nM) (*30*, *31*). Because the L-Ala$^7$-D-Ala$^8$ fragment of CsA makes no direct contact with cyclophilin A, it is reasonable to expect that the bicyclic fragment of TCsA also does not contact the protein. This expectation suggests that the greater binding affinity of TCsA is due to the conformational properties of TCsA induced by the bicyclic fragment, rather than the result of favorable direct contacts between the protein and the bicyclic fragment.

Although we were less certain that the L-Ala$^7$-D-Ala$^8$ region (of CsA) of the cyclophilin A–CsA complex does not contact calcineurin, the binding data suggest that this speculation has merit. The affinity of cyclophilin A–TCsA for calcineurin [$K_i$ = 78 ± 14 nM; inhibition of phosphatase activity (*32*)] is approximately two to three times that of cyclophilin A–CsA for calcineurin ($K_i$ = 198 ± 16 nM) (*33*). In addition, TCsA is twice as potent an inhibitor of the T cell receptor signaling pathway, as judged by its ability to inhibit the induction in a cellular assay of β-galactosidase activity regulated by the nuclear factor of activated T cells (NFAT) [50% inhibition concentration ($IC_{50}$) for TCsA = 2 nM; $IC_{50}$ for CsA = 4 nM] (*34*, *35*). [NFAT activity appears to be dependent on the calcineurin-mediated dephosphorylation of the cytoplasmic component of NFAT (*36*–*38*).]

The collective inhibitory data from biochemical and cellular assays reveal a systematic enhancement in the cyclophilin A– and calcineurin-binding properties of TCsA relative to those of CsA. The enhancement is small (two- to threefold) but statistically meaningful. The basis for the enhancement is most likely the reduced entropic cost of binding resulting from the designed conformational restraints (conformation entropy). Thus, the results demonstrate the feasibility of using structural information to improve the already high affinity interactions provided by a natural system. The degree of enhancement, however, is a reminder of the enormous complexity of receptor-ligand interactions in the solution phase. For example, it is a significant challenge to correctly anticipate the differential effect of the molecular restraint in TCsA on the entropy of solvation or desolvation of the ligand versus that of the receptor-ligand complex (solvation entropy).

The structure-based studies described herein resulted in the preparation of a single target molecule. The TCsA compound is a structural variant of CsA with signaling inhibitory properties greater than those of CsA. These studies suggest that the analysis of a ligand bound to its receptor can provide valuable information for the rational design of new ligands. This capability augurs well for the structure-based design of new cellular probes and therapeutic agents.

