have not had the benefit of molecular guideposts involving deleted or rearranged chromosomes often seen in tumors. Finally, STFs could also make it possible to isolate very large genes or clusters of tightly linked genes.

REFERENCES AND NOTES

1. H. Harris *et al.*, *Nature* **223**, 363 (1969).
2. B. E. Weissman *et al.*, *Science* **236**, 175 (1987).
3. M. Koi *et al.*, *Mol. Carcinog.* **2**, 12 (1989).
4. C. Huxley, Y. Hagino, D. Schlessinger, M. V. Olson, *Genomics* **9**, 742 (1991).
5. S. J. Goss and H. Harris, *J. Cell Sci.* **25**, 17 (1977).
6. M. Mannens *et al.*, *Hum. Genet.* **81**, 41 (1988).
7. H. J. Scrable, D. P. Witte, B. C. Lampkin, W. K. Cavenee, *Nature* **329**, 645 (1987).
8. R. B. Wadey *et al.*, *Oncogene* **5**, 901 (1990).
9. A. E. Reeve, S. A. Sih, A. M. Raizis, A. P. Feinberg, *Mol. Cell. Biol.* **9**, 1799 (1989).
10. M. A. Skinner, R. Vollmer, G. Huper, P. Abbott, J. D. Inglehart, *Cancer Res.* **50**, 2303 (1990).
11. I. U. Ali, R. Lidereau, C. Theillet, R. Callahan, *Science* **238**, 185 (1987).
12. M. Fujimori *et al.*, *Cancer Res.* **51**, 89 (1991).
13. T. Ehlen and L. Dubeau, *Oncogene* **5**, 219 (1990).
14. E. R. Fearon, A. P. Feinberg, S. H. Hamilton, B. Vogelstein, *Nature* **318**, 377 (1985).
15. A. Weston *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5099 (1989).
16. S. F. Dowdy *et al.*, *Science* **254**, 293 (1991).
17. K. M. Call *et al.*, *Cell* **60**, 509 (1990).
18. L. Bonetta *et al.*, *Science* **250**, 994 (1990).
19. W. E. Loh *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1755 (1992).

19a. M. Koi, L. A. Johnson, A. P. Feinberg, unpublished data.

