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Lysosphingolipids Inhibit Protein Kinase C: Implications for the Sphingolipidoses

YUSUF A. HANNUN AND ROBERT M. BELL*

Lysosphingolipids potently and reversibly inhibited protein kinase C activity and binding of phorbol dibutyrate in vitro and in human platelets. As with activation of protein kinase C by phosphatidylserine and *sn*-1,2-diacylglycerol, inhibition was subject to surface dilution. Accordingly, inhibition in mixed micelle assays was dependent on the molar percentage of lysosphingolipids rather than the bulk concentration. Lysosphingolipids inhibited protein kinase C activity at molar percentages similar to those required for activation by phosphatidylserine and *sn*-1,2-diacylglycerol. Since lysosphingolipids accumulate in Krabbe's disease, Gaucher's disease, and other sphingolipidoses, the hypothesis that lysosphingolipid inhibition of protein kinase C represents the missing functional link between the accumulation of sphingolipids and the pathogenesis of these disorders appears to unify existing data. The accumulation of lysosphingolipids would cause progressive dysfunction of signal transduction mechanisms vital for neural transmission, differentiation, development, and proliferation and would eventually lead to cell death.

PROTEIN KINASE C PLAYS IMPORTANT roles in signal transduction, cellular differentiation, and tumor promotion. The interaction of extracellular signals with their receptors leads to the phospholipase C–dependent hydrolysis of inositol phospholipids, especially phosphatidylinositol-4,5-bis-phosphate, resulting in the formation of two second messengers, inositol tris-phosphate (IP_3) and diacylglycerol (DAG) (*1*). IP_3 mobilizes Ca^{2+} from intracellular stores (*2*). DAG activates protein kinase C (*1*). Phorbol diesters, which are potent tumor promoters, also bind to and activate protein kinase C (*3, 4*).

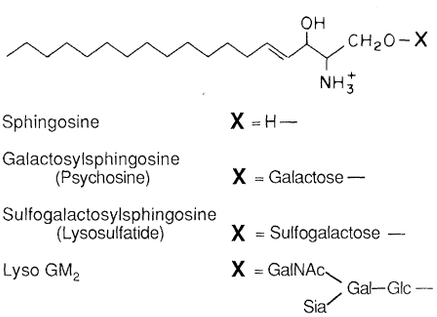

Fig. 1. Structure of lysosphingolipids. Lysosphingolipids are derivatives of the long-chain base sphingosine where the 1-hydroxyl is substituted by different head groups. With the exception of lysosphingomyelin (sphingosylphosphorylcholine) where the linkage is through a phosphodiesteric bond, lysosphingolipids have a glycosidic bond at C-1.

Protein kinase C is found in highest concentrations in the mammalian central nervous system (*5*) where it appears to participate in transduction of neurotransmitter and other signals, receptor regulation (*1, 6*), and neuronal differentiation. Phorbol diester receptors are associated with fetal brain cells that are actively elaborating neurites (*7*). Protein kinase C activity in rat brain rises during the first month of postnatal development (*8*); it also changes in primary neuronal cultures with age (*9*). Localization of protein kinase C by specific antisera to the perinuclear and presynaptic areas (*10*) is consistent with a role in neuronal differentiation and function. Although the precise role of this enzyme in neural development is unknown, the correlations noted suggest that alterations in its level or activity during critical periods in neural development could result in functional and pathologic changes.

Sphingosine was discovered in our laboratory to be a potent and reversible inhibitor of protein kinase C activity and of phorbol diester binding in mixed micellar assays, and also in human platelets, neutrophils, and HL-60 cells (*11*). The critical structural features of sphingosine required for inhibition of protein kinase C were the primary amine and a hydrophobic character (*11*). Since inhibition still occurred when the 1-hydroxyl was substituted, the question of whether lysosphingolipids (Fig. 1) would inhibit protein kinase C arose. This question is of interest for at least two reasons: (i) naturally occurring inhibitors of protein kinase C could be of importance in the regulation of signal transduction; and (ii) lysosphingolipid inhibition of protein kinase C might have functional consequences in the sphingolipidoses.