## REFERENCES AND NOTES


1. I. D. Kuntz, *Science* **257**, 1078 (1992).
2. S. L. Schreiber, *Cell* **70**, 365 (1992).
3. K. Wüthrich *et al.*, *Science* **254**, 953 (1991).
4. W. L. Jorgensen, *ibid.*, p. 954.
5. L. D. Zydowsky *et al.*, *Protein Sci.* **1**, 1092 (1992).
6. D. Yang, M. K. Rosen, S. L. Schreiber, *J. Am. Chem. Soc.* **115**, 819 (1993).
7. M. K. Rosen, D. Yang, P. K. Martin, S. L. Schreiber, *ibid.*, p. 821.
8. M. B. Andrus and S. L. Schreiber, unpublished results.
9. H. R. Loosli *et al.*, *Helv. Chim. Acta* **68**, 682 (1985).
10. H. Kessler, M. Köck, T. Wein, M. Gehrke, *ibid.* **73**, 1818 (1990).
11. An NMR study suggests that a water-soluble CsA derivative exists in a number of slowly interconverting conformations in aqueous solution (*17*), and at least seven CsA conformations are populated in dimethyl sulfoxide (*8*).
12. C. Weber *et al.*, *Biochemistry* **30**, 6563 (1991).
13. S. W. Fesik *et al.*, *ibid.*, p. 6574.
14. R. Wenger, *Transplant. Proc.* **18** (suppl. 5), 213 (1985).
15. S. L. Schreiber, N. J. Anthony, B. D. Dorsey, R. C. Hawley, *Tetrahedron Lett.* **29**, 6577 (1988).
16. J. D. Aebi, D. Guillaume, B. E. Dunlap, D. H. Rich, *J. Med. Chem.* **31**, 1805 (1988).
17. Y. Thériault *et al.*, *Nature* **361**, 88 (1993).
18. G. Pflügl, *ibid.*, p. 91.
19. C. Spitzfaden *et al.*, *FEBS Lett.* **300**, 291 (1992).
20. N. H. Sigal *et al.*, *J. Exp. Med.* **173**, 619 (1991).
21. A. A. Patchett *et al.*, *J. Antibiot.* **45**, 94 (1992).
22. J. Liu *et al.*, *Biochemistry* **31**, 3896 (1992).
23. P. A. Bartlett, G. T. Shea, S. J. Telfer, S. Waterman, in *Molecular Recognition: Chemical and Biological Problems*, S. M. Roberts, Ed. (Royal Society of Chemistry, London, 1989), pp. 182–196; P. A. Bartlett *et al.*, in *Chemistry at the Frontiers of Medicine* (Proceedings of the Robert A. Welch Foundation Conference on Chemical Research XXXV, 1992), pp. 45–68.
24. An epimer of **1** has been reported as a β turn mimic [U. Nagai and K. Sato, *Tetrahedron Lett.* **26**, 647 (1985)].
25. M. Lipton, T. Guo, P. A. Bartlett, personal communication.
26. J. M. Scholtz and P. A. Bartlett, *Synthesis*, 542 (1989).
27. J. E. Baldwin and E. Lee, *Tetrahedron* **42**, 6551 (1986).
28. In the 2D-NOESY spectrum of **7**, a nuclear Overhauser enhancement was observed between the protons at C-8a and C-6, which establishes their cis relation and defines the absolute stereochemistry at C-8a to be **R**.
29. G. Fischer, B. Wittmann-Liebold, K. Lang, T. Kiefhaber, F. X. Schmid, *Nature* **337**, 476 (1989); N. Takahashi, T. Hayano, M. Suzuki, *ibid.*, p. 473.
30. Inhibitory constants were calculated from data obtained from two to three measurements at 10 different concentrations. Determination of $K_i$ values for rotamase inhibition was carried out as described [B. E. Bierer *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9231 (1990)].
31. The binding of TCsA to cyclophilin A is better than that of all of the roughly 100 CsA analogs reported, with the exception of [(Boc)Dab]$^8$-CsA ($K_i$ = 2.4 nM), whose reported affinity for cyclophilin is comparable to that of TCsA [J. L. Kofron, P. Kuzmic, V. Kishore, E. Colon-Bonilla, D. H. Rich, *Biochemistry* **30**, 6127 (1991)].
32. A. S. Manalan and C. B. Klee, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4291 (1983).
33. Inhibitory constants were calculated from seven (TCsA) and four (CsA) measurements with the equation reported in (*22*). The reported values are an average of these measurements and their standard deviation.
34. S. Fiering, J. P. Northrop, G. P. Nolan, G. R. Crabtree, L. A. Herzenberg, *Genes Dev.* **4**, 1823 (1990).
35. Inhibitory constants were calculated from data obtained at seven different concentrations (four to seven measurements at each concentration).
36. W. F. Flanagan, B. Corthésy, R. J. Bram, G. R. Crabtree, *Nature* **352**, 803 (1991).
37. S. L. Schreiber and G. R. Crabtree, *Immunol. Today* **13**, 136 (1992).
38. P. G. McCaffrey, B. A. Perrino, T. R. Soderling, A. Rao, *J. Biol. Chem.* **268**, 3747 (1993).


39. We thank P. J. Belshaw for synthesizing MeBmt, an amino acid used in the synthesis of the hexapeptide **10**. Supported by a grant from the National Institute of General Medical Sciences (GM-38627) and a postdoctoral fellowship from NIH (D.G.A.).

14 June 1993; accepted 18 August 1993


# An Antiviral Soluble Form of the LDL Receptor Induced by Interferon


Dina G. Fischer,* Nathan Tal, Daniela Novick, Sara Barak, Menachem Rubinstein†



**Interferons, which induce several intracellular antiviral proteins, also induce an extracellular soluble protein that inhibits vesicular stomatitis virus (VSV) infection. This 28-kilodalton soluble protein was purified to homogeneity and identified by protein sequencing as the ligand-binding domain of the human 160-kilodalton low density lipoprotein receptor (LDLR). The existence of an antiviral soluble LDLR was confirmed by immunoaffinity chromatography with monoclonal antibody to LDLR. This soluble receptor mediates most of the interferon-triggered antiviral activity against VSV, apparently by interfering with virus assembly or budding, and not by inhibiting virus attachment to cells.**