20. M. Orita, Y. Suzuki, T. Sekiya, K. Hayashi, *Genomics* **5**, 874 (1989).
21. C. G. Cole, P. N. Goodfellow, M. Bobrow, D. R. Bentley, *ibid.* **10**, 826 (1991).
22. A. de Bustros *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5693 (1988).
23. P. N. Schofield *et al.*, *Br. J. Cancer* **63**, 687 (1991).
24. A. E. Fidler, M. A. Maw, M. R. Eccles, A. E. Reeve, *Lancet* **339**, 243 (1992).
25. M. Mannens *et al.*, *Cytogenet. Cell Genet.* **58**, 1967 (1991).
26. I. Henry *et al.*, *Nature* **351**, 665 (1991).
27. Y. Ning *et al.*, *Exp. Cell Res.* **192**, 220 (1991).
28. S. J. Baker, S. Markowitz, E. R. Fearon, J. K. V. Willson, B. Vogelstein, *Science* **249**, 912 (1990).
29. I. Garcia, D. Brandt, J. Weintraub, W. Zhou, M. Aapro, *Cancer Res.* **51**, 294 (1991).
30. E. Redeker and M. Mannens, personal communication.
31. M. Koi, H. Morita, M. Shimizu, M. Oshimura, *Jpn. J. Cancer Res.* **80**, 122 (1989).
32. DNA markers were as follows: A telomere oligonucleotide, $(TTAGGG)_8$ [J. Meyne, R. L. Ratliff, R. K. Moyzis, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7049 (1989)]; H-*ras* [C. Shih and R. A. Weinberg, *Cell* **29**, 161 (1982)]; insulin [G. I. Bell, J. H. Karam, W. J. Rutter, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 5759 (1981)]; IGF2 (detected by PCR; primers were 5′-CCTCTCCTCCTCCACGTCAACCT-3′ and 5′-CGCAGCTCCTGGACAGTGGATAA-3′); H19 (detected by PCR; primers were 5′-AAGGAGCACCTTGGACATCTGGA-3′ and 5′-AACCAGCTGCCACGTCCTGTAAC-3′), D11S724, D11S648, and D11S719 [A. Tanigami *et al.*, *Am. J. Hum. Genet.* **50**, 56 (1992)]; Z104 (P. Little, unpublished data), D11S776, D11S517 (detected by PCR; primers were 5′-GGCTCGGCAGGGACAAATACAGACTC-3′ and 5′-GTGTCAAGTGGGGATGGCATCTTCAGT-3′), and D11S501 [R. B. Wadey, P. F. R. Little, J. Pritchard, J. K. Cowell, *Hum. Genet.* **84**, 417 (1990)]; D11S12 and D11S16 [D. Barker, T. Holm, R. White, *Am. J. Hum. Genet.* **36**, 1159 (1984)]; HBB [R. M. Lawn, E. F. Fritsch, R. C. Parker, G. Blake, T. Maniatis, *Cell* **15**, 1157 (1978)]; TA9 [isolated by inter-Alu PCR (*21*) from A9/74-2]; D11S466, D11S431, and D11S441 [T. Tokino *et al.*, *Am. J. Hum. Genet.* **48**, 258 (1991)]; parathyroid hormone gene [J. Schmidtke *et al.*, *Hum. Genet.* **67**, 428 (1984)]; calcitonin gene [B. D. Nelkin *et al.*, *Biochem. Biophys. Res. Commun.* **123**, 648 (1984)]; WT1 (*18*); catalase [G. A. P. Bruns, T. Glaser, J. F. Gusella, D. Housman, S. H. Orkin, *Am. J. Hum. Genet.* **36**, 25S (1984)]; pSV2neo [P. J. Southern and P. Berg, *J. Mol. Appl. Genet.* **1**, 327 (1982)]; and Alu [P. L. Deininger, D. J. Jolly, C. M. Rubin, T. Friedmann, C. W. Schmid, *J. Mol. Biol.* **151**, 17 (1981)].
33. G. M. Church and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1991 (1984).
34. A. P. Feinberg and B. Vogelstein, *Anal. Biochem.* **137**, 266 (1984).

35. We thank J. Lowe, C. Thompson, D. Law, and S. Emerson for reviewing the manuscript. Supported by NIH grants CA54358 and T32GM07314 and March of Dimes grant 6-FY92-0759.

11 September 1992; accepted 30 December 1992

Import of a Mitochondrial Presequence into Protein-Free Phospholipid Vesicles

Merritt Maduke and David Roise*

A synthetic mitochondrial presequence has been shown to translocate across pure phospholipid bilayers. The presequence was fluorescently labeled so that its association with membranes could be monitored spectroscopically. In the presence of large unilamellar vesicles, the presequence showed time- and potential-dependent protection from reaction with added trypsin and dithionite. The protection was rapidly reversed by treatment of the vesicles with detergent. If the vesicles contained trypsin, the added presequence became sensitive to digestion by the protease. The results show that a mitochondrial presequence can translocate across phospholipid bilayers that lack a hydrophilic translocation pore.

The translocation of proteins across biological membranes is a critical step in the biogenesis of organelles and in the secretion of proteins from prokaryotic and eukaryotic cells. It has been proposed that translocated proteins must pass through a hydrophilic environment in the interior of putative translocator proteins (*1*), but the precise mechanistic role of these proteins has not yet been demonstrated. Alternatively, it has been suggested that translocated proteins may be able to pass directly through the lipid bilayer without the aid of a translocation catalyst (*2*). Support for the role of lipids in protein translocation has come from studies with synthetic targeting sequences. Peptides corresponding to the signal sequences of secreted proteins and to the targeting sequences of mitochondrial and chloroplast proteins have been found to have substantial affinity for phospholipid monolayers and bilayers in model membranes (*3*, *4*). It could be argued that these models do not accurately reflect the behavior of transported proteins in biological systems. However, the recent demonstration that synthetic mitochondrial presequences are rapidly and efficiently imported into isolated mitochondria (*5*, *6*) in a process that involves an initial association of the presequence with the lipid bilayer (*6*) also suggests that affinity for the bilayer is required. At the least, these results suggest that the synthetic molecules can serve as useful probes of the normal protein translocation pathway. In the experiments described here, we have shown that a fluorescently labeled, synthetic mitochondrial presequence can be imported in a potential-dependent manner into pure phospholipid vesicles. The results suggest that mitochondrial presequences may have the inherent ability to pass through phospholipid bilayers and that this ability may be essential for the proper sorting of precursors destined for the mitochondria.