The sphingolipidoses are a group of diseases that arise as a consequence of inborn errors of sphingolipid metabolism (see Fig. 4). These diseases have their onset most commonly in the neonatal period of early childhood with affected patients exhibiting organomegaly or progressive mental and neurologic dysfunction (*12*). Usually there is a deficiency of a lysosomal enzyme involved in the catabolism of a particular sphingolipid, resulting in the accumulation of lipid molecules proximal to the enzymatic lesion. Progressive accumulation of sphingolipids occurs predominantly in the lysosomes of the tissues where synthesis or catabolism (or both) is most active. The lipid-storing cells, specific for each disease, are scattered throughout the involved tissues such as spleen, liver, lung, lymph nodes, bone marrow, and central nervous system. In patients with neurologic disease there is, in addition, neuronal degeneration, loss of cells from the cerebral and cerebellar cortexes, brain atrophy, gliosis, and demyelination. The excessive accumulation of sphingolipids, which are normal cellular lipid constituents, is thought to result in the formation of the lipid-storing cells. However, the molecular basis linking sphingolipid accumulation, the histopathology, and the severe neurodegenerative disease has remained a mystery.

We now report that lysosphingolipids (Fig. 1) are potent inhibitors of protein kinase C activity and of phorbol-diester binding, both in vitro and in human platelets. We propose that lysosphingolipids represent the functional missing link between the accumulation of sphingolipids and the pathogenesis of the sphingolipidoses.

Lysosphingolipids differ from their respective parental sphingolipids by not hav-

Y. A. Hannun, Department of Medicine, Duke University Medical Center, Durham, NC 27710.
R. M. Bell, Department of Biochemistry, Duke University, Medical Center, Durham, NC 27710.

*To whom correspondence should be addressed.

