ETS-1 transcription factor activates the expression of mouse UDP-Gal:GA2/GM2/GD2/GT2 galactosyltransferase gene

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Received 16 July 2004; revised 2 September 2004; accepted 21 September 2004

Available online 7 October 2004

Edited by Felix Wieland

Abstract UDP-Gal:GA2/GM2/GD2/GT2 galactosyltransferase (Gal-T2) transfers galactose to the terminal N-acetylgalactosamine of either the neutral glycolipid GA2 or of the gangliosides GM2, GD2 and GT2. Previous studies revealed a tight regulation of Gal-T2 activity and mRNA expression during development of the rat CNS. Here, we study in PC12 cells the *cis*-acting elements involved in the activation of a fragment of 211 bp around the transcription initiation site of the mouse Gal-T2 promoter. Mutagenesis, competition experiments and functional assays showed that the Ets-1 transcription factor is involved in the activation of the Gal-T2 promoter.

Keywords: Ganglioside; Glycolipid galactosyltransferase promoter; TATA-less promoter; Ets-1 transcription factor

1. Introduction

Gangliosides play important roles in large variety of biological processes, such as cell–cell and cell–extracellular matrix interactions, adhesion, cell differentiation, growth control and receptor-mediated signal transduction [1,2]. Their synthesis is catalyzed by glycosyltransferases that act in succession catalyzing the stepwise transfer of sugar to growing glycolipid oligosaccharides [3–5]. UDP-Gal:GA2/GM2/GD2/GT2 galactosyltransferase (Gal-T2) catalyzes the transfer of galactose residues from the sugar nucleotide donor UDP-Gal to the N-acetylgalactosamine residue of either the neutral glycolipid GA2 or of the gangliosides GM2, GD2 and GT2.

Mouse Gal-T2 is coded by one gene contained into a single exon located in chromosome 17 [6], 500 bp downstream the polyadenylation signal of the S18 ribosomal protein gene (RPS18 gene). The locus contains a number of genes conserved and synthenic in human, rat and mouse genome. Gal-T2 activity and mRNA levels are developmentally regulated in the rat CNS [6,7]. Gal-T2 mRNA is also highly expressed in adult extraneural tissues, particularly testis, which also showed elevated Gal-T2 activity [6]. Altogether, these results strongly suggest that Gal-T2 is under transcriptional control both in neural and extraneural tissues.

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So far, scarce information is available on the mechanisms involved in the transcriptional regulation of Gal-T2. Recently, a 1142 bp 5′ flanking fragment of the mouse Gal-T2 gene with promoter activity located at −550/+9 bp was characterized in mouse neuroblastoma Neuro-2a cells; a combination of DNA pull-down assay and transcription factor array analyses evidenced consensus sequences for the binding of 27 different transcription factors [8]. Here, we study in PC12 cells the *cis*-acting elements involved in the activation of a fragment of 211 bp of the mouse Gal-T2 promoter. PC12 cells were used since they express terminal galactose containing complex gangliosides GM1, GD1a, GD1b and GT1b [9], and hence the transcription factors required for Gal-T2 promoter activity. Results revealed binding sites for Ets-1 transcription factors surrounding the transcription start site. Mutagenesis, competition and overexpression experiments, and functional assays showed that an Ets-1 binding site at −32/−23 bp is critical for the activity of Gal-T2 promoter.

2. Materials and methods

2.1. Plasmid constructs

Fragments −59/+152, −308/+38, −308/−9 and −465/+38 (nucleotide positions are indicated with respect to the transcriptional start site [8]) were amplified by PCR from a 2 kb PCR fragment previously amplified from DNA BAC425018 clone (AF110520) containing the entire mouse Gal-T2 gene and cloned into the *Kpn*I/*Xho*I sites of the pGL3 basic reporter plasmid (Promega). The primers used in PCRs are listed in Table 1. Constructs identities were certified by sequencing. The mouse Ets-1 expressing plasmid pEVRFO Ets-1 and empty pEVRFO were kindly provided by P.K. Brindle, St. Jude Children Research Hospital, Memphis, TN, USA.