The synthetic peptide used in these studies corresponds to the amino-terminal 25 residues that form the presequence of the precursor of yeast cytochrome oxidase subunit IV (CoxIV). The peptide was labeled with *N*,*N*′-dimethyl-*N*-(iodoacetyl)-*N*′-(7-nitro-2,1,3-benzoxadiazol-4-yl)-ethylenediamine (IANBD amide) at its unique cysteine (residue 19) and purified by high-performance liquid chromatography (HPLC). Binding of the labeled presequence to phospholipid vesicles was rapid and resulted in a large enhancement of the fluorescence of the 7-nitro-2,1,3-benzoxadiazolyl (NBD) group. Measurement of this enhancement as a function of the concentration of the vesicles allowed the affinity of the presequence for the vesicles to be determined (Fig. 1).

Translocation of the presequence into the vesicles was assayed by three independent methods. In the first, suspensions of vesicles with bound presequences were treated with trypsin to digest all presequences remaining outside the vesicles. This treatment resulted in a rapid decrease in the fluorescence, because the fluorescent fragment generated by the proteolysis does

Department of Chemistry, University of California, San Diego, La Jolla, CA 92093–0506.

*To whom correspondence should be addressed.

not bind to the vesicles. In the second method, suspensions of vesicles with bound presequences were treated with sodium dithionite to reduce all NBD groups remaining outside the vesicles. The chemical reduction also resulted in a rapid decrease in the fluorescence, because the reduced form of an NBD group is not fluorescent (*7*). Translocated presequences were resistant to either treatment and remained bound to the membrane and highly fluorescent inside the vesicles. In the third approach, trypsin was trapped within the vesicles. We observed the sensitivity of added presequences to the trypsin by following the decrease in fluorescence of the solution as the translocated presequences were digested inside the vesicles and released from the membrane.

Phospholipid vesicles were generated from suspensions of lipid in buffered potassium solutions by extrusion through polycarbonate filters. Valinomycin was added directly to the vesicles, and diluted stock solutions were prepared in the same buffered solution. To generate a membrane potential, we diluted the vesicle solutions 100-fold into an assay solution that contained 100 mM NaCl in place of KCl. Under these conditions, the vesicles express a valinomycin-induced, potassium diffusion potential of about 120 mV, inside negative. The labeled presequence added to these vesicles became progressively less sensitive to treatment with both trypsin and dithionite (Fig. 2). Sensitivity was immediately recovered when the vesicles were treated with Triton X-100 to dissolve the membranes. Control experiments with vesicles diluted into assay solutions that contained potassium (so that no potential was expressed) showed that the presequence had no decrease in sensitivity to the treatments. The progressive protection of the presequence from digestion by trypsin suggests that it had either become irreversibly associated with the surface of the vesicle in a time- and potential-dependent manner or that the presequence had been completely translocated across the lipid bilayer. The coincident loss of sensitivity of the NBD label to reduction by dithionite also suggests that the presequence had been completely translocated across the bilayer, because an NBD group is sensitive to reduction by external dithionite even when it is embedded in the outer leaflet of lipid vesicles (*7*). Additional control experiments with an NBD-labeled CoxIV peptide that contains a two-residue deletion (Δ-11,12) (*8*) showed that the mutant presequence binds only weakly to the vesicles and is not protected from trypsin or dithionite under the conditions described here (*9*).

The presequences that were protected from digestion by trypsin or from reduction by dithionite were tightly associated with the vesicles. When vesicles that had been treated with trypsin or dithionite were subjected to gel filtration, fluorescence migrated with the vesicles only in the cases in which the vesicles had expressed a negative internal electrical potential (Fig. 3). Vesicles without a negative internal potential showed no associated fluorescence. These results are consistent with those of the steady-state fluorescence assays and demonstrate that the protected presequences were irreversibly associated with the vesicles.