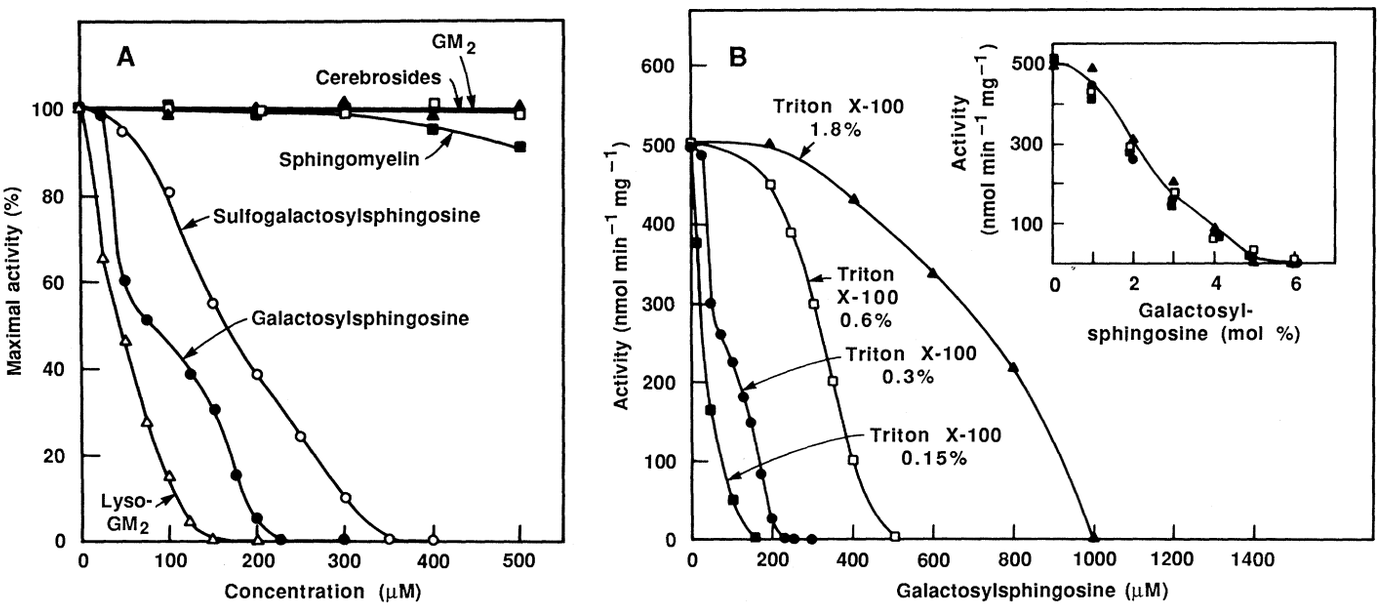

Fig. 2. Inhibition of protein kinase C by lysosphingolipids (**A**) Galactosylsphingosine (●), sulfogalactosylsphingosine (○), and lyso-GM_2 (△) were potent inhibitors of protein kinase C when activity was measured at 6 mol% phosphatidylserine, 2 mol% *sn*-1,2-dioleoylglycerol, and 100 μ*M* Ca^{2+}. These data exemplify the inhibition observed with the rest of the related lysosphingolipids (Table 1). The parental sphingolipids were also evaluated for their effects on protein kinase C activity. They were dried down from chloroform-methanol solutions with the lipid cofactors of protein kinase C and solubilized in Triton X-100. None of the parent compounds inhibited protein kinase C, as shown for cerebrosides, sphingomyelin, and GM_2 (*34*). To further investigate the specificity of inhibition of protein kinase C by lysosphingolipids, the *N*-acetyl derivatives of galactosylsphingosine, sphingosylphosphorylcholine, and lysoGM_2 were prepared as described (*35*) for the *N*-acetyl derivative of sphingosine. The compounds were purified on a cyano high-performance liquid chromatography column and then tested for their effects on protein kinase C activity. None of these *N*-acetyl derivatives inhibited protein kinase C. (**B**) The lysosphingolipids displayed surface dilution kinetics. Galactosylsphingosine was less potent when protein kinase C was assayed at higher concentrations of Triton X-100 mixed micelles containing fixed amounts of phosphatidylserine (6 mol%) and dioleoylglycerol (2 mol%). When the data are plotted as molar percentage of galactosphingosine: Triton X-100 (inset), the inhibition profiles at the different concentrations of Triton X-100 (0.15% w/v, ■; 0.3%, ●; 0.6%, □; and 1.8%, ▲) appeared identical. These results indicate that the lysosphingolipids preferentially partition into the mixed micelles; therefore, effective concentrations should be expressed as the molar percentage of the lipid compounds to Triton X-100 and not as bulk concentrations.

ing the amide-linked fatty acid at the 2-amino position of the sphingoid base (Fig. 1). The lysosphingolipids, therefore, share with sphingosine the two critical structural features required for inhibition of protein kinase C, but differ with respect to the head groups at the 1-hydroxyl position (see Figs. 1 and 4). We prepared lysosphingolipids by base treatment of the respective parental sphingolipids (Table 1), and using a mixed micellar assay (*13*) examined their effects on protein kinase C activity (Table 1 and Fig. 2). All proved to be potent and reversible inhibitors, and none of the intact parental sphingolipids inhibited enzyme activity (Table 1 and Fig. 2A).

The total concentration of galactosylsphingosine required to cause 50% inhibition depended on the number of mixed micelles present (Fig. 2B). Increasing the number of phosphatidylserine (PS), DAG, and Triton X-100 mixed micelles present (indicated by the percentage of Triton X-100) predictably increased the total concentration of galactosylsphingosine required for 50% inhibition. When the data are plotted as the molar percentage of lipid to Triton X-100 (mol%) (inset, Fig. 2B), galactosylsphingosine inhibition appeared equipotent at each level of mixed micelles used. Such data indicate surface dilution, which is a consequence of amphipathic molecules preferentially partitioning into micelles or bilayers (surfaces). Earlier studies with mixed micelles of Triton X-100, PS, and DAG on the activation of protein kinase C indicated that lipid cofactors (PS and DAG) are subject to surface dilution (*13*). Thus, amphipathic inhibitors such as the lysosphingolipids should be subject to surface dilution. It is for this reason that absolute bulk concentrations are not useful and that molar percentage is the appropriate expression of concentration.

The concentrations of the lysosphingolipids that inhibit protein kinase C (1 to 4 mol%) are similar to the concentrations of DAG (1 to 2 mol%) and PS (3 to 6 mol%) required for activation (*13*). Furthermore, inhibition by lysosphingolipids was modulated by the molar percentage of lipid cofactors and by the Ca^{2+} concentration, in a manner similar to sphingosine (*11*). Lysosphingolipids inhibited phorbol diester binding to protein kinase C (Table 1). The molar percentage lysosphingolipid required for 50% inhibition of activity of [^{3}H]PDBu (phorbol dibutyrate) binding differed predictably because different assay conditions were used (Table 1).

