riophage T7 promoter in plasmid pET3B as described [W. Studier and B. A. Moffatt, *J. Mol. Biol.* **189**, 113 (1986); A. H. Rosenberg *et al.*, *Gene* **56**, 125 (1987)]. Soluble *E. coli* extract containing the recombinant TFIID was fractionated by passage through heparin agarose in 0.1 M KCl, followed by step elution with 0.25 M, 0.5 M, and 0.8 M KCl in 20 mM Hepes, pH 7.9, 20% glycerol, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 0.2 mM EDTA. TFIID was assayed by SDS polyacrylamide gel electrophoresis of column fractions. Peak TFIID in the 0.5 M KCl fraction was used in footprinting assays. Empirically determined 1/2, 1, and 2 minimum footprint units were used for footprinting as described (*10*). Recombinant YIID expressed in *E. coli* (*3*) was prepared (*7*) and used at 1/2, 1, and 2 footprint units.

22. Phosphocellulose fractions were prepared as described [J. D. Dignam, P. L. Martin, B. S. Shastry, R. G. Roeder, *Methods Enzymol.* **101**, 582 (1983)]. The in vitro transcription assays were performed as described (*10*) with 1 μg of template plasmid and 2 μl of reticulocyte extracts. Primer extension assays (*10*) were performed with the B1750 oligonucleotide.
23. Q. Zhou and A. J. Berk, unpublished data.
24. L. Wu, D. Rosser, M. C. Schmidt, A. J. Berk, *Nature* **326**, 512 (1987).
25. J. Kadonaga, K. C. Carner, F. R. Masiarz, R. Tjian, *Cell* **51**, 1079 (1987).
26. A. J. Courey and R. Tjian, *ibid.* **55**, 887 (1988).
27. K. A. Wharton, B. Yedvobnick, V. G. Finnerty, S. Artavnis-Tsakonas, *ibid.* **40**, 55 (1985).
28. C. Mariani, V. Pirrotta, E. Manet, *EMBO J.* **4**, 2045 (1985).
29. D. Duboule, M. Haenlin, B. Galliot, E. Mohier, *Mol. Cell. Biol.* **7**, 2003 (1987).
30. Cytoplasmic RNA for Northern analysis was purified from HeLa cells as described [A. Jacobson, *Methods Enzymol.* **152**, 254 (1987)]. Poly(A)$^+$ RNA was enriched by chromatography on oligo (dT)-celluose (Collaborative Research). RNA was purified on a gel consisting of 1% agarose, 6.2% formaldehyde, 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, transferred to nitrocellulose UV–crosslinked, and probed with a $^{32}$P-labeled restriction fragment from the TFIID cDNA.
31. S. J. Collins and M. T. Groudine, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4813 (1983).
32. W. R. Pearson and D. J. Lipman, *ibid.* **85**, 2444 (1988).
33. A. Maxam and W. Gilbert, *Methods Enzymol.* **65**, 499 (1980).
34. We thank R. Gaynor and colleagues for the lambda Zap library; M. Gishinsky for human genomic DNA and cytoplasmic RNA; S. Crews for a *Drosophila* cDNA library and discussions; C. Guthrie and E. Shuster for numerous yeast strains; and P. O'Farrell for discussions. Supported by American Cancer Society (ACS), California division, postdoctoral fellowship and ACS postdoctoral fellowships J-3-89 (to C.C.K.), PF3380 (to P.M.L.), and PF2715 (to M.C.S.) and by National Cancer Institute grant CA25235.

18 April 1990; accepted 22 May 1990

# A cDNA for a Protein That Interacts with the Human Immunodeficiency Virus Tat Transactivator

PETER NELBOCK, PATRICK J. DILLON, ANN PERKINS, CRAIG A. ROSEN*

**The human immunodeficiency virus (HIV) *tat* protein (Tat) is a positive regulator of virus gene expression and replication. Biotinylated Tat was used as a probe to screen a λgt11 fusion protein library, and a complementary DNA encoding a protein that interacts with Tat was cloned. Expression of this protein, designated TBP-1 (for Tat binding protein–1), was observed in a variety of cell lines, with expression being highest in human cells. TBP-1 was localized predominantly in the nucleus, which is consistent with the nuclear localization of Tat. In cotransfection experiments, expression of TBP-1 was able to specifically suppress Tat-mediated transactivation. The strategy described may be useful for direct identification and cloning of genes encoding proteins that associate with other proteins to modulate their activity in a positive or negative fashion.**