The translocation of the presequence to the interior of the vesicles was confirmed by experiments in which trypsin had been trapped inside the vesicles (Fig. 4). The fluorescence of presequences added to these vesicles under conditions in which most of the presequence was bound was initially high but decreased slowly as the presequence was degraded by the internal protease (Fig. 4, lower traces). In control experiments, presequences added to vesicles that had been treated with an equal amount of trypsin after extrusion displayed stable fluorescence and the expected protection of the NBD from reduction by dithionite (Fig. 4, upper traces). These results indicate that the external trypsin was effectively blocked by the inhibitor and that normal import of the presequence occurred. The added presequence was slowly degraded by trypsin-containing vesicles even in the absence of a membrane potential (Fig. 4B, lower trace). This observation suggests that the potential may be responsible only for shifting the equilibrium distribution of the presequence across the membrane in a Nernstian manner and may not be required for the translocation step itself. Digestion of translocated presequences by the trapped protease could also shift the distribution across the membrane even in the absence of a potential.

The kinetics of translocation of the presequence into vesicles can be calculated by measurement of the change in fluorescence

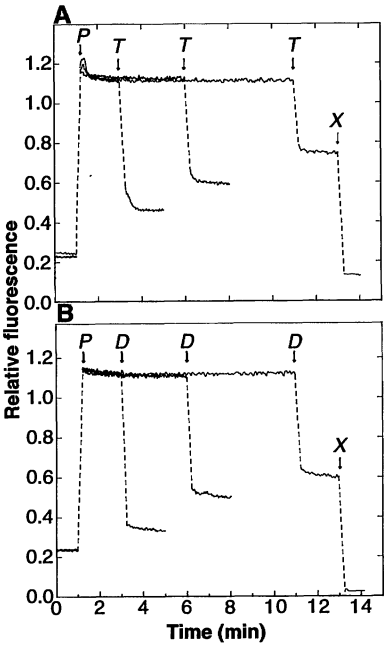

Fig. 2. Time-dependent protection of the fluorescent presequence from trypsin and dithionite. (**A**) and (**B**) each contain superposed traces from three separate experiments. The assays were initiated as described for the binding measurements (Fig. 1) by dilution of vesicles (200 μM final concentration) into 10 mM Na-Hepes (pH 7.0) and 100 mM NaCl. The initial signal is due to light scattering by the vesicles. The labeled presequence was added at 1 min (*P*). Trypsin (*T*, 50 μg) (A) or sodium dithionite (*D*, 10 mM final concentration) (B) was added 2, 5, or 10 min after addition of the presequence. Triton X-100 (*X*, 0.1% final concentration) was added to the longest incubations after the addition of trypsin or dithionite. Because most of the presequence was bound under the conditions used here, the fraction of the initial fluorescence remaining after treatment with either trypsin or dithionite reflects the fraction of the total presequence that was protected. No protection from either trypsin or dithionite was observed at any time if the vesicles were diluted into solutions containing KCl in place of NaCl. The relative fluorescence is a unitless, comparative measure.

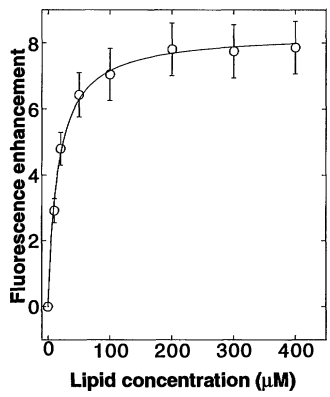

Fig. 1. Binding of the fluorescent presequence to vesicles (*18*). Fluorescence enhancement is defined as $(F - F_0)F_0^{-1}$, where F is the fluorescence of the presequence in the presence of vesicles and F_0 is the fluorescence of the same concentration of presequence in the absence of vesicles but in the presence of trypsin (50 μg). Values of fluorescence were corrected for light scattering by the vesicles. The data were fit to the equation

$$(F - F_0)F_0^{-1} = [(F_{max} - F_0)F_0^{-1}][M]([M] + M_{50})^{-1}$$

where $[M]$ is the concentration of lipid and F_{max} and M_{50} are constants. The best fit gave values of $(F_{max} - F_0)F_0^{-1} = 8.3$ and $M_{50} = 16$ μM. The value of F_{max} corresponds to the fluorescence of the fully bound presequence, and M_{50} is a dissociation constant (*19*). The term M_{50} represents the lipid concentration at which half of the presequence is bound to the vesicles. The line shown was generated from the given values of $(F_{max} - F_0)\ F_0^{-1}$ and M_{50}.