The effect of the lysosphingolipids on human platelets was studied to learn wheth-

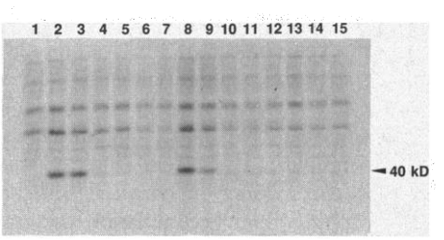

Fig. 3. Inhibition of 40-kD peptide phosphorylation in platelets. The phosphorylation of 40-kD polypeptide in human platelets in response to thrombin was performed as described (*11*). Lysosphingolipids inhibited the phosphorylation of this polypeptide which is known to be effected by protein kinase C. (lane 1) Unstimulated platelets; (lanes 2 to 15) thrombin, 1 unit/ml; (lanes 3 to 7) galactosylsphingosine 5, 10, 25, 50, and 100 μ*M*; (lanes 8 to 11) lysosulfatide 5, 25, 50, and 100 μ*M*; (lanes 12 to 15) lysoGM$_2$ 25, 50, 100, and 200 μ*M*. Galactosylsphingosine (25 μ*M*) did not affect the generation of diacylglycerol second messengers in response to thrombin (*36*). Also, the *N*-acetyl derivatives of galactosylsphingosine and sphingosylphosphorylcholine did not affect 40-kD peptide phosphorylation when tested at concentrations less than 100 μ*M*, indicating that the inhibition by lysosphingolipids was specific and not due to nonspecific detergent actions.