UNLIKE MOST OTHER RETROVIRUSES, HIV gene expression and replication is dependent on the expression of both viral-encoded and cellular transacting regulatory proteins. One such viral regulatory protein, referred to as Tat (*1*), is a positive regulator of HIV gene expression. Tat functions through a sequence known as TAR (*2*), which is located between nucleotides +1 to +43 in the long terminal repeat (LTR) (*3*). In this context, TAR is present in both DNA and the 5′ untranslated region of all viral messages. Although the mechanism for transactivation remains unclear, with both transcriptional (*2–5*) and posttranscriptional (*6, 7*) components being considered, recent studies indicate that Tat interacts with TAR RNA (*8*). Whether this interaction, in itself, is sufficient to bring about transactivation remains unclear, in part because of the lack of an in vitro model to examine Tat function.

Since Tat interacts with TAR RNA, and also elicits a clear transcriptional effect on gene expression, it has been suggested that TAR may function as an RNA enhancer (*9*). We therefore speculated that cellular factors interact with Tat, which in turn mediates transcriptional effects through other interactions within the LTR. With this in mind, we investigated whether a human cDNA encoding a protein that interacts with Tat could be identified. Our approach was similar to that employed for in situ detection of sequence-specific DNA binding proteins (*10*) or fusion proteins that interact with a specific antibody (*11*). Specifically, purified Tat protein was used as a probe to screen a cDNA library. *Escherichia coli*–derived Tat, which was previously shown to be fully functional (*12*), was biotinylated and used as a probe to screen a λgt11 fusion cDNA library prepared from human Jurkat T-cell cDNA (Clonetech). Proteins expressed from the phage were adsorbed onto nitrocellulose, and phage-encoding proteins able to interact with biotinylated Tat were identified by subsequent addition of a streptavidin-coupled alkaline phosphatase conjugate plus a color indicator (*13*). Approximately $2 \times 10^6$ phage were screened, and ten were identified that expressed proteins that interacted with Tat. Tat did not bind to a filter containing a random mix of phage from the library (Fig. 1), but showed preferential binding to one of the ten enriched phage isolates. After enrichment of the ten clones, their cDNA inserts were amplified by the polymerase chain reaction (PCR) (*14*), with flanking primers derived from the λgt11 sequence, and cloned into the appropriate expression vectors.

Examination of the PCR products by Southern (DNA) blot analysis revealed that six of the independent isolates actually represented two different clones (that is, each of these two clones was independently isolated three times) demonstrating the specificity and reproducibility of the screening strategy. A clone from one group, designated TBP-1, for Tat binding protein–1, was subjected to further analysis.

The 1341-nucleotide cDNA was found to contain an open reading frame (ORF) of 404 amino acids (Fig. 2). Visual inspection of the TBP-1 ORF revealed no obvious features common to previously characterized transcriptional or translational regulatory factors. The sequence information suggested that TBP-1 protein was produced from its own AUG codon present at nucleotide 63, suggesting that the full-length cDNA was obtained. Reverse transcription of cellular TBP-1 mRNA followed by primer extension of the cDNA product provides further support that the full-length cDNA was obtained (*15*). The coding capacity of

Department of Molecular Oncology and Virology, Roche Institute of Molecular Biology, Hoffmann–La Roche Inc., Roche Research Center, Nutley, NJ 07110.

*To whom correspondence should be addressed.

