mM $CaCl_2$, 10 mM glucose, 0.001 to 0.01 mM glycine, and 10 mM Hepes, adjusted to pH 7.3 with NaOH and to 310 mOsM with sorbitol. In some experiments 0.1 mM $MgCl_2$ was added. The neuron was continuously perfused at a rate of about 0.1 ml min^{-1} with control or test solution by means of a sewer pipe arrangement (internal diameter, 500 μm); exchange was complete within 1 s. Currents were evoked at 3- to 10-s intervals, filtered in the range 0.2 to 2 kHz, and digitally sampled at 0.5 to 5 kHz. A 10- to 20-mV hyperpolarizing test pulse was used to monitor neuronal input resistance and access resistance throughout the experiment. Sources of drugs were as follows: HA (Sigma or RBI, Natick, MA); CNQX and D-APV (Tocris Neuramin, Bristol, England); glycine and phorbol dibutyrate (Sigma); cimetidine, dimaprit, thioperamide, and *R*-α-methylhistamine (RBI); 2-thiazolylethylamine and impromidine (SmithKline Beecham, Dandenong, Australia); mepyramine and promethazine (gift of D. R. Curtis). Experiments were done at room temperature (24°C).

10. The decay of the control NMDA current in Fig. 2A was fit with two exponentials (τ = 98 ms, 590 ms here) as previously reported [S. Hestrin, P. Sah, R. A. Nicoll, *Neuron* **5**, 247 (1990)]. However, the HA-enhanced current required at least three exponentials (τ = 19, 75, and 425 ms in this case).
11. I accumulated data by applying HA at 1 to 200 μM concentration to each of 10 cells and measuring the increase in peak amplitude of autaptic currents while blocking non-NMDA currents with 10 μM CNQX.
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18. Miniatures were identified visually from sweeps digitally sampled at 1 kHz after filtering at 500 Hz. The non-NMDA channel antagonist CNQX was omitted from external solutions in these experiments because the rapidly rising non-NMDA component of the miniatures provided a landmark with which to align the miniatures for averaging. For the cell in Fig. 3A, the frequency of miniatures during HA application was 1.18 times that during the control period.
19. Rapid steps into NMDA-containing external solution were made by means of an array of glass tubes (internal diameter, 0.5 mm) attached to a piezoelectric multimorph (Philips). Solution flowed continuously through each of the tubes at about 3 ml min^{-1}, completely bathing the cell and its dendritic tree with the solution of choice. The time for solution changes was less than 30 ms, as determined from the junction potential change measured with a cell-free patch electrode. Enhancement due to HA was seen with the application of either 1 mM NMDA (n = 9) or 30 μM to 1 mM L-glutamate (n = 5).
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21. Cultures were incubated for 18 to 43 hours with pertussis toxin, with 0.1% dimethyl sulfoxide as the vehicle. In control cells, taken from the same culture plating and treated with vehicle alone, 100 μM adenosine produced a complete and reversible block of excitatory autaptic transmission (n = 4).
22. In these experiments the HA effect was monitored for up to 1 hour, and, although the strength of autaptic transmission declined markedly during this time [S. D. Hess, P. A. Doroshenko, G. J. Augustine, *Science* **259**, 1169 (1993)], the relative HA enhancement remained constant. However, there was no independent measure to confirm that GTP-γ-S had reached the postsynaptic terminals.
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A Transglutaminase That Converts Interleukin-2 into a Factor Cytotoxic to Oligodendrocytes

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Regenerating optic nerves from fish produce a factor that is cytotoxic to oligodendrocytes. The cytotoxic factor is recognized by antibodies to interleukin-2 (IL-2) and has the apparent molecular size of a dimer of IL-2. An enzyme, identified as a nerve transglutaminase, was purified from regenerating optic nerves of fish and was found to catalyze dimerization of human IL-2. The dimerized IL-2, unlike monomeric IL-2, is cytotoxic to oligodendrocytes from rat brain in culture. The results suggest that posttranslational modification of a cytokine can alter its activity. Under conditions in which oligodendrocytes inhibit neuronal regeneration, dimerization of IL-2 might provide a mechanism to permit nerve growth.