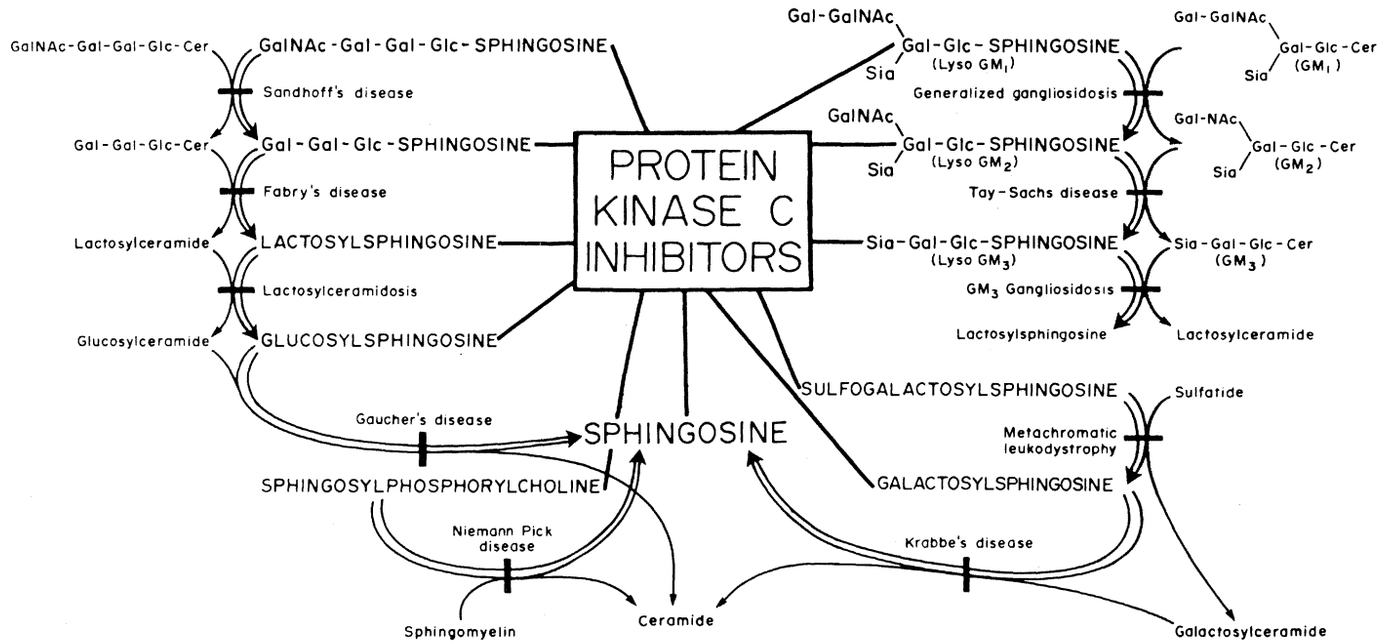

Fig. 4. Proposed metabolic pathways for the degradation of lysosphingolipids. The pathways of sphingolipid catabolism are illustrated (thin arrows). Deficiency of enzymes in these pathways (solid bars) leads to the indicated diseases. It is proposed that the same enzymes also act on lysosphingolipids (double arrows), and therefore, both the parent sphingolipid and the lysosphingolipid would accumulate in enzyme deficient states. Sphingosine and lysosphingolipids differ from the parent sphingolipids in their ability to strongly inhibit protein kinase C, thus interfering in the function of important pathways of signal transduction and cell regulation. Also the cytotoxicity of lysosphingolipids may ultimately lead to permanent tissue damage and cell death.

er these molecules would function in an intact biological system. Protein kinase C activation in platelets leads to the phosphorylation of a 40-kD polypeptide. All the lysosphingolipids tested inhibited this phosphorylation induced by thrombin (Table 1 and Fig. 3), dioctanoylglycerol, or phorbol myristate acetate. The mechanism by which these molecules gain access to the intracellular compartment is still unknown. The lysosphingolipids tested also inhibited phorbol binding to human platelets (Table 1).

These molecules were active in inhibiting protein kinase C in platelets at bulk concentrations (5 to 25 μM), which are comparable to those of dioctanoylglycerol (1 to 10 μM) required for induction of 40-kD phosphorylation (*11*). Since similar amounts of platelets were used, these correspond to a similar surface concentration. Sphingosine inhibition of 40-kD peptide phosphorylation is subject to surface dilution in platelets (*11*). These findings suggest that intracellular accumulation of the lysosphingolipids may lead to significant inhibition of protein kinase C.

The finding that lysosphingolipids are potent inhibitors of protein kinase C may provide the missing biochemical link between sphingolipid accumulation and the pathogenesis of the sphingolipidoses (see Fig. 4). The accumulation of lysosphingolipids could result in progressive and continuous inhibition of protein kinase C, whose dysfunction may lead to deranged cellular function, disruption of normal neural development, and possibly cell death. Since protein kinase C is active in nonneural tissues, the excessive accumulation of lysosphingolipids in other organ systems may also contribute to the systemic derangement seen in the sphingolipidoses.

Psychosine (galactosylsphingosine) has been shown to have a role in the pathogenesis of Krabbe's disease, a progressive and fatal neurologic disease (Fig. 4). In 1972, Miyatake and Suzuki proposed that psychosine is responsible for the pathogenesis (*14*), and in support of the hypothesis, Svennerholm *et al.* (*15*) and Igisu and Suzuki (*15*) demonstrated increased amounts of psychosine (up to 100 times normal) in the white matter of brains of affected patients. From the levels of psychosine (0.1 mmol/kg) and assuming 70% water content, we estimate the tissue concentration of galactosylsphingosine would be about 0.14 mM. These data suggest that galactosylsphingosine concentrations in Krabbe's disease are indeed sufficient to inhibit protein kinase C. Psychosine also accumulates early in the brain of the mutant "twitcher mouse," which has a genetic deficiency of galactoceramidase, and a pathologic picture similar to that of Krabbe's leukodystrophy (*16*).

When added to cultured rat cerebellar explants, psychosine caused rapid degeneration of the cells. Injection of psychosine into rat brain produced serious neural toxicity and was ultimately fatal. Injection of galactosylceramide alone caused globoid cell proliferation (*12*). However, when galactosylceramide and 3% psychosine were injected, significant tissue degeneration over and above the globoid cell reaction occurred (*17*).

The accumulation of psychosine in Krabbe's disease results in the destruction of oligodendroglia followed by demyelination, axonopathy, some neuronal degeneration, and ultimately severe astrocytic gliosis. Thus, psychosine accumulation is believed to account for the progressive neurologic and mental dysfunction of Krabbe's disease. The biochemical target or targets of psychosine, however, had remained a mystery until now. Psychosine inhibition of protein kinase C may represent the missing functional link.