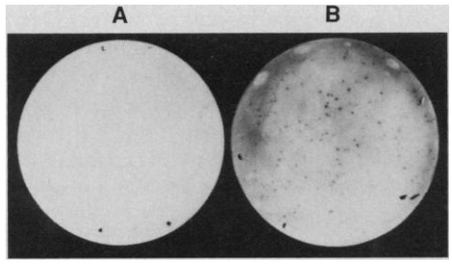


**Fig. 1.** In situ detection of TBP-1 expressed from bacteriophage. Approximately 200 to 300 bacteriophage obtained from the λgt11 cDNA library (**A**) or an enriched TBP-1 isolate (**B**) were mixed with the permissive *E. coli* strain 1090 and plated in top agar containing IPTG. After a 4-hour incubation at 42°C, plates were overlaid with nitrocellulose filters and incubated an additional 3 hours at 37°C. Filters were removed and probed with biotinylated Tat, followed by addition of streptavidin-coupled alkaline phosphatase and the appropriate color indicator (*13*).

```
MSTEEIIQRTRLLDSEIKIMKSEVLRVTHELQAMKDKIKENSEKIKVNKTLPYLVSNVIE

LLDVDPNDQEEDGANIDLDSQRKGKCAVIKTSTRQTYFLPVIGLVDAEKLKPGDLVGVNK

DSYLILETLPTEYDSRVKAMEVDERPTEQYSDIGGLDKQIQELVEAIVLPMNHKEKFENL

GIQPPKGVLMYGPPGTGKTLLARACAAQTKATFLKLAGPQLVQMFIGDGAKLVRDAFALA

KEKAPSIIFIDELDAIGTKRFDSEKAGDREVQRTMLELLNQLDGFQPNTQVKVIAATNRV

DILDPALLRSGRLDRKIEFPMPNEEARARIMQIHSRKMNVSPDVNYEELARCTDDFNGAQ

CKAVCVEAGMIALARGATELTHEDYMEGILEVQAKKKANLQYYA
```

**Fig. 2.** Deduced amino acid sequence from the TBP-1 cDNA. Nucleotide sequence of the cDNA was determined by DNA sequencing (*22*) of the TBP-1 insert cloned in plasmid pBR322. The complete nucleotide sequence is available upon request (*23*).

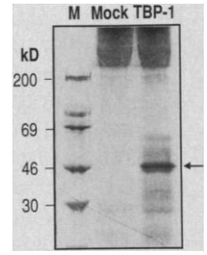


**Fig. 3.** In vitro translation of the TBP-1 cDNA. The TBP-1 cDNA insert was amplified by PCR with a 5′ primer containing the T7 promoter sequence. Amplified DNA was purified and added to an in vitro transcription reaction containing T7 RNA polymerase, and transcribed TBP-1 RNA was used to program a rabbit reticulocyte translation system. The 45-kD protein encoded by TBP-1 RNA is shown.

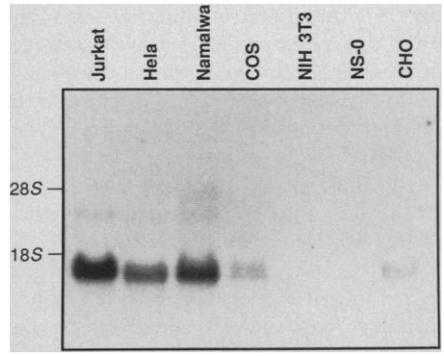


**Fig. 4.** Northern blot analysis of TBP-1 expression. The TBP-1 cDNA probe was nick-translated and hybridized to 20 μg of total cellular RNA prepared from the cell lines shown. RNA was prepared by means of RNAzol (Cinna/Biotecx) per manufacturer's specifications. An overnight exposure of the autoradiogram is shown.

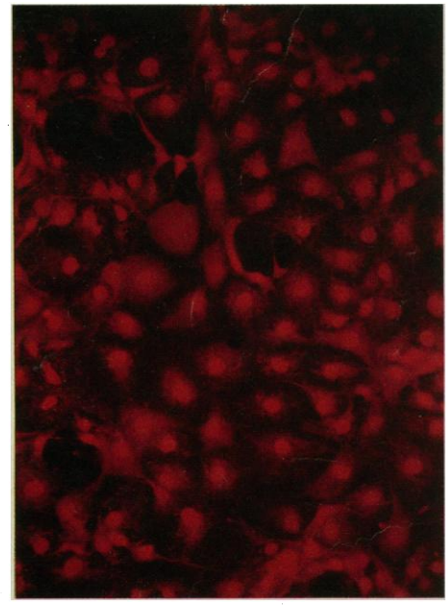

**Fig. 5.** Immunofluorescence demonstrating TBP-1 protein is localized in the nucleus. COS cells were plated at a density of $2 \times 10^5$ per 60-mm dish. The next day cells were fixed (*18*) and incubated overnight with a polyclonal rabbit antisera to TBP-1 (anti–TBP-1) followed by the addition of a rhodamine isothiocyanate–conjugated goat anti–TBP-1. Cells are shown under ultraviolet illumination for immunofluorescence.

this ORF was confirmed by inserting the TBP-1 gene into a transcription vector and using the in vitro transcribed RNA to program a rabbit reticulocyte translation lysate. The TBP-1 RNA programmed the synthesis of an approximate 45-kD protein, as would be expected from the sequence information (Fig. 3).