Interferons (IFNs) are virus- and mitogen-induced cytokines that function by several mechanisms to induce resistance against many types of viral infections. IFNs induce several proteins, some of which have a known role in mediating the antiviral state. Among the antiviral proteins induced are the Mx proteins, 2′,5′-oligoadenylate synthetase, protein kinase (P68), and the RNA binding protein 9-27 (*1*–*4*). Many other IFN-induced proteins have been detected (*5*), but their role in establishing the antiviral state is either indirect or unclear. The majority of IFN-induced proteins are

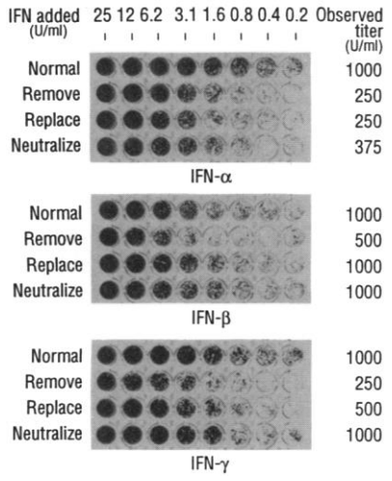


**Fig. 1.** Evidence for formation of AVH in IFN-treated cell cultures. Titration of IFN-α2, -β, and -γ was done on WISH cells in 96-well plates. Cells were incubated overnight with a series of twofold-diluted stocks (1000 U/ml, calibrated against the respective NIH standards) of recombinant human IFNs (final nominal concentration in the well indicated at the top). The next day, neutralizing anti-IFN was added (Neutralize) or the medium was replaced, either by fresh medium lacking IFN (Remove) or fresh medium containing the same concentration of recombinant IFN (Replace). VSV was then added to all wells, including the standard assay row (Normal). The cytopathic effect was scored after 20 to 24 hours and used for calculation of the observed titer (right column), taking the titer in Normal rows as 1000 U/ml.

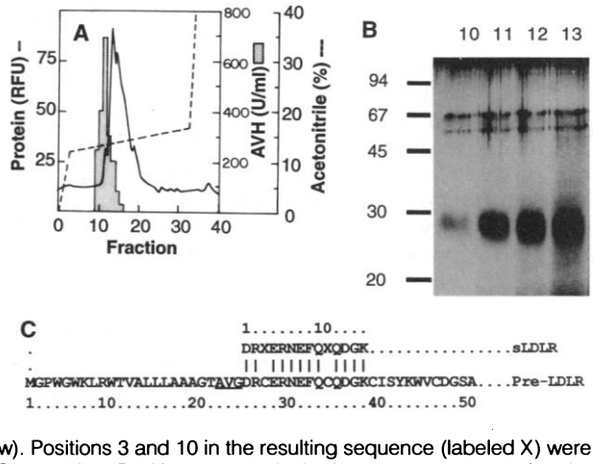


**Fig. 2.** Isolation and identification of AVH as sLDLR. (**A**) The final step of the purification process (*9*), reversed-phase HPLC at pH 7.5, yielded about 2 μg of pure AVH. (**B**) SDS-PAGE of portions of biologically active fractions of the final HPLC step was done under reducing conditions, and the gel was stained with silver. Lane numbers correspond to the HPLC fractions. (**C**) Protein microsequencing was done on a pool (0.6 μg) of portions from HPLC fractions 10 to 12 (upper row), and the sequence was compared with the $NH_2$-terminus of human pre-LDLR (lower row). Positions 3 and 10 in the resulting sequence (labeled X) were not identified, as expected for a Cys residue. Positions 1 to 24 in the lower row correspond to the signal peptide of human LDLR, including the extra three amino acid residues (underlined) (*18*).

either intracellular or membrane-associated. Secreted proteins include $\beta_2$-microglobulin, plasminogen activator, tumor necrosis factor–α (TNF-α) and -β, and a 30-kD protein whose function is unknown (6), none of which have been implicated directly in the antiviral action of IFN. We now present the first example of an IFN-induced extracellular antiviral protein.