Glucosylsphingosine accumulation has been demonstrated in the brains and spleens of Gaucher's disease patients (*18*), and a role for it in the pathogenesis has been suggested (*19*). The levels of glucosylsphingosine in affected tissues (0.06 to 0.19 mmol/kg) (*19*) correspond to concentrations of 0.15 mM (for a 70% water content). These concentrations suggest that sufficient amounts of glucosylsphingosine accumulate in Gaucher's disease to inhibit protein kinase C. The ganglioside lysoGM$_2$ has also been detected (estimated accumulation, 0.04 mM) in the brain of Tay-Sachs patients but it has not been detected in normal brain (*20*).

As with the three diseases discussed, accu-

mulation of the parental lipid could lead to the formation of the lysosphingolipid (Fig. 4). Whenever examined, the same catabolic enzyme functions to degrade both the parent sphingolipid and lysosphingolipid (*21*). Thus, a genetic deficiency in the catabolism of the parent sphingolipid would extend to the lysosphingolipid. A similar dual function has been noted with synthetic enzymes that can utilize either ceramide- or sphingosine-based substrates (*22, 23*). The present information about lysosphingolipid formation and degradation is very limited, but the enzymes involved are most active during the phase of rapid myelination (*21, 22, 24*). Sphingosine, galactosphingosine, glucosylsphingosine, sulfogalactosphingosine, and possibly other lysosphingolipids are highly cytotoxic (*17, 25*).

The data described permit us to propose that the accumulation of the lysosphingolipids as a consequence of an inherited deficiency in the catabolism of sphingolipids causes the observed cytotoxicity and pathophysiology of the sphingolipidoses. The accumulation of the parent sphingolipid may account for the presence of lipid-storing cells in these diseases, but does not explain the serious cell dysfunction and cell death observed, especially in the central nervous system. The lysosphingolipids, in contrast, are cytotoxic metabolites. Moreover, all of the lysosphingolipids have a common target, protein kinase C, by which they could affect cell regulation and cell function by inhibiting signal transduction. Although protein kinase C is present in all tissues, accumulation of the lysosphingolipids may determine the pathologically affected organ systems. Lysosphingolipid accumulation should parallel that of the parental sphingolipids.

Critical tests of the hypothesis require demonstration of elevated levels of lysosphingolipids in the affected tissues of patients having other enzyme deficiencies (Fig. 4), and demonstration of the inhibition of protein kinase C by accumulated lysosphingolipids in affected tissues. Further exploration of the role of protein kinase C in neural development and regulation of central nervous system function is essential to understand how the inhibition of protein kinase C activity by the lysosphingolipids relates to the pathology and functional derangement observed in the sphingolipidoses. The possibility that lysosphingolipids exist as normal cellular constituents and function to regulate protein kinase C activity remains to be investigated (*26*).

Table 1. Inhibition by protein kinase C activity and of phorbol-dibutyrate binding by lysosphingolipids. Lysogangliosides were prepared from their parental gangliosides by hydrolysis in methanolic KOH essentially as described by Neuenhofer *et al.* (*27*). During this preparation, with approximately 90 to 95% conversion, the *N*-acetate of *N*-acetylneuraminic acid is partially hydrolyzed (*27*). Fatty acids were partitioned into heptane, after acidification of the methanolic reaction mixture. Galactosylsphingosine, glucosylsphingosine, and lactosylsphingosine were prepared as described for galactosylsphingosine (*28*). Sphingosylphosphorylcholine was prepared by hydrolysis in HCl-butanol (*29*). The purity of the lysosphingolipid preparations was greater than 95%; the principal contaminants were the parental sphingolipids. This was assessed by thin-layer chromatography (TLC) on silica gel H plates developed in chloroform, methanol, 2*N* NH_4OH (65:35:9) and visualized by ninhydrin reaction for *N*-lyso compounds, by α-naphthol for both gangliosides and lysogangliosides, and by a phospholipid spray (*30*) for sphingosylphosphorylcholine. Lysosphingolipids were quantitated by reaction of the amine group with trinitrobenzenesulfonate (*31*). Gangliosides and lysosphingolipids were also quantitated by reaction with anthrone (*31*). Protein kinase C was purified to near homogeneity (*32*), and activity was measured with a mixed micellar assay (*13*). Lysosphingolipids were added directly in aqueous solution, and allowed to equilibrate with the Triton X-100 (0.3% w/v) mixed micelles containing 6 mol% phosphatidylserine and 2 mol% dioleoylglycerol. Phorbol-dibutryrate binding was measured in a mixed micellar assay that required different conditions (*33*).