The conservation of TBP-1 among different species was examined by Southern blot analysis. A Bam HI digest of DNA from cells of human, simian, murine, and hamster origin revealed that TBP-1 is well conserved and probably present at no more than one or two copies per cell (*15*).

The pattern of TBP-1 RNA expression was examined by Northern (RNA) blot analysis. RNA expression was highest in human cells and barely detected in the murine cell lines NIH 3T3 and NS-0 (Fig. 4). This suggests that expression of TBP-1 is under different species- or tissue-specific regulatory constraints. The 1.4-kb TBP-1 transcript is consistent with the size of the TBP-1 cDNA clone.

The nuclear localization of HIV Tat implies that the localization of TBP-1 should be nuclear if its association with Tat is to be relevant to function. Accordingly, the 5′ and 3′ ends of the TBP-1 cDNA insert were modified and cloned into a bacterial pDS expression vector (*16*). TBP-1 protein was purified (*12, 17*) from *E. coli* to about 85% homogeneity and used as an antigen to produce polyclonal antisera in rabbits. Indirect immunofluorescence study with the antisera against TBP-1 revealed that TBP-1 is indeed a nuclear protein (Fig. 5). Similar localization was observed in all cell lines examined that express TBP-1. Protein immunoblot analysis (*15*) confirmed that TBP-1 is ~45 kD, consistent with the Northern blot and in vitro translation analysis.

To examine the potential functional significance of the TBP-1–Tat interaction in vivo, the TBP-1 insert was placed under control of the SV40 early region promoter, and its ability to affect Tat-mediated transactivation was examined in cotransfection assays. As transactivation has been observed in all cell lines tested, we examined the effect of TBP-1 expression in COS (CV-1, origin minus, SV40) cells and CHO (Chinese hamster ovary) cells. These cell lines support a low, but detectable, level of transactivation. This approach was taken, as it should readily allow for detection of small negative or positive effects of TBP-1 expression on Tat function. Cotransfection of pSV-TBP-1 with a Tat expression plasmid [pSV-Tat (*18*)] and LTR indicator plasmid [pU3R-III (*19*)] resulted in a marked decline in transactivation as the concentration of TBP-1 DNA was increased (Fig. 6). This decrease was specific for transactivation and did not result from competition for a limiting amount of transcriptional regulatory factors, since cotransfection with a similar plasmid containing the SV40 promoter but lacking the TBP-1 insert [pSV-Neo (*20*)] had no effect on transactivation. Similarly, no effect was observed after cotransfection of pSV-TBP-1 with other non-Tat responsive promoters obtained from Rous sarcoma virus, visna virus, or SV40, ruling out the possibility that TBP-1 expression is toxic to cells. The lower level of suppression observed in COS cells, as compared to CHO,

may reflect the already high endogenous level of TBP-1 protein in COS cells.

Using biotinylated Tat as a probe to screen a human cDNA library, we have isolated and characterized a nuclear protein of molecular mass 45 kD that interacts with the HIV Tat transactivator protein. An understanding of the exact mechanism and role for the interaction of TBP-1 with Tat awaits the development of an in vitro system for Tat function. In cotransfection experiments, however, we were able to demonstrate that expression of TBP-1 suppresses Tat function. In this respect, TBP-1 appears to function as a transdominant suppressor of Tat. Thus, one might predict that in the absence of positive regulatory factors, in those cell lines where expression of TBP-1 or a related cellular factor is high, transactivation will be low (*21*).

The findings reported here should not come as a surprise, as one cannot predict that all protein-protein associations will result in a positive effect. Similarly, we might expect to find that one of the other clones identified from this screening strategy is a positive modulator of transactivation. We can only speculate as to how Tat mediates transactivation in an environment of both positive and negative cellular factors. One obvious possibility is that additional cellular factors, interacting with other DNA or RNA cis-acting elements present in the LTR, determine the ability of TBP-1 or other factors to bind in vivo. Therefore, the abundance of any one such factor, either positive or negative, could dictate the path transactivation will follow in a particular cell line. These concepts are not unique to this system. For example, many cis-acting DNA elements contain common sequence motifs, which at least in vitro, bind to the same protein. However, expression of genes under control of these elements is often subject to different regulatory pathways in vivo.

