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Cell death prevention, mitogen-activated protein kinase stimulation, and increased sulfatide concentrations in Schwann cells and oligodendrocytes by prosaposin and prosaptides

(saposin C/glial cells/myelin/apoptosis)

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ABSTRACT **Prosaposin, the precursor of saposins A, B, C, and D, was recently identified as a neurotrophic factor. Herein prosaposin was found to increase sulfatide concentrations in primary and transformed Schwann cells (iSC) and oligodendrocytes (differentiated CG4 cells). Of the four mature saposins, only saposin C was found to increase sulfatide concentrations in these cell types. A similar result was obtained by using peptides (prosaptides) encompassing the neurotrophic sequence located in the saposin C domain. Dose–response curves demonstrated maximal enhancement by saposin C and prosaptides at low nanomolar concentrations (5–10 nM). The increase in sulfatide concentration by a 14-mer prosaptide, TX14(A), in CG4 oligodendrocytes was about 3-fold greater than in primary Schwann cells. A mutant prosaptide with a single amino acid replacement of Asn → Asp was inactive. Prosaptides did not induce cell proliferation of primary Schwann cells, iSC cells, or CG4 oligodendrocytes but nanomolar concentrations of prosaptides prevented cell death of iSC cells and CG4 oligodendrocytes. Immunoblot analysis demonstrated that phosphorylation of both mitogen-activated protein kinase p-42 and p-44 isoforms were enhanced 3- to 5-fold after 5 min of treatment with prosaptides at concentrations of 1–5 nM. These findings suggest that prosaposin and prosaptides bind to a receptor that initiates signal transduction to promote myelin lipid synthesis and prolong cell survival in both Schwann cells and oligodendrocytes. Prosaposin may function as a myelinotrophic factor *in vivo* during development and repair of myelinated nerves explaining the deficiency of myelin observed in prosaposin-deficient mice and humans.**

Prosaposin is a 517-amino acid residue protein encoded by a single locus on human chromosome 10 (1). Proteolytic processing of prosaposin gives rise to four sphingolipid activator proteins named saposins A, B, C, and D that are localized within lysosomes and that activate the hydrolysis of sphingolipids by lysosomal hydrolases. In addition to its role as a lysosomal precursor protein, prosaposin is active as a neurotrophic factor eliciting neuronal differentiation and triggering a signal cascade after binding to a cell surface receptor (2–4). Recently mitogen-activated protein kinase (MAPK) activation has been shown for prosaposin in PC12 cells (4). The neurotrophic region of prosaposin has been localized to a 12-amino acid residue stretch in the amino-terminal portion of the saposin C domain (3). We have described (3) several synthetic peptides derived from this region that are as biologically active as prosaposin; we named these peptides "prosaptides."

Prosaposin and prosaptides are active on a variety of neuronal cells including hippocampal neurons (5, 6), spinal cord α-motor neurons (7), sensory neurons of the dorsal root ganglion (7), cerebellar granule cell neurons (3), and neuroblastoma cells (2–4, 8). In each of these cells, prosaposin or prosaptides stimulate neurite outgrowth in nanomolar concentrations and prevent cell death. In this report we demonstrate that prosaposin and prosaptides stimulate sulfatide synthesis and increase the sulfatide concentration in Schwann cells and oligodendrocytes, indicating that prosaposin and prosaptides are trophic factors for myelin formation.

MATERIALS AND METHODS

Materials. Prosaposin and saposin C were isolated as described (9, 10). Two prosaptides were synthesized from the active region of saposin C; a 22-mer called peptide 769P (CXFLVKEVTKLIDNNKTEKEIL where X equals D-alanine) and a 14-mer called peptide TX14(A) (TXLIDNNATEEILY; where X equals D-alanine). A third mutant peptide 769M (CXFLVKEVTKLIDDNKTEKEIL) was identical to 769P except for a D → N substitution at position 14. All peptides were synthesized commercially at 95% purity (AnaSpec, San Jose CA). The first two peptides were active as neurotrophic agents in neurite outgrowth assays and as stimulators of MAPK, whereas 769M was inactive (4).

