TG Ag antibody (Fig. 5A). Using [3H]sphingomyelin labeled in its ceramide component, the [H] label was recovered both in the ceramide and in the sphingosine fractions after preincubation with CTAP-III and subsequent cytokine activation. Generations of [3H]ceramide and [3H]sphingosine, increased 3.9- to 4.3-fold, were induced by the presence of CTAP-III plus TNFα (Fig. 5B). Preincubation with N-oleylethanolamine, which inhibits the deacylation of ceramide by ceramidases, tended to increase the generation of [3H]ceramide in cells treated with CTAP-III plus TNFα. The amount of [3H]sphingosine was lowered by 72% (Fig. 5B). This suggested that indeed ceramide degradation had been inhibited. Thus, the sphingomyelin delivered to the cells by CTAP-III was rapidly degraded to sphingolipid messengers after cytokine activation.

The TNFα-stimulated hydrolysis of cellular sphingomyelin is catalyzed by acid and neutral sphingomyelinases (A-SMase, N-SMase (25, 26)). CTAP-III (3.3 μM) augmented
the generation of [14C]phosphocholine by 2.1- and 5.9-fold (Fig. 6A). In contrast, in A-SMase-deficient fibroblasts, no increase of [14C]phosphocholine was elicited. In 70Z/3 pre-B cells transfected with mutants of TNF-R55, defective for activation of either A-SMase alone (A212–308/346), or both A- and N-SMase (A205) (20), CTAP-III increased the import of LDL-derived py-sphingomyelin (not shown). The CTAP-III-supplied [14C]sphingomyelin was rapidly hydrolyzed in 70Z/3 cells transfected with the wild type receptor, the generation of [14C]phosphocholine being augmented by 110% and 74% after 150 and 300 s of TNFα stimulation (Fig. 6B). No TNFα-dependent increase of [14C]phosphocholine was observed in the transfectants expressing receptors defective for the activation of A-SMase or of both A- and N-SMase (Fig. 6D). The data show that the sphingomyelin supplied to the cells by the action of CTAP-III was degraded by A-SMase.

In a reconstituted system we tested whether coactivation of the platelets and PBL enhanced the sphingolipid messenger generation by stimulation of the sphingomyelin transfer. Control experiments verified that the supernatants recovered from thrombin-activated platelets increased the transfer of py-sphingomyelin from the LDL particles (1 μg/ml) to the PBL (1 × 10⁶; by 4.7-±1.3-fold compared with the addition of extracellular media from untreated platelets (n = 3)). To exclude that the LDL-associated [3H]sphingomyelin was hydrolyzed by extracellular secretory SMases (27), supernatants recovered from suspensions of the activated platelets, as well as from the TNFα-stimulated PBL, were added to the suspensions of [3H]sphingomyelin-LDL. After the end of a 10-min incubation period, no [3H] was detected in the ceramides and sphingosine fractions of the lipid phases indicating that there was no extracellular hydrolysis of the [3H]sphingomyelin. The generation of the [3H]phospholipids was enhanced by 3.0-(ceramide) and 2.4-fold (sphingosine) due to the presence of the activated platelets (Fig. 6C). The formation of the degradation products was prevented when the anti-β-TG Ag antibody was added. Thus, platelet-secreted β-TG Ag proteins, predominantly CTAP-III, stimulated sphingomyelin import into the PBL, which was subsequently degraded in response to cytokine activation.

**DISCUSSION**

Sphingomyelin represents a key precursor for intracellular messenger molecules implicated in the signaling chains mediating the cellular response to different forms of stress, as well as inducing proliferation, cellular differentiation, and apoptosis (28, 29). Sphingomyelin is also an important constituent of the lateral domains of the plasma membrane known as caveolae (1) and lipid rafts (30). To fulfill these functions, the intrabilayer localization and concentration of sphingomyelin is subject to extensive regulation. The selective import of lipoprotein-derived phospholipids represents a means to rapidly modify the cellular phospholipid composition. We observed that the release of thrombin-activated platelets contained proteins mediating the selective sphingomyelin exchange between lipid vesicles. Following purification of the active proteins, they were identified as the chemokines CTAP-III and its precursor PBP, members of the β-TG Ag family. Under in vitro conditions, CTAP-III is quantitatively the major β-TG Ag protein secreted by the activated platelets. The uptake of py-sphingomyelin into PBL, fibroblasts, and Jurkat cells was strongly increased by physiologically relevant concentrations of CTAP-III. The exchange of py-labeled glycerophospholipids (phosphatidylcholine, phosphatidylethanolamine) remained unchanged by the presence of CTAP-III. Thus, CTAP-III specifically stimulated the sphingomyelin transfer. It represents the first water-soluble protein known to mediate the selective cellular import of sphingomyelin.