Axons in the central nervous system of mammals, unlike those of fish, have a poor capacity for regeneration after axonal injury (*1–3*). Injured central nervous system axons do regenerate, however, when grown in a supportive environment, such as in peripheral nervous system grafts (*4*). One of the environmental elements that might be responsible for the poor ability to regenerate in mammals is oligodendrocyte-mediated inhibition of nerve growth (*5*).

Regenerating optic nerves from fish produce a factor cytotoxic to oligodendrocytes that may act to eliminate mature oligodendrocytes (*6–8*). This cytotoxic factor was recognized by antibodies to IL-2 (*8*). We now present data indicating that a dimer of IL-2, produced by a transglutaminase derived from regenerating fish optic nerves, is a factor cytotoxic to oligodendrocytes.

Because the cytotoxic factor from regenerating fish optic nerves was about twice the molecular size of IL-2 derived from fish lymphocytes (*8*), it seemed possible that it could be a dimer of IL-2 produced by a posttranslational modification. We incubated conditioned medium of regenerating fish optic nerves (CM) (*9*) with human IL-2 (hIL-2) and then visualized proteins in the mixture with antibodies to IL-2. A new immunoreactive band (of 30 kD) was observed in addition to the original 15-kD IL-2 monomer (Fig. 1), suggesting that the new immunoreactive band, which was recognized by antibodies to IL-2, might be a dimer of IL-2 and that the CM contains the substances required for the apparent dimerization. Similar results were observed with mouse IL-2. The new band appeared only when Ca^{2+} was added to the preparation. It did not appear in control experiments in which either the CM or IL-2 was present alone.

We considered the possibility that dimerization of IL-2 occurred by means of a Ca^{2+}-dependent enzyme that is selectively synthesized or activated in regenerating nerves after injury. An enzyme of the cross-linking transglutaminase family (*10, 11*) appeared to be a likely candidate. We raised in rabbits antibodies against a 14–amino acid peptide (KKVKYGQCWVFAGV) (*12*) (SWISS-PROT data bank) that contains the active site of transglutaminases and is conserved among tissues and species. Immunoblotting analysis of the CM with affinity-purified antibodies to this peptide (TG-Ab) revealed a 55-kD immunoreactive protein (Fig. 2). We homogenized regenerating or uninjured fish optic nerves immediately after their excision, collected the supernatants after high-speed centrifugation (HSS), and compared them by immunoblotting analysis with the TG-Ab. Densitometric analysis showed that the 55-kD immunoreactive band was three times as intense in regenerating nerves than in uninjured nerves; therefore, regeneration is associated with increased transglutaminase immunoreactivity.

We purified the nerve transglutaminase to apparent homogeneity from HSS or CM of regenerating fish optic nerves by two steps of affinity chromatography, consisting of columns of poly-L-lysine (PLL) and of TG-Ab. The eluates were subjected to gel

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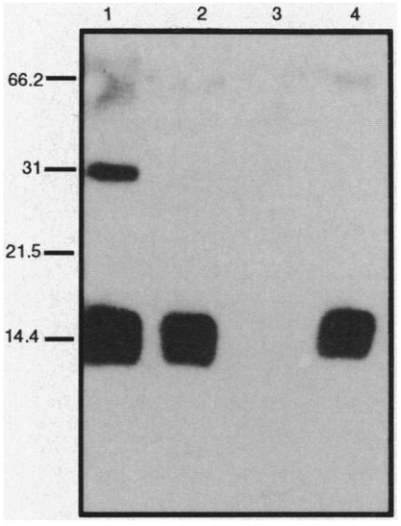

Fig. 1. Dimerization of IL-2 in CM. The CM was prepared as described (*8, 9*). The protein content was determined by the Bradford method (*29*). The CM (6 μg) was incubated for 24 hours at 37°C with hIL-2 (20 ng) in a buffer containing 5 mM $CaCl_2$. The mixture was subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (12% gel) followed by blotting onto a nitrocellulose membrane. Subsequently, the membrane was treated as follows: incubated overnight at 4°C with phosphate-buffered saline (PBS) containing 5% (w/v) milk, washed in PBS, incubated with antibodies to IL-2 for 2 hours at 37°C, washed three times for 5 min in PBS containing 0.05% Tween-20, and incubated with horseradish peroxidase–conjugated goat antibodies to rabbit immunoglobin G for 2 hours at room temperature. Immunoreactive bands were visualized by the chemiluminescence method (ECL, Amersham). Lane 1, CM incubated with hIL-2 in the presence of Ca^{2+}; lane 2, CM incubated with hIL-2 and no Ca^{2+}; lane 3, CM with only Ca^{2+}; and lane 4, hIL-2 alone. Molecular size markers are indicated at the left in kilodaltons.