that results from treatment with either trypsin or dithionite at various times (Fig. 2). An analogous approach was recently used to study the import of a fluorescein derivative of the CoxIV presequence into isolated yeast mitochondria (6). Under conditions similar to those used here, the kinetics with mitochondria were found to be first order, with a half-time ($t_{1/2}$) = 3.5 min. An estimate of the import kinetics from Fig. 2 shows a $t_{1/2}$ of approximately 10 min, a value confirmed by detailed kinetics experiments (9). Thus, the rate of import of the presequence into the lipid vesicles is comparable to that observed with isolated mitochondria.

A variety of studies have examined the role of lipids in the translocation of mitochondrial proteins. Two early reports suggested that apocytochrome C could partly translocate itself across pure lipid bilayers (*10*). Subsequent studies demonstrated that synthetic mitochondrial presequences alone could also interact with lipid bilayers (*4*). Although the effects of electrical potentials on these interactions were somewhat ambiguous [compare, for example, (*11*) with (*12*)], the differences may have resulted from structural and functional variations in the presequences used in each study. Indeed, small changes in the structure of the CoxIV presequence dramatically alter the effects of this presequence on isolated mitochondria (*13*); shorter forms of a presequence may be amphiphilic but may not be efficiently translocated. Recently, a membrane potential was shown to affect the intrinsic steady-state fluorescence of various peptides when they were bound to lipid vesicles (*14*). However, because the transmembrane distribution of the peptides was not evaluated in these studies, explanation of the observations is difficult.

The results here show that the CoxIV presequence can be translocated across a lipid bilayer in a potential-dependent manner. These findings are important for two reasons. First, they show that a peptide of medium length, average hydrophobicity, and with multiple charges can readily pass through the hydrophobic barrier of the lipid bilayer, perhaps by a partial disruption of the bilayer structure (*11*). Second, they suggest an alternative explanation for the ability of precursor proteins to be targeted to mitochondria. If mitochondrially directed presequences behave as membrane-permeant cations, the specificity of import may depend entirely on interactions of the presequence with the electrical potential of the mitochondrial inner membrane. Fluorescent dyes that are membrane-permeant cations are frequently used as specific stains for mitochondria in living cells (*15*). The dyes can diffuse passively across all cellular membranes but accumulate in the mitochondria because of the mitochondrial membrane potential. By analogy, the presequence of a newly synthesized precursor protein may be free to bind nonspecifically, but reversibly, to other cellular membranes but would be irreversibly trapped in the mitochondria upon potential-dependent translocation across the mitochondrial membranes. Although there may be mitochondrial proteins that facilitate this process or maintain the precursor in a conformation that is competent for import (*16*), the primary sorting step may be the potential-dependent translocation of the presequence itself. Once the presequence has been translocated, the rest of the precursor protein must follow, perhaps by a Brownian ratchet mechanism (*17*).

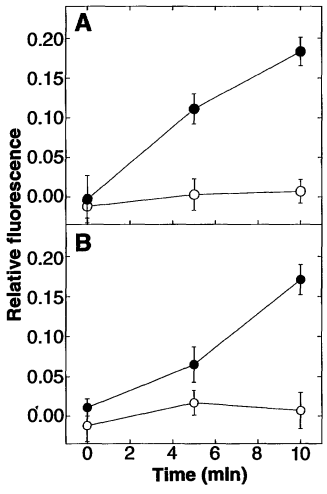

Fig. 3. Gel filtration of the protected presequences. Assays were identical to those described in Fig. 2 except that the vesicles were diluted into either 10 mM Na-Hepes (pH 7.0) and 100 mM KCl (open circles) or 10 mM Na-Hepes (pH 7.0) and 100 mM NaCl (closed circles). After addition of the presequence and subsequent treatment with either (**A**) trypsin or (**B**) dithionite at the indicated times, the solutions (1 ml) were transferred to the top of a column that contained Sephadex G-50 (3 ml) in the same buffer. The solutions were passed rapidly through the column by centrifugation at 1600*g* for 90 s (*20*). The fluorescence of each of the eluted solutions was measured as described in Fig. 1. Values were corrected for background scattering of control vesicles without the presequence.