Cell Culture. Primary Schwann cells were prepared from sciatic nerves isolated from 1-day-old Sprague–Dawley rat pups by the method of Assouline *et al.* (11). At the first passage, Schwann cells were further selected from fibroblasts using anti-fibronectin antibody and rabbit complement. This resulted in approximately 99% pure Schwann cell cultures as assessed by S100 and fibronectin immunofluorescence. The cultures were then routinely cultured in DMEM (glucose at 1 g/liter) containing 10% fetal calf serum, penicillin at 100 units/ml, streptomycin at 100 μg/ml, bovine pitutary extract at 21 μg/ml, and 4 μM forskolin.

iSC cells are a spontaneously transformed line from rat primary Schwann cells (courtesy of L. Bolin, DNA Research Institute, Palo Alto, CA) (12). They were maintained in DMEM (glucose at 1 g/liter), containing 10% horse serum, penicillin at 100 units/ml, and streptomycin at 100 μg/ml and grown at 37°C under humidified 7.5% CO_2 (12). CG4 cells (courtesy of R. Quarles, National Institute of Neurological Disorders and Stroke, Bethesda, MD), an O-2A progenitor cell line (13) were routinely grown in polyornithine-coated plates in 70% DMEM (glucose at 1 g/liter) containing 2 mM glutamine, penicillin at 100 units/ml, streptomycin at 100 μg/ml, N1 supplement, and 30% B104-conditioned medium. The cells were induced to differentiate into

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Abbreviation: MAPK, MAP kinase.

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oligodendrocytes by culturing in medium without B104-conditioned medium prior to sulfatide analysis (13).

Immunoassay for Sulfatide. Sulfatide concentrations were determined by TLC immunostaining with a anti-sulfatide monoclonal antibody (courtesy of Y. Hirabayashi, Riken Frontier, Tokyo). Primary Schwann cells were seeded in 100-mm Petri dishes and grown to confluency. The CG4 progenitor cells were seeded at 8×10^5 cells in 100-mm Petri dishes and cultured for 24 h in DMEM–N1–B104CM. The medium was then changed to serum-free DMEM/F-12 for primary Schwann cells or DMEM–N1 plus 0.5% fetal calf serum for CG4 cells with or without effectors for a further 48 h after which cells were harvested and sonicated in 100 μl of distilled water by sonication. To assess inhibition of sulfatide synthesis, an anti-769P antiserum (3) was added to medium at a concentration of 0.5 μl/ml. This antiserum has been reported to block the stimulation of neurite outgrowth in neuroblastoma cells treated with prosaposin, saposin C, or peptide 769P (3). An aliquot was taken for protein assay and 90 μl of the remaining homogenate was extracted with 5 ml of chloroform/methanol, 2:1 (vol/vol), to obtain the lipid fraction. The lipid fractions were applied to a TLC plate (Polygram SilG; Duren, Germany) and developed with chloroform/methanol/water, 60:35:8 (vol/vol). Immunostaining was performed at 37°C by placing the plate in 10 mM Tris·HCl (pH 7.5) containing 0.15 M NaCl in Tris-buffered saline (TBS) containing 1% ovalbumin and 1% polyvinylpyrrolidone (PVP)-40 and then incubating for 1 h with anti-sulfatide antibody in 1% PVP-40/TBS. The plate was incubated for 1 h with horseradish peroxidase-conjugated anti-mouse IgG in 1% PVP-40/TBS. After a 1-h incubation with antibody, the plate was washed for five 5-min periods with 0.05% Tween 20/TBS. Sulfatide was then quantified by visualizing with an ECL kit (Amersham) and determining the density of the spot with a densitometer (ImageQuant, Molecular Dynamics). A standard curve of pure sulfatide was used (2–50 ng) to quantify the amount of sulfatide relative to cell protein (BCA assay, Sigma). All assays were done in duplicate or triplicate unless otherwise indicated. The results are the mean ± SEM of three experiments.