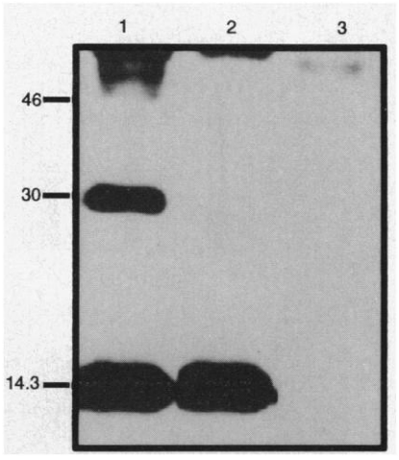

Fig. 4. Dimerization of hIL-2 in the presence of the purified TG_N. Lane 1, purified TG_N incubated with hIL-2 (20 ng); lane 2, hIL-2 (20 ng) only; lane 3, TG_N only. The experiment was performed as described (Fig. 1), but with purified TG_N substituted for CM. All incubations were carried out in the presence of 5 mM Ca^{2+} and heat-inactivated fetal calf serum (0.3%).

electrophoresis and visualized by silver staining. From the TG-Ab eluate, a single band of 55 kD was detected (Fig. 3). A single 55-kD band was also detected by protein immunoblotting with the TG-Ab (Fig. 3B). To confirm that the purified enzyme is a member of the transglutaminase family, we measured incorporation of [^{14}C]-putrescine into *N,N*-dimethylcasein. Both the crude CM and the substances obtained at each purification step catalyzed this reaction (Table 1).

The purified nerve transglutaminase (TG_N) was tested for its ability to dimerize IL-2. In the presence of IL-2 and Ca^{2+}, TG_N displayed the expected dimerization activity. In addition to the IL-2 band, a 30-kD IL-2–immunoreactive band was also observed (Fig. 4). Because the nerve transglutaminase was applied in a purified form, this result suggests that the 30-kD band is, indeed, a covalent dimer of IL-2. About 25% of the IL-2 was dimerized under these conditions.

To determine whether the dimeric IL-2 is cytotoxic to oligodendrocytes, we incubated IL-2 with TG_N in the presence of Ca^{2+} and then applied it to cultures of rat brain oligodendrocytes. Viable oligodendrocytes were assessed by spectroscopy and microscopy (Fig. 5). The number of oligodendrocytes was reduced when the concentration of IL-2 incubated with TG_N in the presence of Ca^{2+} was 100 U/ml (50 ng/ml) but not 10 U/ml (Fig. 5). In the absence of TG_N, IL-2 had no detectable activity. Likewise, neither the addition of TG_N alone nor the addition of a mixture of IL-2 (100 U/ml) and TG_N that had been incubated without Ca^{2+} was cytotoxic to the cultured oligodendrocytes. Because 25% of the original IL-2 was dimerized in the presence of TG_N and 100 U/ml of IL-2 (Fig. 4), we estimate that 25 U/ml (12.5 ng/ml) of dimeric IL-2 is cytotoxic under conditions in which 100 U/ml of monomeric IL-2 is not cytotoxic. This concentration of dimeric IL-2 is

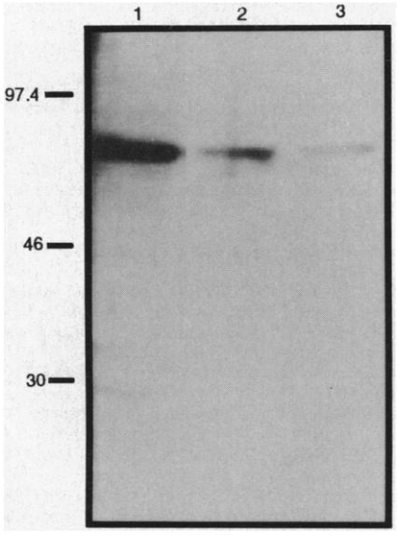

Fig. 2. Immunoreactive transglutaminase in regenerating fish optic nerves. Lane 1, CM (20 μg); lane 2, HSS from regenerating optic nerves (16 μg); and lane 3, HSS from uninjured nerves (20 μg). All preparations were separated by SDS-PAGE (10% gel). Subsequent immunoblotting analysis was conducted with the TG-Ab, following the procedure described in Fig. 1. Molecular size markers are indicated at the left in kilodaltons.