Disease	Lysosphingolipid	Inhibition of protein kinase C*		Inhibition of [^{3}H]PDBu binding to protein kinase C*	
		μ*M*	mol%	μ*M*	mol%
None	Sphingosine	80†	1.9	400‡	9.3
Krabbe's	Galactosylsphingosine	85†	2	500‡	11.6
Gaucher's	Glucosylsphingosine	85†	2	400‡	9.3
Lactosylceramidoses	Lactosylsphingosine	130	3	NT§	
Niemann-Pick	Sphingosylphosphorylcholine	120†	2.8	500‡	11.6
Metachromatic leukodystrophy	Sulfogalactosylsphingosine	150†	3.5	450‡	10.5
	Sulfolactosylsphingosine	NT			
Fabry's	Globotriaosylsphingosine	180	4.2	NT	
	Galabiosylsphingosine	NT		NT	
GM_1 gangliosidosis	LysoGM_1	45	1	300	7.0
Tay-Sach's	LysoGM_2	50†	1.2	300‡	7.0
Sandhoff's	LysoGA_2	60	1.4	NT	
GM_3 gangliosidosis	LysoGM_3	45	1	NT	
Other gangliosidoses					
	Lyso $GD_{1\alpha}$	25†	0.6	NT	
	Lysotrisialoganglioside	20	0.5	NT	
	Lysogloboside	180	4.2	NT	
	Sphingosyltrihexoside	180	4.2	NT	

*Concentrations giving 50% inhibition; 1 mol% = 43 μ*M* under the assay conditions with 0.3% Triton X-100. The potency of lysosphingolipids in inhibiting [^{3}H]phorbol-dibutyrate binding appears to be less than their potency in inhibiting protein kinase C activity because different assay conditions were used (see legend above). †These compounds were also tested for inhibition of 40-kD phosphorylation in human platelets. They all inhibited thrombin-, dioctanoylglycerol-, and phorbol myristate acetate–induced phosphorylation over a concentration range of 10 to 50 μ*M*. ‡These compounds were also tested for their effects on PDBu binding to human platelets. They all inhibited binding over a concentration range of 5 to 50 μ*M*. §NT, not tested.

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26. The finding that lysosphingolipids are potent reversible inhibitors of protein kinase C raises the possibility that these molecules may be normal cellular constituents produced in response to external stimuli or during certain phases of cell development to function as "negative effectors." Cells appear to contain enzymes to produce and degrade the lysosphingolipids. The sphingolipids might play a role analogous to that of phosphatidylinositols. The lysosphingolipids produced in a "sphingosine cycle" would then function as second messengers with protein kinase C as a target. In fact, increased *N*-acylation and deacylation of sphingomyelin occurs in antibody stimulated L-929 cells [R. G. Ulrich and W. T Shearer, *Biochim. Biophys. Res. Commun.* **121**, 605 (1984)].
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HTLV *x* Gene Mutants Exhibit Novel Transcriptional Regulatory Phenotypes

WILLIAM WACHSMAN, ALAN J. CANN, JANICE L. WILLIAMS, DENNIS J. SLAMON, LARRY SOUZA, NEIL P. SHAH, IRVIN S. Y. CHEN

The human T-cell leukemia viruses, HTLV-I and HTLV-II, contain a gene, termed *x*, with transcriptional regulatory function. The properties of the *x* proteins were analyzed by constructing mutant genes containing site-directed deletions and point mutations. The results demonstrate that the amino terminal 17 amino acids of the *x* protein constitute part of a functional domain that is critical for the transcriptional activating properties of the protein. Within this region, substitution of a leucine residue for a proline residue results in major changes in the *trans*-activation phenotype of the protein. The mutant HTLV-II *x* protein, though incapable of activating the HTLV-II long terminal repeat, will block *trans*-activation of the HTLV-II long terminal repeat by the wild-type protein. The altered phenotype of this mutant suggests a potential negative regulatory function of the *x* protein.

