10. A.-M. Partanen and I. Thesleff, *Differentiation* **34**, 25 (1987).
11. R. Rugh, *The Mouse* (Burgess, Minneapolis, MN, 1968).
12. W. Y. Langdon, A. W. Harris, S. Cory, J. M. Adams, *Cell* **47**, 11 (1986).
13. L. M. Corcoran, J. M. Adams, A. R. Dunn, S. Cory, *ibid.* **37**, 113 (1984).
14. R. C. Richmond, M. A. Pereira, J. H. Carter, R. E. Lang, *J. Histochem. Cytochem.* **36**, 179 (1988).
15. M. Shibuya, J. Yokota, Y. Ueyama, *Mol. Cell. Biol.* **5**, 414 (1985).
16. Y. Taya *et al.*, *EMBO J.* **3**, 2943 (1984).
17. B. D. Cowley, F. L. Smardo, J. J. Grantham, J. P. Calvet, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8394 (1987).
18. H. Land, F. Parada, R. A. Weinberg, *Nature* **304**, 596 (1983).
19. M. Rentrop, B. Knapp, H. Winter, J. Schweizer, *Histochem. J.* **18**, 271 (1986).
20. The overall photographs shown in Fig. 1 do occasionally mislead the observer. Besides artifactual signals, for example, on the edges, differences in tissue density may lead to false interpretations. True values for specific in situ hybridization are obtained only if grain densities over individual cells are determined and compared to the sense probe background (Table 1). Our description refers to such values and may therefore occasionally appear at odds with the overall photographs in Fig. 1.
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Perineurium Originates from Fibroblasts: Demonstration in Vitro with a Retroviral Marker

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A cellular sheath, the perineurium, forms a protective barrier around fascicles of nerve fibers throughout the peripheral nervous system. In a study to determine the cellular origin of perineurium, a culture system was used in which perineurium forms after purified populations of sensory neurons, Schwann cells, and fibroblasts are recombined. Before recombination, the Schwann cells or the fibroblasts were labeled by infection with a defective recombinant retrovirus whose gene product, β-galactosidase, is histochemically detectable in the progeny of infected cells. Perineurial cells were labeled when fibroblasts had been infected but not when Schwann cells had been infected. Thus, perineurium arises from fibroblasts in vitro and, by implication, in vivo as well.

IN VERTEBRATE PERIPHERAL NERVES, axons are ensheathed by Schwann cells (SCs), and fascicles of axon-SC units are encircled by perineurium. The perineurium consists of concentric, flattened cellular layers interspersed with clusters of collagen fibrils; individual perineurial cells are laden with vesicles and caveolae, extensively linked by tight junctions, and coated with basal lamina (basement membrane) on much of their surface (*1, 2*). The perineurium is virtually impermeable to all proteins that have been tested and restricts the passage of ions (for example, potassium and calcium) and small molecules (for example, glucose) (*2*). By forming a "tissue-nerve barrier," the perineurium regulates the endoneurial milieu and protects axons and SCs from antigens, toxins, infectious agents, and abrupt changes in ionic composition (*2, 3*). Perineurial protection against infection may be particularly important near body surfaces, where fasicles of sensory fibers frequently become exposed after injury. In addition, the perineurium may engender adjustments in nerve length by contractile mechanisms, as judged by the high incidence of actin filaments and associated subplasmalemmal dense bodies in perineurial cells (*4*).

A long-standing question about the perineurium is: What is its cellular source? Several candidates have been proposed (*2, 5–7*), but the two leading contenders have been SCs and fibroblasts (Fbs). (Occasional Fbs reside among axon-SC units, and Fbs form the cellular layers of the epineurium outside the perineurium.) When perineurium forms within developing (*6*) or regenerating (*7*) nerves in vivo, both Fbs and SCs are invariably present. In vitro, perineurium forms when pure populations of Fbs and SCs are combined with sensory neurons and cultured for several weeks; little or no perineurium forms when neurons are cultured with only Fbs or SCs (*8, 9*). However, it has been difficult to decide between Fbs and SCs as perineurial precursors because perineurial cells exhibit some properties of both cell types but are distinct from each one (*10*). Therefore, although indirect evidence favors the idea that perineurium arises from Fbs rather than SCs (*7, 8*), researchers studying peripheral nerve agree (*1, 2, 5–7, 11, 12*) that direct evidence on this point is lacking.