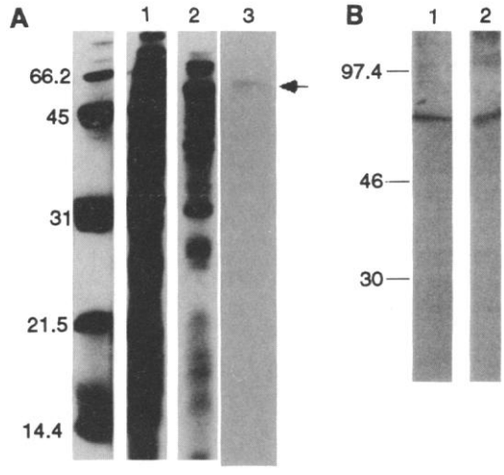

Fig. 3. Purification of TG_N from regenerating fish optic nerves. (**A**) Optic nerves (n = 60) were excised 6 to 7 days after crush injury and homogenized in a buffer containing tris-HCl (10 mM, pH 7.4), $CaCl_2$ (1.5 mM), spermidine (1 mM), aprotinin (25 μg/μl), leupeptide (25 μg/μl), pepstatin (5 μg/μl), and sucrose (0.25 M). After centrifugation (1 hour at 4°C at 150,000*g*) HSS was collected and passed through an affinity column of PLL coupled to agarose. The resulting eluate was subjected to an additional affinity column of TG-Ab. Bound substances were eluted from the column with 0.2 M glycine (pH 2.7) and neutralized with 1 M tris (pH 8.0). After each step, protein content was determined and aliquots were analyzed by SDS-PAGE. The separated proteins were visualized by silver staining. Lane 1, HSS; lane 2, eluate from PLL column; lane 3, eluate from TG-Ab column. (**B**) As in (A), TG_N was purified from CM and subjected to SDS-PAGE. The resulting lane was divided into two and one half (lane 1) was silver stained. The other half (lane 2) was transferred to nitrocellulose membrane and probed with the TG-Ab. Molecular size markers are indicated in kilodaltons to the left of each gel.

