rapid kinetics and would be consistent with all other data. It would be surprising if integrins played a role in synaptic function only at frog motor nerve terminals. However, with the exception of a report that RGD peptides block hippocampal long-term potentiation (*25*), other instances have not been described. Integrins, which can transduce signals in both directions across the cell membrane (*17, 18, 23, 24*), might be involved more generally in cell-cell trophic-inductive interactions, in maintaining synaptic organization, and in regulating synaptic function. Integrins appear also to play a role in the enhancement of transmitter release by hypertonic solutions (*26*) and, if involved in helping regulate transmitter release at central nervous system synapses, might contribute to the excitotoxic response to brain swelling with ischemia.

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6. Sartorius nerve muscle preparations were dissected from mature (4 to 5 cm body length) *Rana pipiens* and held in NFR (116 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $NHCO_3$, 3 mM dextrose, and 5 mM Hepes, pH 7.4) in an apparatus that permitted elongation or shortening of the muscle at both ends simultaneously. EPPs and mEPPs were recorded by means of "floating" microelectrodes consisting of just the distal shank and tip of a microelectrode, filled with 0.6 M K^+ acetate and 5 mM KCl and held on the end of a coiled flexible electrode wire. A suction electrode was used to stimulate the sartorius nerve.
7. Rest length was defined as the muscle length in situ with the frog on its back with its hind limbs extended at approximately 45° from the body axis and extended directly back from the knee. The sarcomere spacing at rest length was 2.2 to 2.3 mm, and 1-mm stretch represented approximately a 3% increase in muscle length.
8. To avoid spontaneous twitching in Ca^{2+}-free Ringer solution, we pretreated the preparation for 15 min with 25 mM μ-conotoxin, which blocks Na^+ channels in muscle, but much less effectively in nerve (*27, 28*), and has no apparent effect on transmitter release or synaptic potentials.
9. Preparations were immersed for 1.5 hours in Ca^{2+}-free Ringer containing 25 mM BAPTA-AM, made from a stock solution of 5 mM in dimethylsulfoxide (DMSO), with 0.02% pluronic acid (Molecular Probes) to facilitate loading of the dye and 20 mM tetrakis(2-pyridylmethyl) ethylenediamine (TPEN, Molecular Probes) to chelate heavy metals, according to techniques worked out by Robitaille and Charlton (*28*). The -AM form of the buffer is membrane-permeant but becomes trapped and concentrated inside the terminal into the Ca^{2+}-binding form after esterification. Controls were treated with the same final concentration of DMSO, pluronic acid, and TPEN.
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21. Preparations were treated for 1.5 hours in LD Ringer solution, then returned to NFR for 1 hour before testing, at 1:100 serum dilution throughout. The antigen used to generate the P25 immunoglobulin G was a β_1 fusion protein of about 50% of the full sequence.
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A VAMP-Binding Protein from *Aplysia* Required for Neurotransmitter Release

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Before the fusion of synaptic vesicles with the plasma membrane, a protein complex is thought to form between VAMP—an integral membrane protein of the vesicle—and two proteins associated with the plasma membrane, SNAP-25 and syntaxin. The yeast two-hybrid interaction cloning system has now been used to identify additional proteins from *Aplysia* that interact directly with VAMP. A 33-kilodalton membrane protein, termed VAP-33 (VAMP-associated protein of 33 kilodaltons), was identified whose corresponding messenger RNA was detected only in the central nervous system and the gill of *Aplysia*. Presynaptic injection of antibodies specific for VAP-33 inhibited synaptic transmission, which suggests that VAP-33 is required for the exocytosis of neurotransmitter.

A fundamental property of all neurons that interact by means of chemical synaptic transmission is that neurotransmitter is stored in synaptic vesicles in the nerve terminal and released by exocytosis in response to an increase in presynaptic Ca^{2+} (*1*). Transmitter release can be modulated by activity, and such modulation is a common feature of synaptic plasticity that is thought to contribute to memory formation. Significant progress toward a molecular description of transmitter release has been achieved by identifying and analyzing proteins associated with synaptic vesicles (*2, 3*). The purification of vesicle-associated proteins and the isolation of their cognate genes (*4*) have been facilitated by the abundance of synaptic vesicles in the brain. Other proteins have been identified by their ability to interact physically with vesicle-associated proteins in immune complexes (*5, 6*). However, conventional biochemical binding assays and immunoprecipitation methods rely on the formation of relatively stable, high-affinity protein-protein complexes; they do not detect weaker or more transient interactions. To identify proteins important for vesicle exocytosis that bind more weakly to components of previously identified synaptic complexes, we have used the two-hybrid system developed in yeast (*7*). With this approach, we have now identified a protein from *Aplysia californica* that specifically interacts with the synaptic vesicle protein VAMP (also termed synaptobrevin) (*8*).