If the function of TBP-1 on transactivation is truly negative, then one might expect that in cell lines such as Jurkat or Namalwa, where the expression of TBP-1 appears to be high (at least at the level of RNA expression), as is the level of transactivation, that either additional positive modulators of transactivation are in greater abundance or that interaction with other cellular proteins present in this environment prevents the association of TBP-1 with Tat. We can also speculate that TBP-1, or cellular factors with similar function, contributes to the establishment of latent HIV infections. An understanding of those events that determine the basis for interaction of positive or negative cellular factors with Tat may provide insight toward a means of controlling this process, ultimately allowing the blocking of transactivation at the cellular level.

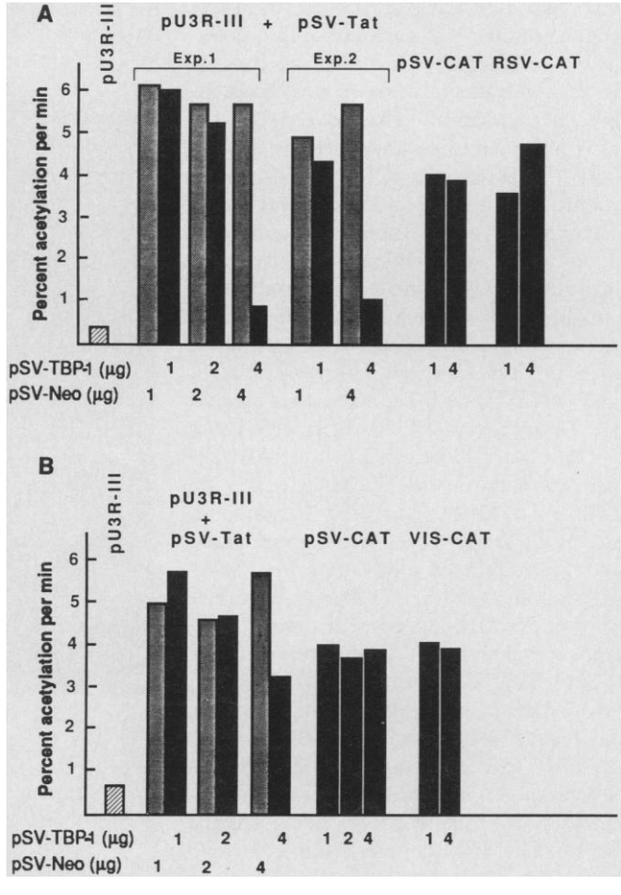


**Fig. 6.** Effect of TBP-1 expression in cotransfection assays. Plasmid pSV–TBP-1, expressing TBP-1 under control of the SV40 promoter, was cotransfected with the plasmid DNAs shown into CHO (**A**) or COS (**B**) cells. Chloramphenicol acetyltransferase (CAT) assays were performed 48 hours after transfection. The percent acetylation per minute of [$^{14}$C]-chloramphenicol to acetylated products was determined. To distinguish between true repression due to TBP-1 expression and down-regulation resulting from competition for transcription factors, the indicator plasmids were cotransfected with similar amounts of plasmid pSV-Neo, which contains the same transcriptional regulatory sequences as pSV-TBP-1, but lacks the TBP-1 gene. Cotransfection with the indicated amount of plasmid (in micrograms) pSV-TBP-1 (black) or pSV-Neo (shaded). Plasmid pU3R-III (*1*) expresses CAT under the control of the HIV-1 LTR; pSV-tat (*18*) expresses the HIV-1 *tat* gene under control of the SV40 promoter; pSV2 CAT (*24*), VISCAT, and RSVCAT express the *cat* gene under control of the SV40, visna, or Rous sarcoma virus transcriptional regulatory sequences, respectively.