2.2. Cell culture, transfection and luciferase assays

PC12 rat pheocromocitoma cells were grown as Gil et al. [10] and transfected using LipofectAmine (Gibco-BRL) with 500 ng of each promoter construct and 80 ng of phRL-SV40 *renilla* luciferase as a control for transfection efficiency. Two days later, they were harvested with Passive Lysis Buffer (Promega). The relative *firefly* luciferase activity was normalized to the *renilla* luciferase activity (Dual-Luciferase reporter assay system, Promega). For each condition, at least three independent experiments were performed in triplicate.

2.3. Electrophoretic mobility shift assay

Total cell extracts were obtained from PC12 cells according to the methods of Kumar and Chambon [11]. Twenty μg of total PC12 cell extract was incubated for 20 min on ice with 20 μl of reaction buffer containing 20 mM Tris, pH 7.5, 2 mM DTT, 0.5 mM PMSF,

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doi:10.1016/j.febslet.2004.09.066

Table 1
Primer sequences used for PCR cloning

Designation	Sequence
+152 Gal-T2	5′ TCTCTCGAGACCCATGCGCGGGGCTCTGG 3′
−9 Gal-T2	5′ TCTCTCGAGCATGCCTCTCTAGGCCGGAA 3′
+38 Gal-T2	5′ TCTCTCGAGGCACGCGGAACTCCGCTCCATCCC 3′
−59 Gal-T2	5′ TCTGGTACCGACCTATGTGAAATGCACGCC 3′
−308 Gal-T2	5′ TCTGGTACCTGCCTTAGGTGCAGCAAACTGGGCGG 3′
−465 Gal-T2	5′ TCTGGTACCAGTACTTAATGCCACAGCGATCC 3′
−109 Gal-T2	5′ TCTGGTACCCAGCCTGGTATTCCGCG 3′
−59 mEts	5′ TCTGGTACCGACCTATGTGAAATGCACGCCGCTTGTCGGCAGATCTCCTAGAGAGGC 3′

Underlined are restriction sites introduced at the 5′ ends for further cloning.

Table 2
Oligonucleotides used for EMSA experiments

Designation	Sequence
−44/−12	5′ CACGCCGCTTGTCGGCTTCCGGCCTAGAGAGGC 3′
44/−12 mEts-1	5′ CACGCCGCTTGTCGGCAGATCTCCTAGAGAGGC 3′
−8/+25	5′ ATGGGAAGGTGCGGGGAGGGACGGGATGGAGCG 3′
−8/+25 mSp1	5′ ATGGGAAGGTGCGAAGCTTGACGGGATGGAGCG 3′
GABP	5′ GGGCTGCTTGAGGAAGTATAAGAAT 3′
PEA3/Ets-1	5′ GATCTCGAGCAGCAACTTCGA 3′
Sp1	5′ TTATTCGATCGGGGCGGGGCGAGC 3′
c-Myc	5′ GGAAGCAGACCACGTGGTCTGCTTCC 3′
USF1	5′ CACCCGGTCACGTGGCCTACACC 3′
SRE	5′ GGATGTCCATATTAGGACATCT 3′
NF-1	5′ TTTTGGATTGAAGCCAATATGATAA 3′
TFIID	5′ GCAGAGCATATAAGGTGAGGTAGGA 3′
Oct-1	5′ TGTCGAATG AAATCACTAGAA 3′
NSI	5′ GACCTATGTGAAATGCACGCCGCTTGTCGGCTT 3′
NSII	5′ CCGGCCTAGAGAGGCATGATGGGAAGGTGCGGG 3′

Primers were annealed and 5′ end labeled by incubation with γ^{32}P-ATP and T4 polynucleotide kinase.

1 mM orthovanadate, 5 μg/ml leupeptine, 5 μg/ml aprotinin, 20% glycerol, and 1 μg salmon sperm DNA. The radiolabeled probes (Table 2) were added (14000 cpm/pmol) and the incubation system was continued for another 20 min. In competition experiments, the nuclear extracts were pre-incubated during 20 min with the indicated molar excess of unlabeled probe. DNA-protein complexes were analyzed on non-denaturing 5% polyacrylamide gels using 0.5× TBE as running buffer. Oligonucleotides used as electrophoretic mobility shift assay (EMSA) probes are listed in Table 2.