^{35}S Incorporation Assay. iSC cells were seeded in six-well plates at 8×10^4 cells per well. After a 4-h incubation, the medium was replaced with serum-free low-sulfate DMEM/F-12 containing [^{35}S]sulfate (5 μCi/ml; 1 Ci = 37 GBq) with or without effectors. After 48 h, ^{35}S incorporation into sulfatide was determined as described by Cardwell (14). The results are the mean ± SEM of three experiments.

Cell Death Assays. iSC cells were plated in six-well plates at 2,000 cells per well and cultured in serum-free DMEM/F-12 medium with or without effectors up to 72 h. CG4 cells were induced to differentiate into oligodendrocytes for 2 days in DMEM–N1 medium; the medium was then changed to DMEM (no fetal calf serum), TX14(A) was added, and dead cells were assayed by the trypan blue exclusion assay 24 h later as described (3). The results are the mean ± SEM of three experiments.

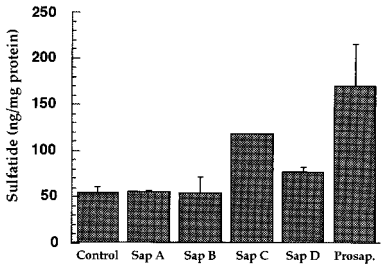

FIG. 1. Enhancement of sulfatide content in primary Schwann cells by prosaposin and saposin A–D. Primary Schwann cells were incubated in medium containing prosaposin (250 ng/ml) or individual saposins (50 ng/ml) for 48 h, and sulfatide concentrations were determined.

Cell Proliferation Assays. Primary Schwann cells and iSC cells were plated in 96-well plates at 8,000 cells per well and 4,000 cells per well, respectively. Effectors were added and serially diluted 1:2 to give a concentration curve. The cells were incubated for 48 and 72 h and cell proliferation was assessed by an MTT assay kit (Promega). The results given are the average ± SEM of duplicate experiments.

MAPK Phosphorylation Assay. Prior to the addition of effectors, cells were grown to approximately 85% confluency and then primary cells and iSC cells were placed in serum-free medium for 6 and 18 h, respectively. After stimulation with effectors MAPK phosphorylation was assayed by using a commercial kit (Upstate Biotechnology) as described (4). Autoradiographs were scanned with ImageQuant and the densitometric data were expressed as a ratio of phosphorylated to unphosphorylated MAPK. The results are representative of three or more experiments.

RESULTS

To determine whether prosaposin stimulates myelin lipid synthesis, we assessed the effects of prosaposin and saposins on sulfatide concentration in primary Schwann cells. Fig. 1 shows the change in sulfatide content of primary Schwann cells after treatment with prosaposin or saposins A–D. TLC immunostaining analysis revealed a 2- to 3-fold increase in sulfatide concentration after 2 days in cells treated with saposin C (4 nM) compared with controls. Saposins A, B, and D were inactive. We then tested whether prosaptides increased the sulfatide concentration in primary Schwann cells. As shown in Fig. 2, TX14(A) increased the sulfatide concentration in primary Schwann cells in a dose-