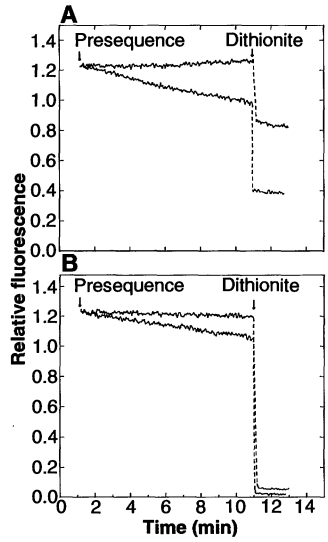

Fig. 4. Sensitivity of the presequence to digestion by trapped trypsin. Assays were performed as described in Fig. 2 except that trypsin was added to the solution used to hydrate and extrude the lipids (lower traces) or, as a control, trypsin was added to the vesicle solution after the extrusion step (upper traces). Soybean trypsin inhibitor (50 μg, sixfold molar excess over trypsin) was included in each assay solution to inhibit the untrapped trypsin. The effectiveness of the inhibitor is apparent in the controls with external trypsin. The traces have been corrected for a small amount of light scattering. (**A**) The vesicles were diluted into 10 mM Na-Hepes (pH 7.0) and 100 mM NaCl to generate a membrane potential. (**B**) The vesicles were diluted into 10 mM Na-Hepes (pH 7.0) and 100 mM KCl so that no membrane potential was generated.

REFERENCES AND NOTES

1. G. Blobel, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1496 (1980); S. J. Singer, P. A. Maher, M. P. Yaffe, *ibid.* **84**, 1015 (1987); S. M. Simon and G. Blobel, *Cell* **65**, 371 (1991); C. V. Nicchitta, G. Migliaccio, G. Blobel, *ibid.*, p. 587; M. Wiedmann, D. Goerlich, E. Hartmann, T. V. Kurzchalia, T. A. Rapoport, *FEBS Lett.* **257**, 263 (1989); U. C. Krieg, A. E. Johnson, P. Walter, *J. Cell Biol.* **109**, 2033 (1989); D. Gorlich, E. Hartmann, S. Prehn, T. A. Rapoport, *Nature* **357**, 47 (1992); K. V. Kellaris, S. Bowen, R. Gilmore, *J. Cell Biol.* **114**, 21 (1991); D. Vestweber, J. Brunner, A. Baker, G. Schatz, *Nature* **341**, 205 (1989); T. Sollner *et al.*, *ibid.* **355**, 84 (1992).
2. G. von Heijne and C. Blomberg, *Eur. J. Biochem.* **97**, 175 (1979); W. Wickner, *Annu. Rev. Biochem.* **48**, 23 (1979); D. M. Engelman and T. A. Steitz, *Cell* **23**, 411 (1981).
3. L. M. Gierasch, *Biochemistry* **28**, 923 (1989); R. van't Hof, R. A. Demel, K. Keegstra, B. de Kruijff, *FEBS Lett.* **291**, 350 (1991); S. M. Theg and F. J. Geske, *Biochemistry* **31**, 5053 (1992).
4. D. Roise and G. Schatz, *J. Biol. Chem.* **263**, 4509 (1988).
5. H. Ono and S. Tuboi, *ibid.*, p. 3188; S. M. Glaser and M. G. Cumsky, *ibid.* **265**, 8817 (1990); Y. K. Pak and H. Weiner, *ibid.*, p. 14298; S. Furuya, K. Mihara, S. Aimoto, T. Omura, *EMBO J.* **10**, 1759 (1991).
6. D. Roise, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 608 (1992).
7. J. C. McIntyre and R. G. Sleight, *Biochemistry* **30**, 11819 (1991).
8. D. Roise *et al.*, *EMBO J.* **7**, 649 (1988).
9. M. Maduke and D. Roise, unpublished data.
10. M. E. Dumont and F. M. Richards, *J. Biol. Chem.* **259**, 4147 (1984); A. Rietveld and B. de Kruijff, *ibid.*, p. 6704.
11. D. Roise, S. J. Horvath, J. M. Tomich, J. H. Richards, G. Schatz, *EMBO J.* **5**, 1327 (1986).
12. I. S. Skerjanc, G. C. Shore, J. R. Silvius, *ibid.* **6**, 3117 (1987).
13. S. M. Glaser and M. G. Cumsky, *J. Biol. Chem.* **265**, 8808 (1990).
14. A. I. de Kroon, J. de Gier, B. de Kruijff, *Biochim. Biophys. Acta* **1068**, 111 (1991).
15. L. B. Chen *et al.*, *Cold Spring Harbor Symp. Quant. Biol.* **46**, 141 (1982); L. B. Chen, *Annu. Rev. Cell Biol.* **4**, 155 (1988).
16. K. P. Baker and G. Schatz, *Nature* **349**, 205