2.4. Site-directed mutagenesis

For mutation of Sp1 site, two sets of primary PCRs were run using +152/−59 pGL3 plasmid as DNA template and the following pairs of primers: −8/+25 mSp1 forward/+152 Gal-T2 and −8/+25 mSp1 reverse/−59 Gal-T2 (Tables 1 and 2). The secondary PCR was carried out using the combined gel-purified primary PCR products as template and +152 Gal-T2 and −59 GalT-2 as set of primers. For mutation of Ets-1 site, −59/+152 pGL3 was used as DNA template and −59 mEts-1 and +152 Gal-T2 as primers. PCR products were gel-purified, digested with *Xho*I and *Kpn*I and back cloned into the pGL3 basic vector to generate the −59/+152 mSP1pGL3 and −59/+152 mEts-1pGL3 plasmids. Mutations were confirmed by sequence analysis.

3. Results

3.1. Deletion analysis and activity of the Gal-T2 promoter region

Different genomic DNA fragments of the 5′ upstream region from the transcription start site [8] of the mouse Gal-T2 gene were cloned in pGL3 basic reporter vector and transfected to PC12 cells. A −465/+38 fragment showed about 1500-fold higher promoter activity than the empty pGL3 (Fig. 1). The fragment −308/+38 showed a slightly reduced promoter ac-

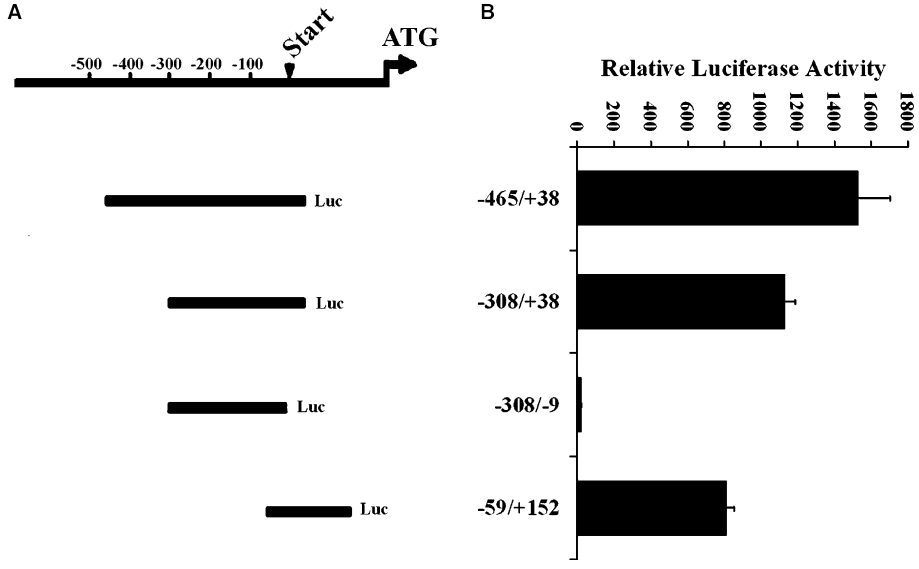

Fig. 1. Deletion analysis and activity of Gal-T2 promoter. (A) PC12 cells were transfected with the indicated DNA fragments from the mouse Gal-T2 promoter cloned in pGL3 basic vector. (B) Promoter activity of the DNA fragments, as determined by *firefly* luciferase activity normalized to *renilla* activity.

tivity, suggesting the presence of positive regulator sequence(s) in the region −308/−465. Deletion of 46 bp from the 3′ end of the −308/+38 fragment (clone −308/−9) markedly reduced the promoter activity, suggesting that *cis*-acting elements located in the region −8/+38 are necessary for the basal promoter activity. On the other hand, deletion of 400 bp of the 5′ region but extending the 3′ region to +152 (−59/+152) resulted in a fragment with about 800-fold activation capacity.

A computerized search (http://www.genomatix.de) for putative *cis*-acting elements in the −59/+152 region revealed binding sites for Brn-2, Ets-1, Staf, Mzf1, Sp1/GC box, KLF6, and E-box. As in other glycosyltransferases [4], no TATA box, CAAT or INR sequences were present in this fragment.