The titration of IFN is done by preincubation of cells with a series of twofold dilutions of IFN, followed by addition of virus, usually without prior removal of the IFN (*7*). We observed that removal of the IFN-containing medium before addition of vesicular stomatitis virus (VSV) reduces the antiviral titer. Furthermore, replacement of the culture medium with a fresh, IFN-containing medium before addition of virus did not fully restore the titer, whereas neutralization of IFN at that stage had little effect on the titer (Fig. 1). These findings, observed with several types of cells, including WISH amnion cells, fibroblasts (SV80), and HeLa cells, led us to conclude that IFN-treated cells produce an extracellular antiviral factor distinct from IFN. It was tentatively named antiviral helper factor (AVH). Calculation of the titers after replacement of IFN indicated that at least half of the total antiviral activity elicited by IFN-α and IFN-γ against VSV is mediated by AVH (Fig. 1).

Concentrated serum-free cell culture supernatants of IFN-γ–treated WISH cells were used as a source of crude AVH for further studies. A quantitative bioassay of AVH, resembling in principle the assay of IFN (*7*), was developed. Serial twofold-diluted crude AVH, mixed with neutralizing monoclonal antibody to IFN-γ (anti–IFN-γ) (*8*), was added to WISH cells in 96-well plates, followed immediately by addition of VSV. After 18 to 24 hours, the antiviral activity was scored by the extent of inhibition of the viral cytopathic effect and also by a virus yield reduction assay. The assay was calibrated against National Institutes of Health (NIH) standards of either IFN-α or IFN-β, whereas IFN-γ exhibits a very low antiviral activity under these assay conditions. This bioassay was used to determine that AVH was nondialyzable and was sensitive to heat, low pH, and trypsin. Size exclusion high-performance liquid chromatography (HPLC) gave a single ~40-kD peak of activity. It was therefore concluded that AVH is a protein. Several types of human cells (for example, WISH, HeLa, and foreskin fibroblasts) were tested as producers of AVH, and all produced comparable amounts of activity in response to IFN-γ induction.

Concentrated, serum-free culture supernatant of IFN-γ–treated WISH cells (typically 20 liters, 2.5 g of protein) was then fractionated by a series of chromatographic steps (9). Each step was monitored by the bioassay and by measurement of protein content. The inducer IFN-γ (pI = 8.5) was eliminated in the first chromatographic (TSK-DEAE) step. Analysis by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions of portions from the final HPLC step gave a single band of 28 kD, coinciding with the biological activity (Fig. 2). The specific activity of this preparation was at least $1.5 \times 10^6$ U/mg.

Protein microsequence analysis of the HPLC-purified AVH (0.6 μg) gave a single sequence consisting of 14 amino acid residues in amounts of 12 to 2 pmol (initial yield 51%). Only cycles 3 and 10, which were later found to correspond to Cys residues, were ambiguous. A comparison of the sequence to the NBRF protein data bank showed 100% identity with the $NH_2$-terminal sequence of the human low density lipoprotein receptor (LDLR, Fig. 2C). The 164-kD LDLR consists of 822 amino acid residues, of which 750 comprise the ectodomain, 22 residues form a single transmembrane domain, and 50 COOH-terminal residues are intracellular. The ligand-binding domain of the receptor consists of the $NH_2$-terminal 292 amino acid residues, which are arranged in seven Cys-rich imperfect repeats (*10*). The molecular weight of AVH and its $NH_2$-terminal sequence indicate that it probably corresponds to the entire 292-residue ligand-binding domain of LDLR. Hence, AVH is a soluble form of LDLR (sLDLR), comprising the ligand-binding domain of this receptor. The $NH_2$-terminal sequence of sLDLR was shorter by three amino acid residues than that of the membrane-associated LDLR (Fig. 2). The observed $NH_2$-terminus of sLDLR is a perfect cleavage site of a signal peptidase (*11*). In contrast, the $NH_2$-terminus of human LDLR has been predicted only by homology to bovine LDLR (*10*) and never confirmed by protein sequencing. Therefore, it is pos-

Department of Molecular Genetics and Virology, Weizmann Institute of Science, Rehovot 76100, Israel.

*Present address: InterPharm Laboratories, Kiryat Weizmann, Ness Ziona 76100, Israel.
†To whom correspondence should be addressed.

sible that the membrane-bound human LDLR is also shorter by three amino acid residues at the $NH_2$-terminus.