## REFERENCES AND NOTES


1. J. Sodroski, R. Patarca, C. Rosen, F. Wong-Staal, W. Haseltine, *Science* **229**, 74 (1985); S. K. Arya, C. Guo, S. F. Josephs, F. Wong-Staal, *ibid.*, p. 69.
2. C. A. Rosen, J. G. Sodroski, W. A. Haseltine, *Cell* **41**, 813 (1985); J. A. Garcia, D. Harrich, L. Pearson, R. Mitsuyasu, R. Gaynor, *EMBO J.* **7**, 3143 (1988); J. A. Garcia *et al.*, *ibid.* **8**, 765 (1989).
3. J. Hauber and B. R. Cullen, *J. Virol.* **62**, 673 (1988).
4. B. M. Peterlin, P. A. Luciw, P. J. Barr, M. D. Walker, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9734 (1986); A. Jakobovits, D. H. Smith, E. B. Jakobovits, D. J. Capon, *Mol. Cell. Biol.* **8**, 2555 (1988); M. J. Selby, E. S. Bain, P. A. Luciw, B. M. Peterlin, *Genes Dev.* **3**, 547 (1989); M. A. Muesing, D. H. Smith, D. J. Capon, *Cell* **48**, 691 (1987); S. Feng and E. C. Holland, *Nature* **334**, 165 (1988); J. Hauber, A. Perkins, E. P. Heimer, B. R. Cullen, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6364 (1987); K. T. Jeang, P. R. Shank, A. Kumar, *ibid.* **85**, 8291 (1988); A. P. Rice and M. B. Mathews, *Nature* **332**, 551 (1988); M. Laspia, A. Rice, M. Mathews, *Cell* **59**, 283 (1989).
5. B.R. Cullen, *Cell* **46**, 973 (1986).
6. C. M. Wright, B. K. Felber, H. Paskalis, G. N. Pavlakis, *Science* **234**, 988 (1986).
7. M. B. Feinberg, R. F. Jarrett, A. Aldovini, R. C. Gallo, F. Wong-Staal, *Cell* **46**, 807 (1986); C. A. Rosen *et al.*, *Nature* **319**, 555 (1986); M. Braddock *et al.*, *Cell* **58**, 269 (1989); B. K. Felber and G. N. Pavlakis, *Science* **239**, 184 (1988).
8. C. Dingwall *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6925 (1989); W. E. G. Muller *et al.*, *J. Biol. Chem.* **265**, 3803 (1990).
9. P. Sharp and R. Marciniak, *Cell* **59**, 229 (1989).
10. C. R. Vinson, K. L. LaMarco, P. F. Johnson, W. H. Landschulz, S. L. McKnight, *Genes Dev.* **2**, 801 (1988); H. Singh, J. H. Bowitz, A. S. Baldwin, Jr., P. A. Sharp, *Cell* **52**, 415 (1988).
11. R. A. Young and R. W. Davis, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1194 (1983); J. M. Sikela and W. E. Hahn, *ibid.* **84**, 3038 (1987).
12. R. Gentz, C. H. Chen, C. A. Rosen, *ibid.* **86**, 821 (1989).
13. Recombinant λgt11 phage were used to infect *E. coli* strain 1090 and plated at a density sufficient to produce $2 \times 10^4$ plaques per 150-mm agar plate. Plates were incubated for 3 hours at 42°C. After this time, nitrocellulose filters, soaked in 10 mM isopropyl β-D-thiogalactoside (IPTG) and dried for 15 min, were placed on the plates for 3 hours at 37°C. Filters were rinsed in TBS buffer [50 mM tris-HCl (pH 7.5) and 150 mM NaCl] for 5 min at room temperature. Biotinylated, purified Tat protein was used to probe the blotted plaques for Tat-binding activity. Routinely 30 μg of Tat was biotinylated in Hepes buffer (pH 7.5) for 3 hours at room temperature in a mixture of biotin-*N*-hydroxysuccinimide ester: purified Tat at 1:10 (w/w). The reaction was terminated by addition of 1 M $NH_4Cl$ to a final concentration of 0.1 M. The free biotin was removed by overnight dialysis against TBS buffer at 4°C. To increase the level of binding between ligand and recombinant fusion protein, we put the nitrocellulose filters through a denaturation-renaturation step using 6 M guanidinium hydrochloride. The filters were first incubated two times 10 min each in 6 M guanidinium hydrochloride solution and then four times, 5 min each, in 3 M guanidinium hydrochloride solution. After blocking for 30 min in binding buffer plus 5% dry milk, the filters were washed three times for 5 min each in binding buffer. The protein-protein binding reaction with the biotinylated protein (1 to 5 μg) was done in binding buffer + 1% dry milk for 1 to 12 hours at 37°C. The