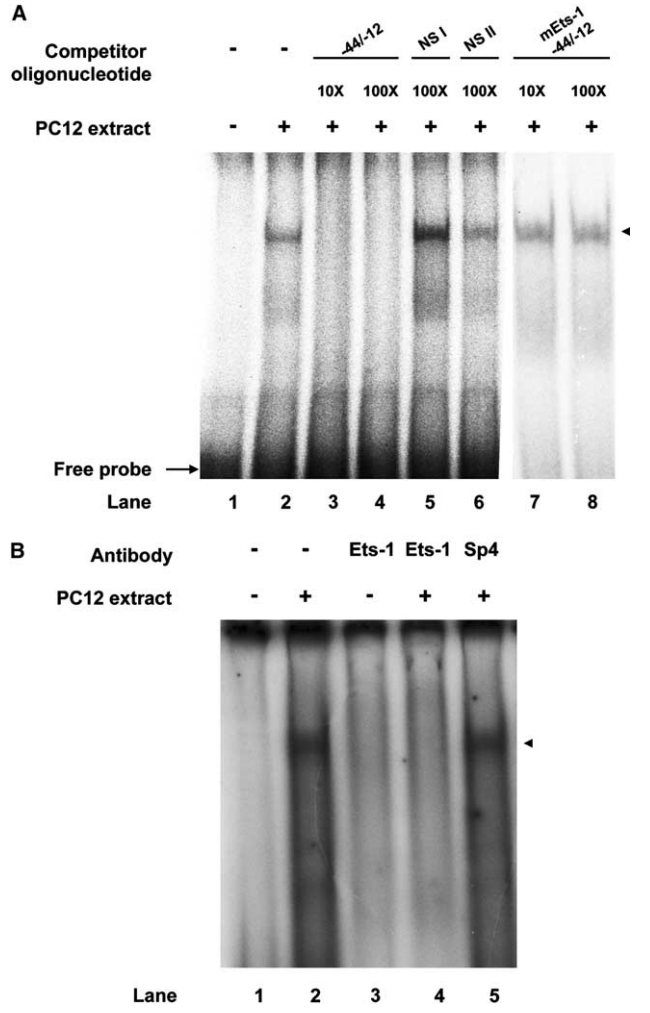

Fig. 2. EMSA analyses of protein complexes interacting with the −44/−12 region. (A) The 5′ end ^{32}P labeled −44/−12 oligonucleotide was incubated without (lane 1) or with 20 g of PC12 extract in the absence (lane 2) or in the presence of the unlabeled competitor at 10 (lane 3) or 100 (lane 4) molar excess. Competition with two different not related unlabeled oligonucleotides at 100 molar excess (lanes 5 and 6), or with an oligonucleotide containing a mutation in the Ets-1 motif at 10 (lane 7) and 100 (lane 8) molar excess is also shown. Arrowhead marks the position of the complex. (B) The 5′ end ^{32}P labeled −44/−12 oligonucleotide was incubated without (lane 1) or with 20 μg of PC12 extract (lane 2), or incubated with anti-Ets-1 antibody in the absence (lane 3) or presence of 20 μg of PC12 extract (lane 4). As a control of specificity, the probe was incubated with anti-Sp4 antibody and 20 μg of PC12 extract (lane 5).

3.2. Electrophoretic mobility shift analysis reveals protein binding to Ets-1 like sites

EMSA experiments with a −44/−12 oligonucleotide as probe containing a copy of the Ets-1 site at −32/−23 (Fig. 2A) revealed one major complex (lane 2), which was specifically competed with a 10- and 100-fold excess of an unlabeled competitor oligonucleotide with identical sequence (lanes 3 and 4) but not with 100-fold molar excess of unrelated oligonucleotides (lanes 5 and 6). The relevance of the Ets motif present at −32/−23 was confirmed by mutation of the stringently conserved CCGGAA consensus sequence to AGATCT (mEts-1). The mutated oligonucleotide was no longer an effective competitor (lanes 7 and 8), indicating that the Ets-1 sequence was critical for interaction with the protein in the extract. Fig. 2B shows that complex formation was reduced in the presence of anti-Ets-1 antibody. Reduction was specific for this antibody, since an unrelated antibody (anti-Sp4) was without effect. No supershifting effect was observed in these experiments, suggesting failure of binding of the probe to the antibody-protein complex or of antibody to the protein-oligonucleotide complex. Oligonucleotides containing binding sites for TFIID, Oct-1, USF1, Sp1, NF-1, c-Myc and SRE (Fig. 3, lanes 3, 4, 6, 8, 9, 10 and 11) failed to compete. On the other hand, GABP and PEA3/Ets-1 effectively competed with the −44/−12 oligonucleotide (Fig. 3, lanes 5 and 7, respectively). These results indicate that the Ets-1 binding site is