Immunoaffinity purification with C7, a monoclonal antibody to the ligand-binding domain of bovine LDLR (*12*), was used to confirm the linkage between sLDLR and the antiviral activity. Crude concentrated sLDLR (AVH) was loaded onto a C7 column, the column was washed and eluted at a high pH, and the eluted fractions were tested for antiviral activity. Very low activity was recovered in the eluted fractions, probably because of inefficient binding of sLDLR in the crude supernatant to the C7 column. The experiment was then repeated with partially purified sLDLR from the first step of chromatography (TSK-DEAE) (*9*). All the antiviral activity was bound to the column and then eluted at pH 11 with a recovery of 32% and an 80-fold purification (Fig. 3). Analysis of the eluted fractions by SDS-PAGE under nonreducing conditions gave more than one protein band. However, protein immunoblotting with C7 revealed a specific 28-kD band, detectable only in the eluted fractions in which sLDLR was enriched (Fig. 3, inset). These results confirmed the existence of a 28-kD sLDLR that displayed antiviral activity. The 28-kD band could not be identified in the column load, probably because of its low abundance and interference by comigrating proteins in the gel.

Cells treated with either purified sLDLR or IFN and then with VSV did not show budding of viral particles, as determined by transmission electron microscopy. Neutralizing monoclonal antibodies to IFN-α (*13*) and IFN-γ (*8*), and a polyclonal antibody to IFN-β had no effect on the antiviral activity of sLDLR. Unlike known IFNs, no protection from VSV was seen when cells were preincubated with sLDLR for 6 to 18 hours and washed before addition of VSV. Therefore, we concluded that the antiviral action of sLDLR is independent and different from that of IFNs.

Initially we assumed that LDLR is the VSV receptor and that sLDLR effectively competes for binding to the virus. To test this theory, we examined fibroblasts of familial hypercholesterolemia (FH) patients, who have null mutations in their LDLR gene and therefore express neither LDLR nor sLDLR. Two such fibroblast types, FH350-3 (*14*) and S-233, were as sensitive to VSV as were foreskin fibroblasts of normal individuals. Therefore, we concluded that LDLR is not the VSV receptor, or at least not the only receptor. The FH cells could be protected from VSV, both by various IFNs and by sLDLR. As expected, and unlike other cell types (Fig. 1), replacement of the culture media with fresh IFN before addition of VSV had no effect on the antiviral titer as determined in FH cells, confirming that induction of sLDLR contributes to the overall IFN-induced antiviral activity in normal cells.

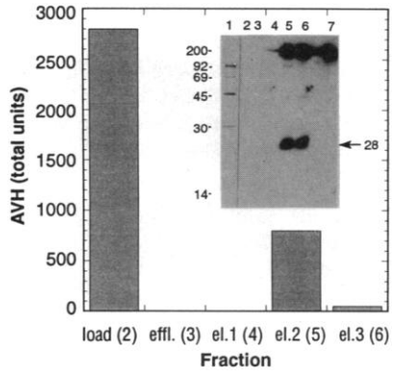


**Fig. 3.** Immunoaffinity chromatography of sLDLR on a C7 column. Partially purified AVH (load, 2800 U, 42 mg, consisting of the 200 mM NaCl peak from the TSK-DEAE step) (*9*) was loaded on a C7 agarose column (0.6 ml). The column was washed and then eluted at pH 11. Portions of each fraction were subjected to bioassay of antiviral activity. The numbers in parentheses correspond to lane numbers in the inset. Portions containing equal amounts of protein were analyzed by SDS-PAGE (12% acrylamide, nonreducing conditions) followed by protein immunoblotting with C7 and $^{125}$I-labeled protein A (inset). Lane 1, molecular size markers (indicated on the left margin); lane 2, load; lane 3, effluent; lanes 4 to 6, elution 1 to 3, respectively; lane 7, C7. The 28-kD sLDLR, observed in elution 2 and 3, is indicated at the right. The intense band below the 200-kD marker represents C7 that had leaked from the column and reacted with $^{125}$I-labeled protein A.