To resolve this issue, we have paired two recently developed techniques. The first is a method whereby separately cultured neurons, Fbs, and SCs can be recombined to form a perineurium that exhibits many attributes present in vivo, that is, basal lamina, collagen fibrils, multiple cellular laminae, junctions of several types, and characteristic vesicles and caveolae (*8, 9*). The second is a method to permanently label the progeny of individual cells (*13, 14*). This method uses a recombinant retrovirus in which the *Escherichia coli lacZ* gene is substituted for retroviral structural genes. When the retrovirus infects a dividing cell, the *lacZ* gene integrates into the host genome and is inherited by daughter cells. The *lacZ*-encoded β-galactosidase, which we designate lacZ, catalyzes a histochemical reaction that yields a blue product visible by light microscopy and an electron-dense product detectable by electron microscopy. Most important for our purposes, the marker is not diluted by the numerous cell divisions that occur as the cultures develop. We were therefore able to prepare neuron-SC-Fb cultures containing either lacZ-positive Fbs or lacZ-positive SCs and then determine which combination gave rise to lacZ-positive perineurium.

Dorsal root ganglia from 15-day rat fetuses were dissociated, placed in culture, and treated with an antimitotic agent to kill SCs and Fbs, leaving a purified neuronal population (*15*). The SCs cultured from separate ganglia were freed of Fbs as described (*15*), then resuspended and added to the neurons, whereupon they proliferated in response to a mitogen that axons present (*16*). Because the retrovirus infects only mitotically active cells (*17*), SCs were infected soon after addition to neurons to take advantage of the wave of SC proliferation that normally occurs; as expected, many axon-related SCs (~10% of the population) but no neurons became lacZ-positive. Embryonic Fbs were cultured from cranial periosteum and infected separately, then added to the neuron-SC cultures 3 weeks later when the neuritic outgrowth had become populated with SCs. After assembly of the neuron-SC-Fb cultures, the cells were given differentiation-supporting medium in which SCs myelinate axons (*15*). Recognizable perineurium formed by 6 weeks of culture, although full differentiation requires additional weeks (*9*). Retroviral infection did not retard development, as demonstrated by extensive myelination and perineurium formation in numerous cultures. After 6 to 8 weeks, the cultures were stained for lacZ (*13*) and prepared for microscopy (*9, 18*).

Our consistent result was that infected Fbs but not infected SCs gave rise to lacZ-positive perineurial cells. In whole mounts of cultures containing neurons, uninfected

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Table 1. Incidence of lacZ-positive perineurial cells in retrovirus-infected cultures. Areas containing numerous lacZ-positive cells associated with well-developed fascicles were selected in whole mounts and sectioned. Regions within the sections were photographed for cell identification (*19*).

Culture type	LacZ-positive regions examined	Regions with LacZ-positive perineurial cells
Neuron, infected SC, and uninfected Fb	18	0
Neuron, uninfected SC, and infected Fb	23	22

SCs, and infected Fbs, lacZ-positive cells appeared to embrace fascicles (Fig. 1, a and b). Sections of such areas demonstrated that the marked cells were present at the fascicle perimeter, where perineurial cells are normally situated, but absent from the fascicle interior (Fig. 1c). Electron microscopy of these cultures confirmed the identity of the lacZ-positive fascicle-associated cells as perineurial (Fig. 1d), and showed that SCs were, as expected, essentially lacZ-negative. In contrast, in cultures containing neurons, infected SCs, and uninfected Fbs, lacZ-positive cells were found sequestered within fascicles (Fig. 1, e to g). Electron microscopy of these cultures confirmed the presence of substantial reaction product in the cytoplasm of SCs but not of neurons, Fbs, or perineurial cells (Fig. 1h). Table 1 summarizes results obtained from 41 lacZ-positive regions in eight cultures from three separate experiments (*19*). From these results, we conclude that perineurium is derived from Fbs but not from SCs.

In light of this conclusion, previous experiments on perineurial development can be reinterpreted and new experiments designed. Some examples follow. (i) Previous experiments demonstrated that periosteal Fbs can substitute for nerve-derived Fbs to form perineurium in vitro (*9*). This observation was interpreted as indicating either plasticity of Fb fate (if Fbs formed perineurium) or the existence of inducer substances from Fbs of different sources (if SCs formed perineurium). Our result supports the first of these alternatives. (ii) Similarly, when segments of sciatic nerve are transplanted into spinal roots or vice versa, the perineurium that forms is more typical of the host region than of the donor area (*12*). A likely interpretation now is that Fbs respond differentially to region-specific organizing influences. (iii) Perineural tumors ("perineuriomas") have been described as a discrete entity, distinct from schwannomas (tumors of SCs) and neurofibromas (which include proliferating Fbs) (*20*). Our result implicates Fbs as the stem cells of these tumors. (iv) In that perineurial cells are coated with basal lamina, linked by tight junctions (*1, 2*), and rich in cytoplasmic contractile elements (*4*), they resemble specialized populations of Fbs, called myofibroblasts, which are found in healing wounds, around seminiferous tubules, and beneath intestinal epithelium (*21*). Our finding that perineurial cells are derived from Fbs supports the supposition (*4*) that they are a type of myofibroblast and encourages a search for factors common to myofibroblast-bearing tissues that promote this pathway of differentiation. (v) Finally, our method of cell marking by retroviral-mediated gene transfer should be useful for directly testing the inference that Fbs form perineurium in vivo and, more generally, for studying the fates of cells that can be infected in isolation and then combined with