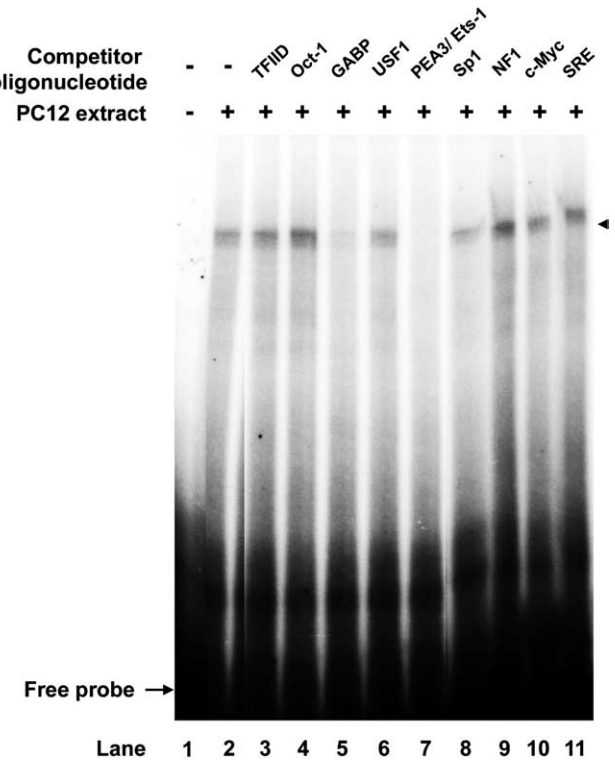

Fig. 3. Competition analyses of protein complex formation with oligonucleotides containing binding sites for various transcription factors. The oligonucleotide probe containing −44/−12 Gal-T2 sequences was incubated without (lane 1) or with 20 μg of PC12 extract (lanes 2–11). In lanes 3–11, the PC12 cell extract was preincubated with unlabeled oligonucleotides that contain the binding site for TFIID (lane 3), Oct-1 (lane 4), GABP (lane 5), USF1 (lane 6), PEA3/Ets-1 (lane 7), Sp1 (lane 8), NF-1 (lane 9), c-Myc (lane 10), and SRE (lane 11) transcription factors.

involved in the formation of the complex with −44/−12 oligonucleotide.

3.3. Gal-T2 promoter activity increases upon binding of proteins to the Ets-1 like sequence

To test the functional importance of the Ets binding sites, the −59/+152 fragment was mutated into the Ets-1 site and assayed for their promoter activity. A site-specific mutation in the Ets-1 site reduced the promoter activity by approximately 90% (Fig. 4), indicating that this site is implicated in the transcriptional control of mouse Gal-T2 gene expression and contributes to its basal activity. The relevance of the Ets-1 site was confirmed by overexpressing the Ets1 transcription factor, which resulted in a 2.5-fold increase of promoter activity (Fig. 4).

3.4. Overexpression of Ets-1 in CHO-K1 cells results in increased expression of complex gangliosides

CHO-K1 cells lack expression of complex gangliosides due to a lack of GalNAc-T activity. However, stable transfection of GalNAc-T renders these cells capable of converting a fraction of GM2 into GD1a, indicating that they express Gal-T2 and excess of Sial-T4 activities. Transient transfection of Ets-1 to these cells resulted, after metabolic labeling during 12 h with ^{14}C-galactose (2 μCi), in ≈11% higher conversion of GM2 to GD1a, indicating that in transfected cells more glycosylation intermediates were moved downstream the pathway GM3 → GM2 → GM1 → GD1a (not shown). The simplest interpretation of this result is that overexpression of Ets-1 resulted in increased Gal-T2 activity and hence increased conversion of intermediates to GD1a.

4. Discussion

A 211 bp region (−59/+152) of the proximal promoter of the mouse Gal-T2 gene characterizes for the absence of typical mammalian TATA box, CCAAT box or INR (initiator) sequences close to the transcription initiation site and for the presence of putative sites for interaction with various *trans*-acting factors such as Ets-1, Sp1 and Brn-2. A data bank search revealed conservation of Ets-1, Sp1, and KLF6 binding sites in putative Gal-T2 promoters of human, rat and mouse.