THE HUMAN T-CELL LEUKEMIA VIRUS (HTLV), types I and II, and bovine leukemia virus (BLV) comprise a distinct group of oncogenic retroviruses with common structural and functional features. All are etiologically associated with lymphoid malignancies: HTLV-I with adult T-cell leukemia (*1*), HTLV-II with some cases of variant T-cell hairy-cell leukemia (*2*), and BLV with enzootic leukosis in cattle and sheep (*3*). Studies in vitro show that HTLV-I and HTLV-II immortalize normal T cells from humans, primates, and rodents (*4*). Because they lack sequences homologous to a known oncogene (*5*), and because they exhibit a random pattern of viral integration (*6*), HTLV-I, HTLV-II, and BLV appear to induce leukemogenesis by a mechanism distinct from that of other groups of oncogenic retroviruses.

Sequences unique to HTLV and BLV, termed the X region, are located at the 3′ end of the viral genome (*5*). We have shown through mutational studies that the HTLV-II X region is required for efficient replication of the virus, and that it acts in *trans* to increase the level of proviral transcription (*7*). At least two proteins appear to be encoded by different but overlapping X region open reading frames (*8*). One of these proteins is produced by the *x* gene (also variously termed the *x-lor* or *tat* gene) in HTLV-I-, HTLV-II-, and BLV-infected cells; its size is 40 kD ($p40^{xI}$), 37 kD ($p37^{xII}$), and 34 kD in these cells, respectively (*9–11*).

Transient cotransfection assays have been used to show that the *x* protein enhances transcription in *trans* (*trans*-activation) from the viral long terminal repeat (LTR) (*12–15*). In addition, the HTLV-II *x* protein, $p37^{xII}$, *trans*-activates heterologous promoters: the HTLV-I LTR and the E1A-inducible early region promoter, EIII, from adenovirus (*16*). The adenovirus EIII promoter is normally dependent on the adenovirus E1A protein for efficient transcription. The E1A gene is essential for efficient adenovirus replication (*17*) and is also necessary for the oncogenic properties of adenovirus in rodent cells (*18*). The functional analogies between *x* and E1A provide further support for a role of *x* in cellular transformation by HTLV. Together, these data support the hypothesis that the *x* gene participates in cellular immortalization by inducing abnormal expression of cellular genes.

The results of our present studies on the mutagenesis of $p40^{xI}$ and $p37^{xII}$ implicate the amino terminus of the molecule as a functional domain for *trans*-activation. Our data demonstrate that a single amino acid substitution at position 5 of the amino terminus in both $p37^{xII}$ and $p40^{xI}$ alters the phenotype of *trans*-activation for various promoters. In addition, the mutant HTLV-II *x* protein inhibits the action of the wild-type protein in *trans*-activation of the HTLV-II LTR.

The *x* gene messenger RNA (mRNA) is composed of three exons, of which the last two are used to encode the *x* protein (*19, 20*). To obtain efficient *x* protein expression, we previously developed constructs of the HTLV-I and HTLV-II *x* genes (termed *x*I and *x*II) that, after transfection, express the native *x* protein without the need for RNA splicing (*21*). These *x* constructs were generated by linking a synthetic DNA oligonucleotide, which encodes amino acids 1 through 17 of the HTLV-I or HTLV-II *x* protein, to the bulk of *x* gene sequences obtained from clones of the respective provirus. The expression vector p91023-B (*22*) was used to express these constructs, yielding high levels of *x* protein in transfected COS cells.

To test whether the amino terminus of $p37^{xII}$ was critical for *trans*-activation, we modified the wild-type *x*II construct by deleting codons 2 through 17 in the oligonucleotide linker (Fig. 1). Expression of this mutant construct, *x*IIΔ(2–17), resulted in production of an appropriately smaller protein of approximately 35 kD (Fig. 2). When cotransfected with the promoter/CAT

W. Wachsman, A. J. Cann, J. L. Williams, D. J. Slamon, N. P. Shah, I. S. Y. Chen, Division of Hematology-Oncology, Department of Medicine, Jonsson Comprehensive Cancer Center, UCLA School of Medicine, Los Angeles, CA 90024.
L. Souza, Amgen Biologicals, 1900 Oak Terrace Lane, Thousand Oaks, CA 91320.