In another set of experiments, the step at which sLDLR mediates antiviral activity was determined. Incubation of WISH cells with VSV for only 5 min and removal of the unbound virus resulted in a complete cytopathic effect after 18 to 24 hours. Addition of purified sLDLR to WISH cells 30 min after infection by VSV gave the same level of protection as obtained when sLDLR was added before the virus. This result was obtained both by a cytopathic effect reduction assay and by measurement of the yield of VSV proteins in the culture supernatants (Fig. 4). The same results were obtained in FH350-3 cells. It was therefore concluded that sLDLR does not act by inhibiting virus binding and that it probably exerts its antiviral effect at a later step. Soluble LDLR probably affects virus assembly or budding, because the yield of VSV proteins, as measured in cell extracts rather than in culture supernatants, was not affected by sLDLR.

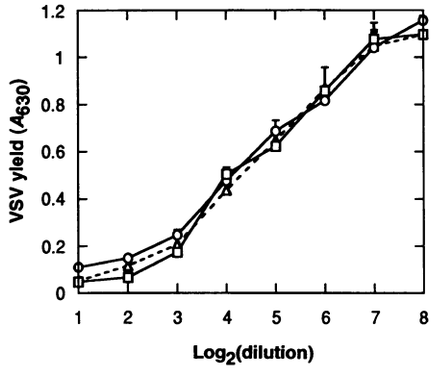


**Fig. 4.** Virus yield reduction assays of IFN and sLDLR. Preformed monolayers of human WISH cells in 96-well plates were treated with a serial twofold dilution of either recombinant IFN-α2 (20 U/ml in dilution 1) (△) or sLDLR (20 U/ml in dilution 1) (□), followed immediately by addition of VSV. In addition, the cells were first treated with VSV, and after 30 min sLDLR (20 U/ml in dilution 1) (○) was added. The VSV yield was determined by enzyme-linked immunosorbent assay with rabbit anti-VSV and horseradish peroxidase–conjugated goat antibodies to rabbit anti-VSV. Maximal yield (no IFN or sLDLR) was (1.29 ± 0.03) $A_{630}$. Background reading [no virus (0.084 ± 0.002) $A_{630}$] was subtracted from all readings.

The induction of all known antiviral proteins by IFNs takes place at the transcriptional level. Therefore, we measured the concentration of cell surface LDLR after incubation with various IFNs. We reacted the cells with C7 and either $^{125}$I-labeled protein A or fluorescein isothiocyanate–labeled second antibody. A dose-dependent induction of cell-associated LDLR protein, up to threefold over the basal level, was seen in WISH cells but not in several other cell types. IFN-γ was the most effective inducer, whereas IFN-β gave the lowest induction. Maximal induction was obtained after incubation with excess (100 U/ml) IFN-γ for about 20 hours, a kinetics similar to that obtained with sLDLR induction. Half-maximal induction, measured after 20 hours, required about 5 U of IFN-γ per milliliter. The induction of cell surface LDLR by IFN on WISH cells may be the mechanism by which a reduction of serum cholesterol occurs in IFN-treated patients (*15*); however, the type of cells or tissues that respond in this way remains to be established.

Soluble proteins corresponding to ligand-binding domains of many polypeptide hormone receptors and cytokine receptors have been described, including soluble receptors to insulin, epidermal growth factor, interleukin-2 (IL-2), growth hormone, IFN-γ, IL-6, TNF, IL-4, and complement (*16*). Therefore, it appears that the existence of soluble variants of cell surface receptors in body fluids is a general phenomenon, and sLDLR may be another member of this growing family of proteins. The function of these different soluble receptors is as yet unknown, and the antiviral

activity of sLDLR indicates that such proteins may perform independent functions that cannot be predicted from the functions of their membrane-associated analogs.

Previous studies have shown that some viruses code for soluble receptor-like molecules that block their respective cytokines, for example, IFN-γ, IL-1, and TNF. Such soluble receptors assist virus infection by suppressing host defense mechanisms (*17*). It now appears that host organisms make use of a similar type of molecule for the opposite role of controlling virus infections. Understanding the mechanism of action of sLDLR will help in determining its possible involvement in other types of viral infections.