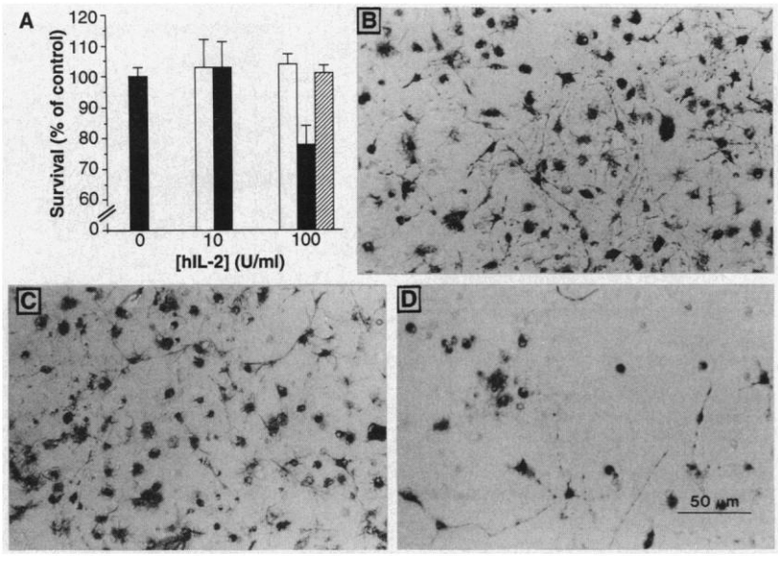

Fig. 5. Cytotoxic effect of dimeric IL-2 on oligodendrocytes. Oligodendrocyte cultures derived from neonatal rat brains were prepared (*30, 31*), seeded in wells coated with PLL (20 μg/μl; Sigma) for 72 hours, and then treated for 48 hours. Cell viability was assessed by the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma) (*32*). (**A**) Oligodendrocytes incubated with hIL-2 (10 U/ml and 100 U/ml) and: TG_N only (hatched bar), TG_N and Ca^{2+} (black bar), or Ca^{2+} only (open bar). Results are expressed as percentage survival (mean ± SD) relative to that of untreated cells and were analyzed by analysis of variance ($n = 3$, $P = 0.02$, $F = 7.83$). Fisher comparison analysis revealed that the group incubated with hIL-2 and TG_N in the presence of Ca^{2+} (containing the dimeric hIL-2) is significantly different at 95% from all other incubated groups. Photographs show MTT-stained cultures incubated with (**B**) hIL-2 only (100 U/ml), (**C**) TG_N only, and (**D**) a mixture of hIL-2 (100 U/ml) and TG_N in the presence of Ca^{2+}.

Table 1. Transglutaminase was purified from medium conditioned by regenerating fish optic nerves. The yield, obtained from two preparations, is given by the mean ± SD of the percentage of total protein in crude CM. Activity characteristic of transglutaminase was measured by incorporation of [^{14}C]-putrescine into *N,N*-dimethylcasein (*20*). The purified enzyme eluted from the TG-Ab affinity column with glycine was dialyzed for 2 hours in the presence of *N,N*-dimethylcasein (1 mg/ml) before being added to the reaction mixture. The reaction was initiated by addition of crude CM (2 to 25 μg), PLL eluate (0.5 to 5 μg), or TG-Ab eluate (0.7 to 10 ng) followed by incubation for 20 min at 37°C and terminated by the addition of ice-cold trichloroacetic acid (final concentration, 5%). Specific activity values in counts per minute (cpm) are mean ± SD ($n = 3$, two purifications, three assays). The specific activity of the TG-Ab eluate is about 200 times that of the original CM. Because the enzyme appears to be pure (Fig. 4), this relatively small increase in activity might be explained by the loss of activity in the course of dialysis or the presence of traces of glycine which competes with putrescine.

Preparation	Yield of protein (%)	Specific activity of [^{14}C]putrescine per microgram of protein (cpm)
CM	100	2,500 ± 1,200
	Eluate	
PLL	24.1 ± 2.6	8,420 ± 350
TG-Ab	0.035 ± 0.005	502,000 ± 115,000

within the concentration range (1 to 100 U/ml) of the physiological activity of IL-2 (*13, 14*).

The transglutaminases are a large family of enzymes that are prevalent in a variety of cells and tissues (*15–19*) and are known to function in the stabilization of intra- and extracellular molecules in various physiological and pathological processes (*10, 11*). In the nervous system, the physiological role and substrates of the enzyme remain unclear. Cytosolic transglutaminases increase in activity after axotomy of fish optic nerves (*20*) and of peripheral nerves (*21, 22*) and are implicated in the regulation of growth and differentiation of several cells, including neuronal cells (*23, 24*).

The transglutaminase we have purified from the fish nervous system has characteristics common to all transglutaminases: its activity is Ca^{2+}-dependent, it is recognized by an antibody to a conserved active-site region of transglutaminases, and it has an activity common to all transglutaminases (*10, 11*). The discovery of TG_N in the present study may allow the preparation of a factor cytotoxic to oligodendrocytes of any species. Conversion of IL-2 to a cytotoxic form might be a way by which mammalian nerves could overcome an obstacle to regeneration imposed by mature oligodendrocytes (*5*). In fish, although oligodendrocyte-like cells are permissive to neuronal growth (*25*), adult optic nerves are permissive to growth of embryonic neurons (*26, 27*) but not permissive to growth of adult neurons unless they undergo postinjury changes (*28*) that include production of the factor cytotoxic to oligodendrocytes (*8*).

The present study provides evidence for a mechanism in which an existing active factor is converted to a form having a different type of activity. It remains to be seen whether this is a unique case or an example of a more general mechanism.

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