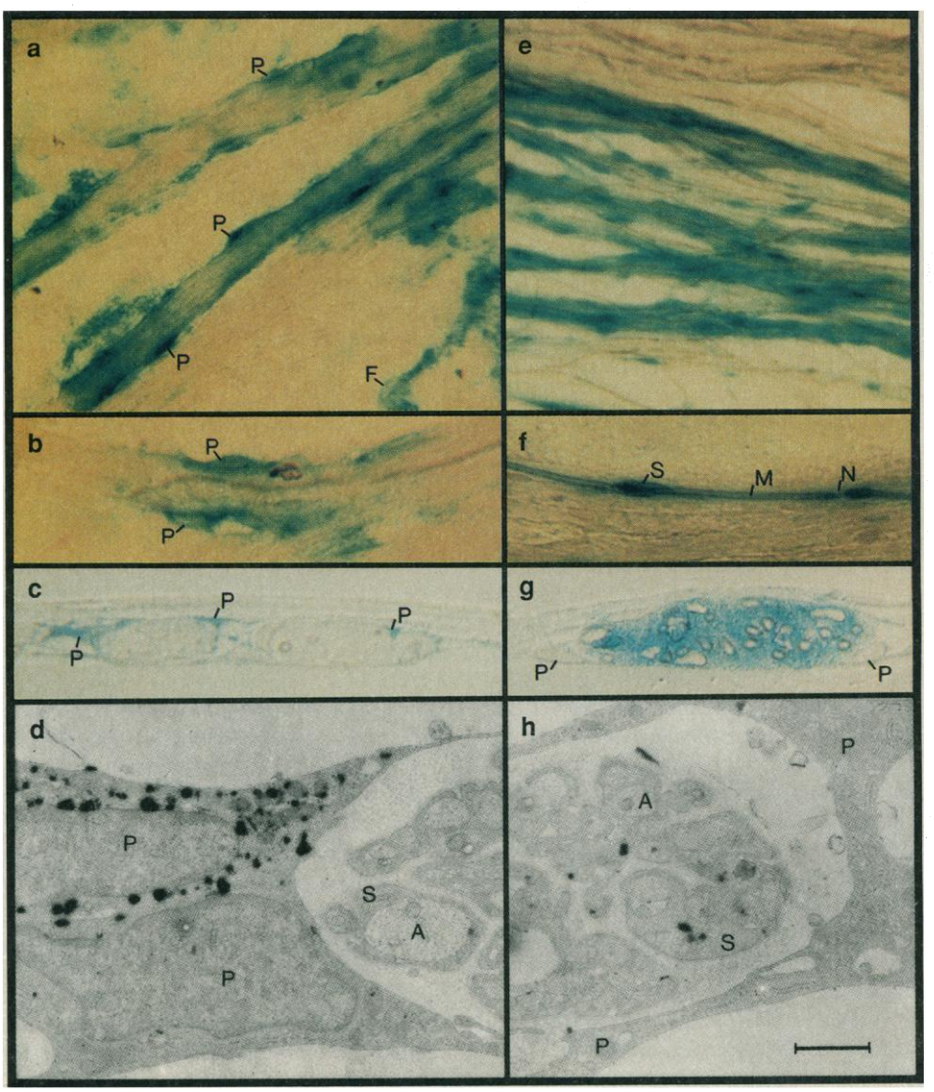

Fig. 1. LacZ-positive perineurial cells are found in cultures derived from retrovirus-infected Fbs and uninfected SCs (**a** to **d**), but not in cultures derived from infected SCs and uninfected Fbs (**e** to **h**). (a and e) Micrographs of whole mounts showing areas containing lacZ-positive cells. (b and f) Higher magnification micrographs of single fascicles in whole mounts. (c and g) Sections (2 μm thick) of labeled fascicles that had been identified in whole mounts, photographed with Nomarski optics. (d and h) Electron micrographs of thin sections serial to regions identified as lacZ-positive in 2-μm-thick sections. LacZ reaction product is blue in light micrographs and appears as black splotches in electron micrographs. In cultures prepared with infected Fbs, labeled cells embracing fascicles (a) were tentatively identified as perineurial in whole mounts (b), and their identity was confirmed in semithin (c) and thin (d) sections. In cultures prepared with infected SCs (e to h), numerous myelinating and nonmyelinating lacZ-positive SCs were found within fascicles, but perineurial cells were lacZ-negative. Abbreviations: A, axon; F, fibroblast; M, myelinated axon; N, node of Ranvier; P, perineurial cell; and S, Schwann cell. Bar is 60 μm for (a) and (e), 25 μm for (b) and (f), 15 μm for (c) and (g), and 1 μm for (d) and (h).

other cell populations, either in vitro or in vivo (*22*).

REFERENCES AND NOTES

1. P. K. Thomas, *J. Anat.* **98**, 35 (1963); W. E. Burkel, *Anat. Rec.* **158**, 177 (1967).
2. P. K. Thomas and Y. Olsson, in *Peripheral Neuropathy*, P. J. Dyck, P. K. Thomas, E. H. Lambert, R. P. Bunge, Eds. (Saunders, Philadelphia, 1984), vol. 1, pp. 97–120.
3. K. Krnjevic, *Q. J. Exp. Physiol.* **39**, 55 (1954); W. D. Waggener, S. M. Bunn, J. Beggs, *J. Neuropathol. Exp. Neurol.* **24**, 430 (1965); Y. Olsson and T. S. Reese, *ibid.* **30**, 105 (1971); E. Reale, L. Luciano, M. Spitzner, *J. Neurocytol.* **4**, 261 (1975); K. Akert, C. Sandri, E. R. Weibel, K. Peper, H. Moor, *Cell Tissue Res.* **165**, 28 (1976).
4. M. H. Ross and E. J. Reith, *Science* **165**, 604 (1969).
5. P. Masson, *Rev. Can. Biol.* **1**, 209 (1942); T. R. Shanthaveerappa and G. H. Bourne, *J. Anat.* **96**, 527 (1962); J. H. Morris, A. R. Hudson, G. Weddell, *Z. Zelforsch. Mikrosk. Anat.* **124**, 76 (1972); F. N. Low, in *The Peripheral Nerve*, D. N. Landon, Ed. (Chapman and Hall, London, 1976), pp. 159–187.
6. H. J. Gamble and A. S. Breathnach, *J. Anat.* **99**, 573 (1965).
7. D. Denny-Brown, *Arch. Neurol. Psychiatry* **55**, 171 (1945); P. K. Thomas and S. Bhagat, *Acta Neuropathol.* **43**, 185 (1978); F. Scaravilli, *J. Anat.* **139**, 411 (1984).