## REFERENCES AND NOTES


1. M. A. Horisberger and M. C. Gunst, *Virology* **180**, 185 (1991).
2. I. M. Kerr and R. E. Brown, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 256 (1978).
3. K. Ohtsuki, M. Nakamura, T. Koike, N. Ishida, S. Baron, *Nature* **287**, 65 (1980).
4. P. Constantoulakis *et al.*, *Science* **259**, 1314 (1993).
5. J. Weil, C. J. Epstein, L. B. Epstein, *Nature* **301**, 437 (1983).
6. A. Dolei, F. Ameglio, M. R. Capobianchi, R. Tosi, *Antiviral Res.* **1**, 367 (1981); C. M. Jones, L. Varesio, R. B. Herberman, S. Pestka, *J. Interferon Res.* **2**, 377 (1982); T. L. Gerrard, D. R. Dyer, J. C. Enterline, K. C. Zoon, *ibid.* **9**, 115 (1989); D. Wallach and T. Hahn, *Cell. Immunol.* **76**, 390 (1983); A. D. Luster, R. L. Weinshank, R. Feinman, J. V. Ravetch, *J. Biol. Chem.* **263**, 12036 (1988).
7. S. Rubinstein, P. C. Familletti, S. Pestka, *J. Virol.* **37**, 755 (1981).
8. D. Novick, Z. Eshhar, D. G. Fischer, J. Friedlander, M. Rubinstein, *EMBO J.* **2**, 1527 (1983).
9. Human WISH cells were grown to confluency on Fibracell discs (Sterilin, Aldershot, United Kingdom) in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), in spinner flasks. The serum was then removed, and the cells were induced with IFN-γ (30 U/ml, 18 to 24 hours, 37°C) in MEM supplemented with a protein-free serum substitute (ADC-1, 1:50; Biological Industries, Beit Haemek, Israel), Hepes (20 mM), and insulin (0.2 μg/ml). Culture medium was collected, clarified, and concentrated by ultrafiltration (exclusion size, 10,000). The cell culture was repeatedly induced by IFN-γ and harvested at 24-hour intervals. The concentrated cell culture supernatant was adjusted to sodium borate buffer (pH 8, 20 mM; buffer A) and fractionated on a TSK-DEAE column (2.5 by 33 cm; Merck, Darmstat, Germany) by a stepwise increase of NaCl in buffer A. The protein peak that eluted with 200 mM salt contained antiviral activity. It was pooled, desalted, and loaded on a hydroxyapatite Biogel HTP column (2.5 by 4 cm; Bio-Rad, Richmond, CA). The column was washed with water, and activity was eluted by 15 mM sodium phosphate, pH 6.8. The concentrated active peak was fractionated by an anion exchange HPLC column (1 by 15 cm; Superformance-TMAE-650S, Merck), in a manner similar to the TSK-DEAE step. The active peak was brought to 1.5 M NaCl, loaded on a phenyl Sepharose column (1.6 by 6.5 cm; Pharmacia, Uppsala, Sweden), pre-equilibrated with 1.5 M NaCl in buffer A, and the unbound active protein peak was collected. It was then loaded on an Aquapore RP-300 reversed-phase HPLC column (4.6 by 30 mm; Applied Biosystems, Foster City, CA) in Hepes buffer (pH 7.5, 20 mM) and resolved by an acetonitrile gradient. Active fractions were pooled, diluted, and rechromatographed on the Aquapore RP-300 column (Fig. 2A). Protein concentration was monitored either at 280 nm or by reaction with fluorescamine. Antiviral activity was determined in each fraction in the presence of neutralizing anti-IFN-γ.
10. T. Yamamoto *et al.*, *Cell* **39**, 27 (1984); T. C. Südhof, J. L. Goldstein, M. S. Brown, D. W. Russell, *Science* **228**, 815 (1985); M. S. Brown and J. L. Goldstein, *ibid.* **232**, 34 (1986).
11. G. Von Heijne, *Eur. J. Biochem.* **133**, 17 (1983).
12. U. Beisiegel, W. J. Schneider, J. L. Goldstein, R. G. W. Anderson, M. S. Brown, *J. Biol. Chem.* **256**, 11923 (1981).
13. D. Novick, Z. Eshhar, M. Rubinstein, *J. Immunol.* **129**, 2244 (1982).
14. D. Landsberger *et al.*, *Am. J. Hum. Genet.* **50**, 427 (1992).
15. M. Hawkins *et al.*, *Cancer Res.* **45**, 5914 (1985); E. R. Massaro, E. C. Borden, M. J. Hawkins, D. A. Wiebe, E. Shrago, *J. Interferon Res.* **6**, 655 (1986); I. B. Rosenzweig, D. A. Wiebe, E. C. Borden, B. Storer, E. S. Shrago, *Atherosclerosis* **67**, 261 (1987); E. A. Olsen, G. R. Lichtenstein, W. E. Wilkinson, *J. Am. Acad. Dermatol.* **19**, 286 (1988); F. Boue *et al.*, *Cancer Immunol. Immunother.* **32**, 67 (1990).
16. J. R. Gavin III, D. N. Buell, J. Roth, *Science* **178**, 168 (1972); W. Weber, G. N. Gill, J. Spiess, *ibid.* **224**, 294 (1984); L. A. Rubin *et al.*, *J. Immunol.* **135**, 3172 (1985); D. W. Leung *et al.*, *Nature* **330**, 537 (1987); D. Novick, H. Engelmann, D. Wallach, M. Rubinstein, *J. Exp. Med.* **170**, 1409 (1989); H. Engelmann, D. Aderka, M. Rubinstein, D. Rotman, D. Wallach, *J. Biol. Chem.* **264**, 11974 (1989); B. Mosley *et al.*, *Cell* **59**, 335 (1989); H. F. Weisman *et al.*, *Science* **249**, 146 (1990).
17. C. A. Smith *et al.*, *Science* **248**, 1019 (1990); L. R. Gooding, *Cell* **71**, 5 (1992).
18. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; and W, Trp.