biotinylated complexes were incubated with streptavidin-coupled alkaline phosphatase for 1 hour and detected by incubation with the color reagents *p*-nitroblue tetrazolium (0.33 mg/ml) and BCIP (0.16 mg/ml) in 0.1 M tris-HCl (pH 9.5), 0.1 M NaCl, and 50 mM $MgCl_2$ at room temperature.
14. R. K. Saiki *et al.*, *Science* **239**, 487 (1988); R. K. Saiki *et al.*, *ibid.* **230**, 1350 (1985).
15. P. Nelbock and C. A. Rosen, unpublished observations.
16. D. Stueber *et al.*, *EMBO J.* **3**, 3143 (1984).
17. H. Bujard *et al.*, *Methods Enzymol.* **155**, 416 (1987).
18. S. Ruben *et al.*, *J. Virol.* **63**, 1 (1989).
19. J. Sodroski *et al.*, *Science* **227**, 171 (1985).
20. P. J. Southern and P. Berg, *J. Mol. Appl. Genet.* **1**, 327 (1982).
21. G. N. Pavlakis, B. K. Felber, C. M. Wright in *Human Retroviruses and AIDS: Approaches to Prevention and Therapy*, D. Bolognesi, Ed. (Liss, New York, 1988).
22. F. Sanger, A. R. Coulson, B. G. Barrel, A. J. H. Smith, B. A. Roe, *J. Mol. Biol.* **143**, 161 (1980).
23. The sequence has been deposited with GenBank, accession number M34079.
24. C. M. Gorman, L. F. Moffat, B. M. Howard, *Mol. Cell. Biol.* **2**, 1044 (1982).
25. We thank S. Ruben for helpful discussion and C.-H. Chen for purification of TBP-1 protein.

12 March 1990; accepted 26 April 1990

# Intracellular Calcium Release Mediated by Sphingosine Derivatives Generated in Cells

TARUN K. GHOSH, JUNHUI BIAN, DONALD L. GILL*

**Soluble and hydrophobic lipid breakdown products have a variety of important signaling roles in cells. Here sphingoid bases derived in cells from sphingolipid breakdown are shown to have a potent and direct effect in mediating calcium release from intracellular stores. Sphingosine must be enzymically converted within the cell to a product believed to be sphingosine-1-phosphate, which thereafter effects calcium release from a pool including the inositol 1,4,5-trisphosphate–sensitive calcium pool. The sensitivity, molecular specificity, and reversibility of the effect on calcium movements closely parallel sphingoid base–mediated inhibition of protein kinase C. Generation of sphingoid bases in cells may activate a dual signaling pathway involving regulation of calcium and protein kinase C, comparable perhaps to the phosphatidylinositol and calcium signaling pathway.**

A NUMBER OF PRODUCTS OF CELLULAR lipid breakdown have fundamental roles in signal transduction pathways. The soluble phosphoinositide headgroup, inositol 1,4,5-trisphosphate ($IP_3$) is a major $Ca^{2+}$ signal mediator (*1*) and interacts with intracellular receptors now known to function as $Ca^{2+}$ channels that allow $Ca^{2+}$ release from intracellular pools (*2*). Hydrophobic lipid breakdown products are also major signal mediators, including diacylglycerol (*3*), arachidonic acid, prostaglandins and related derivatives (*4*), and the breakdown products of sphingolipids (*5, 6*). In the latter case, evidence has revealed sphingosine and its derivatives to be powerful inhibitors of protein kinase C (PKC) activity (*5–9*), opposing the action of diacylglycerol, and suggesting that they participate as endogenous modulators of cell function (*5*). Sphingosine is also a potent inhibitor of several calmodulin-activated enzymes, including $Ca^{2+}$-calmodulin–dependent protein kinase (*10*). Moreover, sphingosine mediates and modifies a multitude of responses in cells, including platelet aggregation, the neutrophil respiratory burst, killer cell activation, insulin- and growth factor–induced responses, and the growth and differentiation of many cell types (*6*). We now describe a new action of sphingosine derivatives in mediating rapid and profound translocation of $Ca^{2+}$ from intracellular stores.