8. M. B. Bunge, A. K. Williams, P. M. Wood, J. Uitto, J. Jeffrey, *J. Cell Biol.* **84**, 184 (1980).
9. A. K. Williams, M. B. Bunge, P. M. Wood, *ibid.* **95**, 2a (1982); M. B. Bunge, A. K. Williams, P. M. Wood, in preparation.
10. For example, perineurial cells resemble SCs but not Fbs in bearing a basal lamina; they are like Fbs but unlike SCs in their capacity to synthesize fibronectin; and they resemble neither Fbs nor SCs in their abundance of caveolae and their interconnections by tight junctions (*2, 12*).
11. J. Peltonen, S. Jaakkola, I. Virtanen, L. Pelliniemi, *Lab. Invest.* **57**, 480 (1987).
12. A. Radek, P. K. Thomas, R. H. M. King. *J. Anat.* **147**, 207 (1986).
13. J. R. Sanes, J. L. R. Rubenstein, J.-F. Nicolas, *EMBO J.* **5**, 3133 (1986). Sanes *et al.* detail the construction of the recombinant retrovirus (LZ1) used here and document its use as a lineage tracer in rodent embryos.
14. J. Price, D. Turner, C. Cepko, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 156 (1987); G. E. Gray, J. C. Glover, J. Majors, J. R. Sanes, *ibid.* **85**, 7356 (1988); M. B. Luskin, A. L. Pearlman, J. R. Sanes, *Neuron* **1**, 635 (1988); C. Walsh and C. L. Cepko, *Science* **241**, 1342 (1988).
15. C. F. Eldridge, M. B. Bunge, R. P. Bunge, P. M. Wood, *J. Cell Biol.* **105**, 1023 (1987).
16. P. M. Wood and R. P. Bunge, *Nature* **256**, 662 (1975).
17. R. Weiss, N. Teich, H. Varmus, J. Coffin, Eds., *RNS Tumor Viruses* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982).
18. R. Ratner *et al.*, *J. Cell Biol.* **103**, 159 (1986).
19. Color slides of semithin plastic sections from both types of cultures were projected, and definitively labeled lacZ-positive cells were identified; cells with only a few or unevenly distributed blue granules were not considered to be lacZ-positive as there is some diffusion of reaction product.
20. S. S. Lazarus and L. D. Trombetta, *Cancer* **41**, 1823 (1978); K. M. Weidenheim and W. G. Campbell, *Virchows Arch. A* **408**, 375 (1986).
21. Y. Clermont, *Exp. Cell Res.* **15**, 438 (1958); M. H. Ross, *Am. J. Anat.* **121**, 523 (1967); G. Gabbiani, C. Chaponnier, I. Hüttner, *J. Cell Biol.* **76**, 561 (1978); G. Gabbiani, in *Connective Tissue Research: Chemistry, Biology and Physiology* (Liss, New York, 1981), pp. 183–194; N. C. Joyce, M. F. Haire, G. E. Palade, *Gastroenterology* **92**, 68 (1987).
22. Lymphocytes have been infected by a retrovirus in vitro and then reintroduced in vivo to study their fate [G. Keller, C. Paige, E. Gilboa, E. F. Wagner, *Nature* **318**, 149 (1985)]. In these studies, however, populations of infected cells were identified by DNA blot analysis, whereas our methods allow histochemical identification of individual cells.