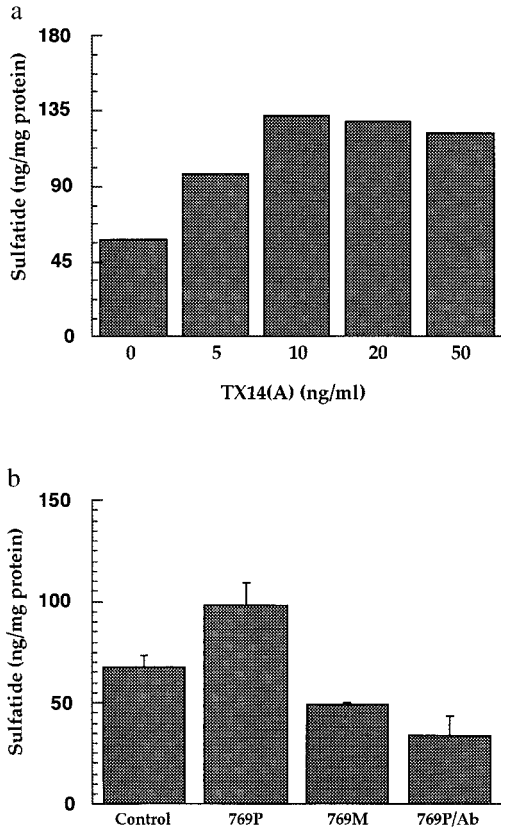

FIG. 2. Effect of prosaptides on sulfatide content in primary Schwann cells. (*a*) Effect of the 14-mer prosaptide, TX14(A). The cells were cultured in medium containing TX14(A) at concentrations from 0 to 50 ng/ml for 48 h and duplicate values were averaged. (*b*) Effects of 769P and 769M and an antiserum raised against 769P. Sulfatide concentrations were determined.

dependent manner with a maximum stimulation at 10 ng/ml (7 nM). Prosaptide 769P gave a similar increase; however, the mutant peptide 769M was inactive. In addition, an antibody against 769P blocked the increase by this peptide.

A similar assay was performed in the glial cell line CG4. As shown in Fig. 3, sulfatide concentration in CG4 cells that had differentiated into oligodendrocytes was enhanced about 6-fold compared with controls containing TX14(A) at 10 ng/ml. The dose–response curve appeared to be bimodal in both Schwann cells and CG4 oligodendrocytes.

The sulfatide concentration in untreated primary Schwann and CG4 oligodendrocytes was 50–60 ng/mg of protein and 300–400 ng/mg of protein, respectively. In iSC cells the sulfatide concentration was 3–5 ng/mg of protein, which was too low to assay by the immunostaining method. In iSC cells the incorporation of [^{35}S]sulfate into sulfatide was determined and a dose–response curve demonstrated that saposin C and TX14(A) enhanced incorporation 20–30% greater than controls (data not shown).

To further examine the effect of TX14(A) on Schwann cells and CG4 oligodendrocytes, we studied the effect of the peptide on cell proliferation and cell death prevention. Both forskolin and bovine pituitary extract, used as positive controls, stimulated primary Schwann cell growth, but TX14(A) did not induce proliferation at concentrations up to 50 nM even in the presence of 50 μM forskolin (Fig. 4). However, TX14(A) prevented cell death when cells were placed in low serum. This effect was evident at concentrations of 1 and 5 nM compared with controls ($P < 0.05$), both in iSC cells and CG4 oligodendrocytes (Fig. 5).

Since prosaptides were found to stimulate MAPK phosphorylation in PC12 cells (4), we investigated whether prosaptide TX14(A) stimulated MAPK phosphorylation in primary Schwann cells. As shown in Fig. 6, TX14(A) increased MAPK phosphorylation of both p42 and p44 isoforms by approximately 3-fold and 5-fold, respectively at 5 nM TX14(A). Similar phosphorylations of MAPK were observed when iSC cells were treated with TX14(A) (Fig. 6*c*). No enhancement of MAPK phosphorylation was observed when cells were treated with 769M (data not shown).

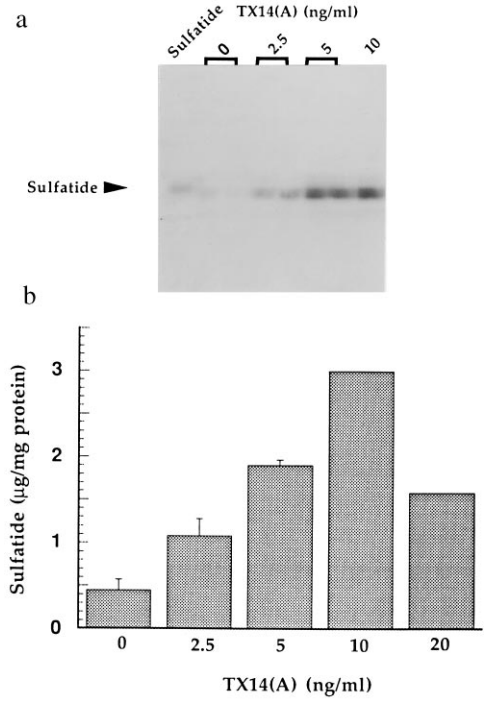

FIG. 3. Effect of TX14(A) on sulfatide content in CG4 oligodendrocyte cells. (*a*) The cells were cultured for 48 h in medium containing TX14(A) at concentrations from 0 to 20 ng/ml. Control lane contains 5 ng of sulfatide. (*b*) Graphical representation of results shown in *a*.