(1991); N. Pfanner, J. Rassow, I. J. van der Klei, W. Neupert, *Cell* **68**, 999 (1992); M. J. Gething and J. Sambrook, *Nature* **355**, 33 (1992).
17. S. M. Simon, C. S. Peskin, G. F. Oster, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 3770 (1992).
18. The presequence Met-Leu-Ser-Leu-Arg-Gln-Ser-Ile-Arg-Phe-Phe-Lys-Pro-Ala-Thr-Arg-Thr-Leu-Cys-Ser-Ser-Arg-Tyr-Leu-Leu-$CONH_2$ was synthesized as in (*11*) and was *S*-alkylated with IANBD amide (Molecular Probes) at Cys^{19} as in (*6*). The labeled presequence was purified by HPLC [Vydac C_{18} column (0.46 cm by 25 cm; Western Analytical Products, Temecula, CA); 25 to 50% gradient of acetonitrile in 0.2% aqueous trifluoroacetic acid]. The concentration of the presequence was determined by amino acid analysis. The labeled presequence had properties similar to those of an acetoxy-NBD derivative of the same presequence (*21*), except that the label was much less sensitive to hydrolysis. Large unilamellar vesicles [50 mM in 10 mM Na-Hepes (pH 7.0) and 100 mM KCl] were generated as in (*19*). Vesicles contained 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocho-line:1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol [4:1 (w:w)]. For the vesicles that contained trypsin, trypsin that had been treated with *N*-tosyl-L-phenylalanine chloromethyl ketone (2 mg ml^{-1}) was included in the solution used to hydrate the lipids. Control vesicles received an identical addition of trypsin after the extrusion step. Valinomycin (9 μM final concentration) was added directly to the concentrated lipid solution. Diluted stocks of the vesicles were made in the same buffered solution. For binding and import experiments, vesicles were diluted 100-fold to the indicated concentrations in 10 mM Na-Hepes (pH 7.0) and 100 mM NaCl or KCl (1 ml) in a fluorescence cuvette at 20°C. After 1 min, the NBD-labeled presequence was added (20 nM final concentration, from a 20 μM stock solution in 50% ethanol). The solution was mixed with the needle of a syringe, and the fluorescence was immediately measured on an SLM-AMINCO SPF-500C spectrofluorometer (excitation, 485 nm; emission, 540 nm; 5-nm bandpass).
19. S. T. Swanson and D. Roise, *Biochemistry* **31**, 5746 (1992).
20. H. Penefsky, *Methods Enzymol.* **56**, 527 (1979).
21. S. Frey and L. K. Tamm, *Biochem. J.* **272**, 713 (1990).
22. We thank J. Kyte, S. Emr, and S. T. Swanson for comments on the manuscript, as well as L. Saunders, J. Zehnder, and S. Kohler. Supported by National Science Foundation grant DCB 90-06691 (D.R.) and National Institutes of Health training grant T32-GM08326 (M.M.).

24 September 1992; accepted 21 January 1993

Neuronal Differentiation Rescued by Implantation of *Weaver* Granule Cell Precursors into Wild-Type Cerebellar Cortex

Wei-Qiang Gao* and Mary E. Hatten*

The migration of postmitotic neurons away from compact, germinal zones is a critical step in neuronal differentiation in the developing brain. To study the molecular signals necessary for cerebellar granule cell migration in situ, precursor cells from the neurological mutant mouse *weaver*, an animal with phenotypic defects in migration, were implanted into the external germinal layer (EGL) of wild-type cerebellar cortex. In this region, labeled *weaver* precursor cells of the EGL progressed through all stages of granule neuron differentiation, including the extension of parallel fibers, migration through the molecular and Purkinje cell layers, positioning in the internal granule cell layer, and extension of dendrites. Thus, the *weaver* gene acts nonautonomously in vivo, and local cell interactions may induce early steps in neuronal differentiation that are required for granule cell migration.