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A Yeast Actin-Binding Protein Is Encoded by *SAC6*, a Gene Found by Suppression of an Actin Mutation

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The protein encoded by *SAC6*, a gene that can mutate to suppress a temperature-sensitive defect in the yeast actin gene, has been identified as a 67-kilodalton actin-binding protein (ABP 67) that associates with all identifiable actin structures. This finding demonstrates the in vivo functional importance of the actin–ABP 67 interaction previously established in vitro and illustrates the use of suppressor analysis to identify physically interacting proteins.

THE EUKARYOTIC CYTOSKELETON IS a dynamic structure characterized by complexity in function, organization, and in the number of protein components. Although these features make understanding the cytoskeleton a fascinating problem, they also provide a considerable challenge. Whereas it has been possible to identify many cytoskeletal constituents, and to determine the functional capacity of these proteins in vitro, it has proven much more difficult to demonstrate functional association of the proteins and the relevance of their biochemical activities in vivo.

Drugs and microinjected antibodies have been useful for probing function in the cytoskeleton, but each of these approaches has limitations. Drug action can be complex (*1*), making interpretation of effects difficult, and although drugs acting on tubulin and actin are known, no drugs targeting associated proteins have been identified. Antibody microinjection has established a role for myosin in cytokinesis (*2*); however, general applicability of this approach is limited by problems of antibody accessibility, and by the difficulty of obtaining inactivating antisera and antisera specific for protein subdomains.

An alternative way to probe functional interactions in the cytoskeleton is through genetics (*3*). Mutants defective in genes encoding cytoskeletal proteins can be isolated and the effects of these mutations can be studied both in vivo and in vitro. A variety of genetic approaches can then be used to identify genes encoding interacting components, and the effects of mutations in these can in turn be studied both in vivo and in vitro.

An important advantage of identifying genes by mutant phenotypes is that the mutant phenotype implicates the biological role of the protein encoded by the gene. Difficulty, however, is usually encountered in determining how the gene product functions on a biochemical level. Conversely, when proteins are identified on the basis of physical interactions and biochemical activities in vitro, difficulty is often encountered in establishing the in vivo function of the protein. We report here a biochemical and genetic study on the actin cytoskeleton of yeast that demonstrates the value of the combined approach.

Saccharomyces cerevisiae has a single essential actin gene (*ACT1*), which has been cloned and sequenced (*4*) and found to encode a protein 90% identical to vertebrate actins. Temperature-conditional lethal (Ts^-) mutations in this gene have been isolated and characterized (*5, 6*), and six genes (*SAC1, 2, 3, 4, 5*, and *6*) that can mutate to suppress the Ts^- defect due to the *act1-1* mutation have been identified (*7, 8*). Genetic evidence suggests that these genes encode components of the actin cytoskeleton. For example, mutations in *SAC6* and *ACT1* can suppress each other's defects. Thus, whereas *act1 sac6* double mutants grow well at all temperatures, *act1* $SAC6^+$ and $ACT1^+$ *sac6* single mutants do not (*8*). Furthermore, *act1* $SAC6^+$ and $ACT1^+$ *sac6*

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