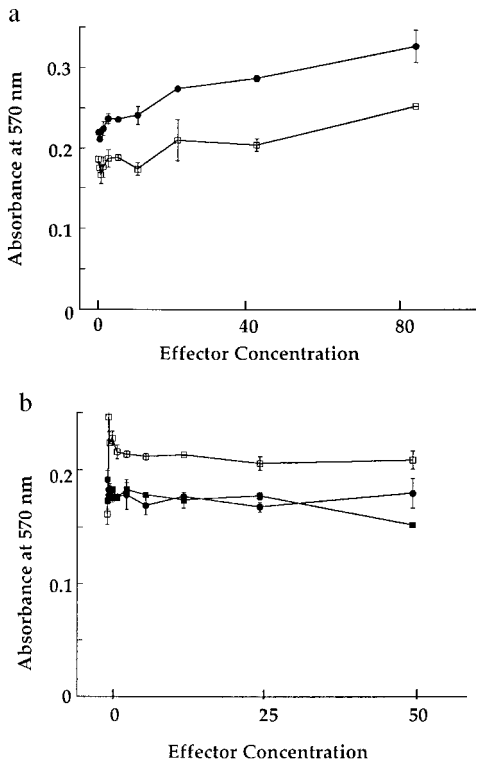

FIG. 4. Effect of TX14(A) on growth of primary Schwann cells. (*a*) Cells were incubated for 48 h in DMEM with 10% fetal calf serum and bovien pituitary extract (0–84 μg/ml) in the absence (open square) or presence (solid circle) of 50 μM forskolin. (*b*) Cells were incubated for 48 h in DMEM with 10% fetal calf serum and 0–50 μM forskolin (open squares) or 0–50 nM TX14(A) in the absence (solid circle) or presence (solid square) of 50 μM forskolin.

DISCUSSION

Thus far the cells that have been reported to respond to prosaposin or prosaptides have been neuronal in origin. However, reports of prosaposin deficiency have emphasized hypomyelination as a pathological hallmark. Hartzer *et al.* (15) reported the clinical description of a child who presented with severe clonic-tonic seizures beginning at birth, muscle fasciculations, and hypotonia and cortical hypomyelination suggestive on radiological examination. Myelin degeneration was also noted on a peripheral nerve biopsy. Subsequently, total prosaposin deficiency due to a transversion of the initiation codon of prosaposin cDNA in both alleles was found in studies of this patients cells (16).

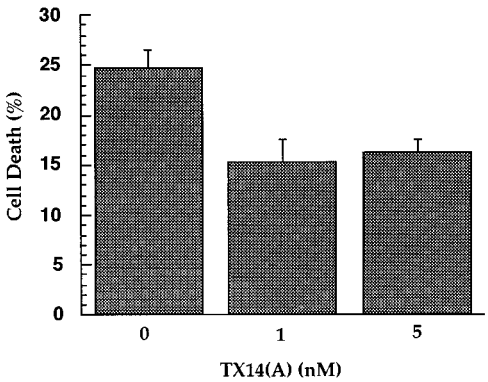

FIG. 5. Prevention of cell death in iSC Schwann cells by TX14(A). The 14-mer prosaptide was added at 1 or 5 nM. After 48 h of culture, trypan blue-positive cells were scored.