19. We thank R. Eisenstadt for technical assistance. Fibroblast S-233 was provided by S. Eisenberg, Hadassa Medical School, Hebrew University, Jerusalem. Supported by a grant from Interlab Inc., Ness-Ziona, Israel. M.R. is the Edna and Maurice Weiss Professor of Interferon Research.

16 April 1993; accepted 16 July 1993


# Long-Term Synaptic Facilitation in the Absence of Short-Term Facilitation in *Aplysia* Neurons


Nigel J. Emptage and Thomas J. Carew*



**Serotonin (5-HT) induces both short-term and long-term facilitation of the identified synaptic connections between sensory and motor neurons of *Aplysia*. Three independent experimental approaches showed that long-term facilitation can normally be expressed in the absence of short-term facilitation: (i) The 5-HT antagonist cyproheptadine blocked the induction of short-term but not long-term facilitation; (ii) concentrations of 5-HT below threshold for the induction of short-term facilitation nonetheless induced long-term facilitation; and (iii) localized application of 5-HT to the sensory neuron cell body and proximal synapses induced long-term facilitation in distal synapses that were not exposed to 5-HT and had not expressed short-term facilitation. These results suggest that short-term and long-term synaptic facilitation are induced in parallel in the sensory neurons and that the short-term process, because it is induced and expressed at the synapse, can occur locally, but the long-term process, because of its dependence on a nuclear signal, is expressed throughout the neuron.**


It is widely accepted that there are two principal forms of memory storage: short-term memory (STM) and long-term memory (LTM). STM describes a process that retains information temporarily, after which it is thought to become incorporated or transferred into a more stable, long-term store (*1*). One way to address the interdependence between STM and LTM is to examine these processes mechanistically. We have used the identified synaptic connections between tail sensory neurons (SNs) and motor neurons (MNs) of *Aplysia*, which exhibit both short-term and long-term synaptic facilitation following learning (*2*), as a model system to explore the relation between STM and LTM. Using three independent experimental approaches, we found that the induction of the short-term synaptic process is not necessary for the induction of the long-term process.

Considerable evidence indicates that 5-HT participates in both short-term and long-term synaptic facilitation in *Aplysia* (*2*). Montarolo *et al.* (*3*) showed that in cell cultures of SNs and MNs a single application of 5-HT produces short-term facilitation, whereas repeated 5-HT applications produce long-term facilitation. We found that in the intact central nervous system (CNS) a single 5-HT application (5 μM) produced short-term facilitation and multiple applications produced long-term facilitation of the monosynaptic excitatory postsynaptic potential (EPSP) between the tail SNs and MNs (*4*) (Fig. 1A). An analysis of variance (ANOVA) revealed a significant overall effect of 5-HT ($F_{1,5} = 5.84$, $P < 0.008$) (*5*). Subsequent planned comparisons showed that a single 5-HT application produced significant short-term facilitation (mean increase = 157.1%, $t_5 = 2.14$, $P < 0.04$, one-tailed), and five applications produced significant long-term fa-


Departments of Psychology and Biology, Yale University, New Haven, CT 06520.

*To whom correspondence should be addressed.