Well characterized phospholipid transfer proteins with a high affinity toward specific phospholipids are those promoting the intracellular exchange of phosphatidylcholine (31) and of phosphatidylinositol (32). Most other phospholipid-translocating proteins such as, for example, the plasma phospholipid transfer protein (33) catalyze the movements of a considerable variety of structurally different (phospholipids. The amino acid sequences of the known phospholipid transfer proteins do not exhibit substantial homologies (34). A data base analysis, including the phospholipid binding proteins mentioned above, did not yield any considerable homologies with the CTAP-III sequence (35). Previous data on the structure of the β-TG Ag proteins might allow some predictions regarding the putative phospholipid binding site within the CTAP-III molecules.

The N-terminally extended forms of the β-TG Ag proteins (CTAP-III, PBP) accelerated the sphingomyelin transfer more efficiently than did the proteolytically truncated derivative NAP-2. A synthetic peptide encompassing the N-terminal amino acids cleaved from CTAP-III to yield NAP-2, when present alone, did not affect cellular sphingomyelin uptake. Accordingly, this stretch of sequence in CTAP-III is necessary for the optimal stimulation of sphingomyelin exchange, but does not itself mediate the transfer. The N-terminal amino acids of CTAP-III were previously shown to be involved in stabilizing the association of CTAP-III into homo-oligomers (predominantly dimers) (36). In line with this view, dimer formation in NAP-2 is less favored than in CTAP-III (37, 38). Dimerization of CXC chemokines is accompanied by the formation of a shallow hydrophobic groove in these molecules (39, 40), which could accommodate the sphingomyelin molecule during the transfer reaction. The role of this hydrophobic compartment as potential sphingomyelin binding site will be tested in further studies.

LDL are the particles with the highest sphingomyelin content among the plasma lipoproteins and might, therefore, provide substantial proportions of the sphingomyelin delivered to the cells by means of CTAP-III in vivo. Our findings exclude that the enhancement of sphingomyelin import by CTAP-III is mediated by the endocytosis of the lipoproteins. In the case of stimulation of phospholipid uptake via endocytosis, one would expect that the transfer of all phospholipid fractions present in the lipoproteins should be enhanced by the chemokine. However, this was not the case. The chemokine increased the cellular uptake of py-sphingomyelin and acted as transfer protein for the sphingolipid under in vitro conditions but did not enhance the transfer of other py-phospholipids. Furthermore, after incubation of Jurkat cells with LDL particles double labeled with [3H]phosphatidylcholine plus [14C]sphingomyelin, CTAP-III selectively increased the amount of cell-associated 14C. The cellular uptake of LDL-derived sphingomyelin was also independent of the interaction of the lipoprotein with the classic LDL receptor, indicating that the effect of CTAP-III is unrelated to the LDL receptor pathway. The results reveal a previously unrecognized function of LDL: its role as a selective donor for sphingomyelin.

The sphingomyelin translocation across the aqueous medium as catalyzed by the water-soluble CTAP-III might occur either exclusively within a hydrophobic environment or at least partially proceed through the water phase. The temperature dependence of the sphingomyelin transfer promoted by CTAP-III yielded a value for the activation energy of 7 kcal/mol. This value is considerably lower than the activation energy for sphingomyelin diffusion across an aqueous medium, which is 21–25 kcal/mol (41). Within the transfer process, the desorp-
tion of the sphingomyelin molecule from the donor particle represents the step requiring most of the energy. The low activation energy measured suggests that the desorption of the sphingomyelin molecule is greatly facilitated by CTAP-III, with the translocation pathway proceeding mostly in a hydrophobic environment. In principle, the stimulation of the sphingomyelin uptake may be facilitated by the physical interactions of the β-TG Ag proteins with the plasma membrane of the cellular acceptors. No protein receptors for PBP and CTAP-III have so far been characterized. Platelet factor-4, a chemokine present-acceptors. No protein receptors for PBP and CTAP-III have so far been characterized. Platelet factor-4, a chemokine present-

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