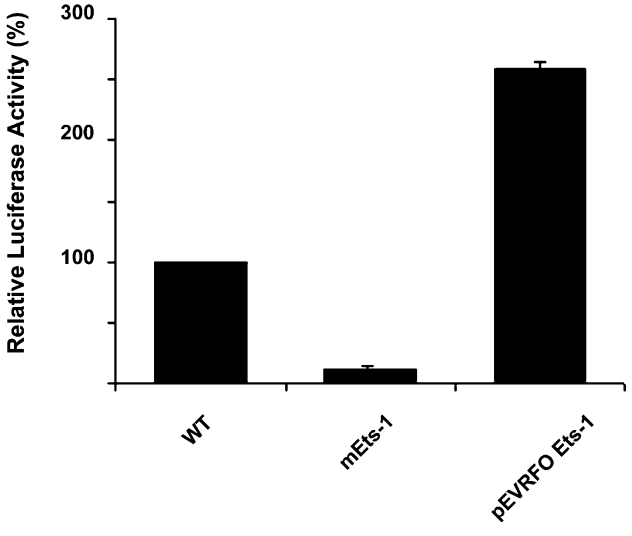

Fig. 4. Functional relevance of Ets-1 site of the −59/+152 fragment. PC12 cells were transfected with either the wild type −59/+152 luciferase construct (WT) or with the same construct but with the Ets-1 site mutated (mEts-1), or co-transfected with WT construct and mouse Ets-1 expressing plasmid pEVRFO Ets-1.

DNAse I protection and EMSA assays revealed two specific DNA-protein complexes. The involved *cis*-acting sequences show homology to binding sites for members of the Ets and Sp transcription factor families. Site directed mutagenesis on the −59/+152 fragment revealed that the Sp1 sites were of minor relevance for the functional activity of the promoter (not shown), while the Ets-1 site at −32/−23 bp (GTCGGC-TTCCGGCCTAGA) was critical for the promoter activity. The Ets-1 site is located in a position at which TATA box elements locate in TATA box containing promoters. It is noticeable that an Ets-1 element (TASS, GCCGGTTTCCT-AGA) similarly located was described as important for the functional activity of the promoter of β1,4 GalT-1 acting on the synthesis of glycoprotein oligosaccharides and lactose [12]. Transient overexpression of Ets-1 in a CHO-K1 cell clone expressing low amounts of GD1a increased the synthesis of this ganglioside by about 11%. Considering an efficiency of transfection of about 30%, this would mean about 33% activation of GD1a synthesis in the transfected cells, evidence in favor of the functionality of Ets-1 in vivo. The Ets superfamily is a structural class of *trans*-acting phosphoproteins that binds to purine-rich (GGAA/T) DNA sequences and functions as transcription factors [13]. Most of the Ets proteins bind DNA as monomers. However, their transcriptional activity is modulated by other partner factors [14]. In our case, the Ets site could play a role in the binding of other proteins necessary for recruitment and positioning of the transcription initiation complex [15].

Ets proteins play important roles in different biological processes, including cellular proliferation, differentiation, development, transformation, immune response and apoptosis. It is interesting to note that particularly Ets-1 increased in several mouse brain areas during days 7 and 18 of post-natal development [16], a period characterized for active sinaptogenesis and synthesis of glycolipids. A concomitant conversion of simple (GM3, GD3) to complex (GM1, GD1a, GD1b, GT1b) gangliosides also occurs during this period due to the upregulation of the activities of GalNAc-T and Gal-T2 transferases, both necessary for synthesis of the tetrasaccharide chain of complex gangliosides [17].

Acknowledgements: This work was supported in part by Grants from SECyT-Universidad Nacional de Córdoba (H.J.F.M.), "Ramon Carrillo-Arturo Oñativia" (to J.L.D. and H.J.F.M.), 75197–554001 from Howard Hughes Medical Institute of USA (to H.J.F.M.), 10087 from Mitzutani Foundation (to H.J.F.M.), and 01-5185 from FONCYT (to H.J.F.M.). The authors thank N. Koritschoner for help in initial studies, G. Panzetta for critical reading of the manuscript and P. Crespo for help with lipid analyses. The technical assistance of G. Schachner and S. Deza is also acknowledged. J.L.D and H.J.F.M. are Career Investigators, and L.P.R. Fellow of CONICET, Argentina.

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