Although a system of radial glial fibers provides an important guidance system for neuronal migrations in the central nervous system (CNS) (*1*, *2*), the molecular signals that induce postmitotic precursor cells to migrate away from germinal zones of the brain are not known. To identify the signals that induce granule neuron differentiation and migration in the developing cerebellar cortex, we used the neurological mutant mouse *weaver* as a model system. In this animal, cerebellar granule cell precursors proliferate normally in the superficial layer of the EGL (*3*, *4*) but fail to extend neurites (*5*) or to migrate away from the EGL (*1*, *6*, *7*).

To establish the dynamics of wild-type granule cell development in situ, dye-labeled, wild-type EGL precursor cells were implanted into wild-type EGL, and the differentiation of labeled cells was followed by fluorescence microscopy (*8*). Between 24 and 48 hours after implantation, labeled precursor cells moved into the deeper aspect of the EGL and initiated parallel fiber extension (Fig. 1A). These cells also generated axons 100 to 200 μm in length before the elaboration of a short, migratory process that descended perpendicular to the plane of axon outgrowth. Over the next 24 to 48 hours, labeled cells migrated through the molecular layer (ML) and Purkinje cell layer (PCL) and settled in the internal granule cell layer (IGL). Within the IGL, the labeled cells began to resemble granule cells morphologically (*1*, *3*): They extended a T-shaped axon into the ML and formed numerous short dendrites (Fig. 1B). Visualization of labeled cells provided evidence that axon extension occurs before the inward migration of the soma of granule cells. This growth matches the order of granule

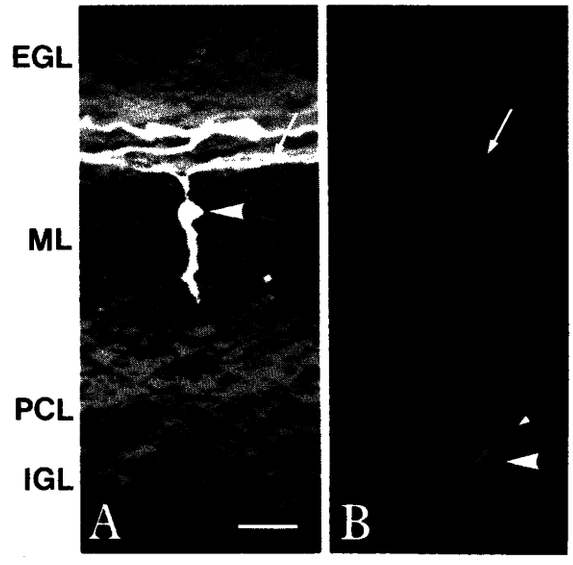

Fig. 1. Differentiation of dye-labeled wild-type EGL precursor cells after implantation into the EGL of early postnatal cerebellar cortex. (**A**) Pseudocolored confocal image. Three days after implantation, labeled cells extended long parallel fibers in the plane parallel to the pial surface (small arrow) and a migratory process (large arrowhead) perpendicular to the pial surface. Labeled cells began to move into the deeper aspect of the EGL 24 to 48 hours after implantation. Individual cells required 6 to 12 hours to transit the molecular layer (ML). (**B**) Fluorescence microscopy image. Six days after implantation, the cell soma of a labeled cell (large arrowhead) was located deep to the Purkinje cell layer (PCL), in the internal granule cell layer (IGL). The axon of the labeled neuron extended toward the molecular layer, forming a T shape characteristic of mature granule cells at the site where the axon bifurcates into the parallel fibers. Within the IGL, the cell extended short, dendritic processes (small arrowhead). Whereas the lipophilic dye PKH-26 stained both the cell body and the processes, fluorescent microbeads were confined to the cell soma (large arrowhead). Bar, 15 μm for both (A) and (B).

Department of Pathology, Center for Neurobiology and Behavior, College of Physicians and Surgeons, Columbia University, New York, NY 10032.

*Present address: Laboratory of Developmental Neurobiology, Rockefeller University, 1230 York Avenue, New York, NY 10021–6399.