The movements of intracellular $Ca^{2+}$, the mechanisms of second messenger–activated $Ca^{2+}$ transfer, and the nature of mobilizable $Ca^{2+}$ pools have been studied extensively with saponin-permeabilized cells (*11, 12*). We used permeabilized cells of the $DDT_1MF$-2 smooth muscle cell line (*11–13*) and observed that addition of 30 μM sphingosine induced a large release of $Ca^{2+}$ accumulated within intracellular $Ca^{2+}$-pumping organelles (Fig. 1A). The effect was seen after a lag of 30 s and thereafter was rapid. A similar release of $Ca^{2+}$ was also induced by sphingosylphosphorylcholine (SPC); however, in this case the effect was almost instantaneous. In contrast, addition of up to 100 μM *N*-acetyl sphingosine did not induce any release of $Ca^{2+}$. The structures of these compounds are shown in Fig. 1B. Sphingosine and SPC are both highly effective in blocking PKC activity (*8*). The charged 2-amino position of sphingosine is important for PKC inhibition, no inhibition being observed if this position is acylated, as in *N*-acetyl sphingosine (*5, 7, 8, 14*); thus, we saw an identical specificity profile for $Ca^{2+}$ release. This correspondence of specificity with PKC inhibition (*7, 14*) was extended by observations revealing no effect on $Ca^{2+}$ release of ceramide or sphingomyelin (the respective long-chain *N*-acyl derivatives of sphingosine and SPC) and showing dihydrosphingosine to be equally effective as sphingosine.

The effect of sphingosine on $Ca^{2+}$ release was potent (Fig. 1C); concentrations as low as 0.3 μM induced significant release; the 50% effective concentration ($EC_{50}$) for sphingosine was 1.6 μM. The $EC_{50}$ for SPC was slightly higher, 2.8 μM. In vitro, the effects of sphingosine and its derivatives on PKC activity appear less potent ($EC_{50}$ values of 80 and 120 μM for sphingosine and SPC, respectively). However, lipid dilution effects of these hydrophobic molecules in the mixed micelle assay contributed to a considerably reduced apparent sensitivity (*5, 7, 8*). In vivo, the blocking actions of sphingosine on activation of neutrophils by phorbol ester or on growth of Chinese hamster ovary cells (both effects believed mediated by PKC) are half-maximal between 1 and 2 μM (*14*). Such effects, measured under almost identical cell concentrations as we used here ($5 \times 10^5$ cells per milliliter), have a sensitivity very similar to activation of $Ca^{2+}$ release. If measurements could be performed after yet further dilutions of cells, the sensitivity

**Table 1.** Influence of SPH and SPC on mannose-6-phosphatase (Man-6-Pase) activity in isolated RER vesicles from $DDT_1MF$-2 cells. Membranes used were the B3 (RER-enriched) fraction isolated from cells (*17, 19*). Activity was assessed by colorimetrically measuring inorganic phosphate ($P_i$) released from glucose-6-phosphate (Glc-6-P) or mannose-6-phosphate in the intracellular medium used for $Ca^{2+}$ transport assays (Fig. 1) containing RER vesicles (100 μg/ml), 0.8 mM glucose-6-phosphate or mannose-6-phosphate and either 50 μM sphingosine or SPC, or 0.25% Triton X-100 (TX-100). The assay proceeded for 12 min at 37°C, and liberated $P_i$ was determined by the method of Chen *et al.* (*30*). Results are means ± SD of six determinations. The limit of sensitivity was approximately 1 nmol of $P_i$ per milligram of membrane protein per minute.

| Addition to RER vesicles | Enzyme activity (nmol/mg/min) | |
|---|---|---|
| | Glc-6-Pase | Man-6-Pase |
| None | 38.3 ± 4.3 | <1.0 |
| SPC | 35.0 ± 2.3 | <1.0 |
| SPH | 28.3 ± 0.8 | <1.0 |
| TX-100 | 73.3 ± 1.8 | 71.6 ± 9.9 |
| TX-100 + SPC | 71.8 ± 2.2 | 66.7 ± 1.8 |
| TX-100 + SPH | 67.0 ± 2.9 | 73.3 ± 2.6 |

Department of Biological Chemistry, University of Maryland School of Medicine, Baltimore, MD 21201.

*To whom correspondence should be addressed.