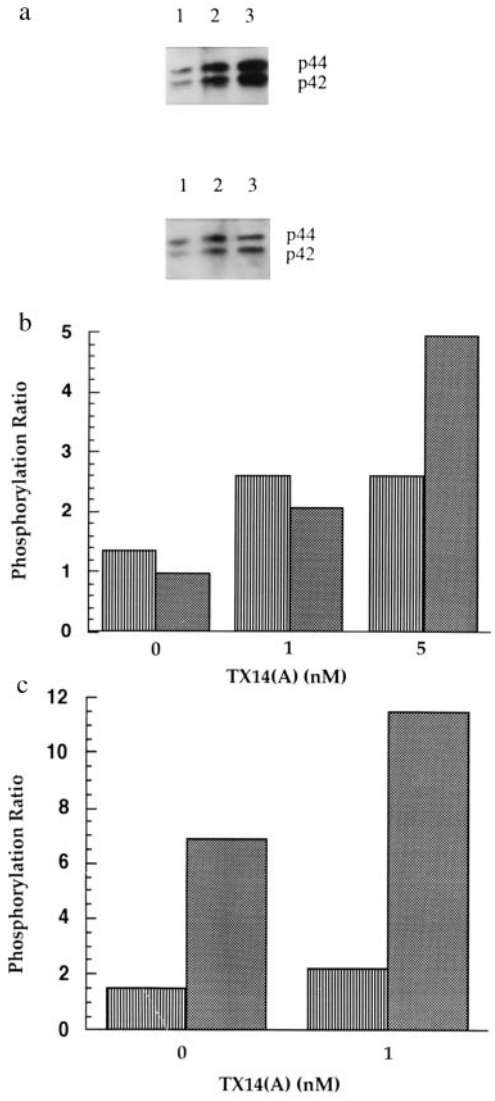

FIG. 6. Phosphorylation of MAPK in primary Schwann and iSC cells after stimulation with TX14(A). (*a Upper*) Western blot of MAPK phosphorylation in primary Schwann cell lysates with a polyclonal antibody that recognizes phosphorylated MAPK p42 and p44. (*Lower*) Western blot of MAPK phosphorylation in primary Schwann cell lysates with a polyclonal antibody that recognizes unphosphorylated MAPK p42 and p44. Lanes: 1, control; 2, TX14(A) at 1 nM; 3, TX14(A) at 5 nM. (*b*) Ratio of phosphorylated MAPK to unphosphorylated MAPK in primary Schwann cells by densitometric analysis. (*c*) Ratio of phosphorylated MAPK to unphosphorylated MAPK in iSC Schwann cells by densitometric analysis.

Both central and peripheral deficiency of myelin was documented in a prosaposin-deficient transgenic knockout mouse (17). The phenotype was that of severe central and peripheral hypomyelination noted after 1 month of age combined with neuronal and parenchymal lipid storage. On pathological inspection central myelin was severely deficient and hypomyelination of the sciatic nerve was evident.

Recently, it was demonstrated that prosaposin mRNA levels increase nearly 6-fold 7 days after sciatic nerve crush (18). The timing of this response is coincident with nerve regeneration including remyelination. It has been reported that application of prosaposin to the cut end of sciatic nerves prevents the dieback of myelinated axons normally observed after transection (6). These observations indicate that prosaposin may play an important role in development and injury repair of both central and peripheral myelinated nerves.

Our present demonstration that prosaposin, saposin C, and prosaptides stimulate MAPK phosphorylation and increase the sulfatide content in both Schwann cells and in oligodendrocytes suggests that hypomyelination in the prosaposin-deficient human and in the transgenic prosaposin knockout mouse is due to a deficiency of the trophic action of prosaposin on myelination. Although the increased content of sulfatide after treatment could also be due to decreased degradation, the demonstration of increase sulfate incorporation into sulfatide, the rate and magnitude of the sulfatide increase, and phosphorylation of MAPK indicate enhanced synthesis as the basis for the increase. Prosaposin and prosaptides appear to induce both differentiation of neuronal cells (2, 3, 8) and synthesis of gangliosides (19); the present study indicates that Schwann cells and oligodendrocytes respond similarly. Studies are underway in experimental animals to determine the efficacy of prosaptides as therapeutic agents for the treatment of demyelinating peripheral neuropathy and to ascertain their effects upon myelin synthesis in central demyelinating models.

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