The partial complementary DNA (cDNA) clones isolated in this screen contained an open reading frame encoding a 19-kD polypeptide. With a polymerase chain reaction (PCR)–based strategy, additional cDNAs were isolated that contained a single long open reading frame encoding a putative 260–amino acid polypeptide with a predicted molecular mass of 30 kD (Fig. 1A). The recombinant protein expressed in bacteria migrated at 33 kD on SDS–polyacrylamide gel electrophoresis; the polypeptide was therefore referred to as VAP-33 (VAMP-associated protein of 33 kD).

When expressed as a bacterial fusion protein with glutathione-S-transferase, VAP-33 bound specifically to in vitro–translated

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VAMP, suggesting that the interaction detected in yeast was direct and not mediated by additional factors (9) (Fig. 1B). In vitro–translated VAMP also bound to GST-syntaxin fusion protein, but not to GST alone or to an *Aplysia* transcription factor GST fusion. Northern (RNA) blot hybridization analysis indicated that the VAP-33 gene was expressed only in the central nervous system (CNS) and the gill (*10*) (Fig. 1C). The gill is a highly innervated tissue that contains the cell bodies of many peripheral nerves, which may account for the transcripts identified in this organ.

The primary structure of VAP-33 shows no overall similarity to previously described proteins. A FASTA comparison of the sequence with GeneBank identified regions of similarity with a major sperm protein (*11*); however, the extracellular location of this sperm protein and the expression pattern of VAP-33 suggest that this sequence similarity does not reflect a functional homology. VAP-33 has no hydrophobic NH_2-terminal signal sequence, but the COOH-terminus is highly hydrophobic, characteristic of a class of integral membrane proteins typified by cytochrome b_5 and which also includes two SNARE (soluble NSF receptor) proteins, syntaxin and VAMP (*12*).

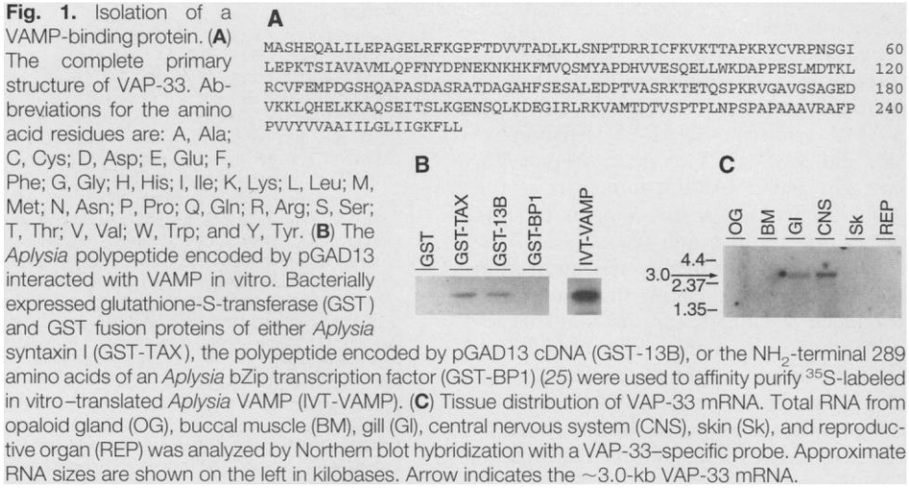

Fig. 1. Isolation of a VAMP-binding protein. (**A**) The complete primary structure of VAP-33. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. (**B**) The *Aplysia* polypeptide encoded by pGAD13 interacted with VAMP in vitro. Bacterially expressed glutathione-S-transferase (GST) and GST fusion proteins of either *Aplysia* syntaxin I (GST-TAX), the polypeptide encoded by pGAD13 cDNA (GST-13B), or the NH_2-terminal 289 amino acids of an *Aplysia* bZip transcription factor (GST-BP1) (*25*) were used to affinity purify ^{35}S-labeled in vitro–translated *Aplysia* VAMP (IVT-VAMP). (**C**) Tissue distribution of VAP-33 mRNA. Total RNA from opaloid gland (OG), buccal muscle (BM), gill (GI), central nervous system (CNS), skin (Sk), and reproductive organ (REP) was analyzed by Northern blot hybridization with a VAP-33–specific probe. Approximate RNA sizes are shown on the left in kilobases. Arrow indicates the ~3.0-kb VAP-33 mRNA.

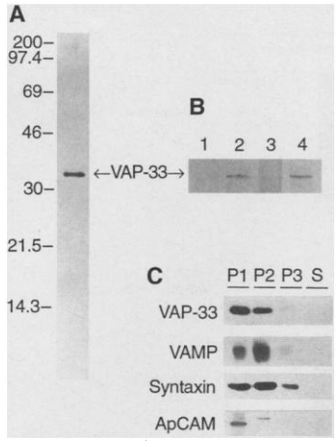

Fig. 2. Antiserum to VAP-33 recognizes a single 33-kD membrane protein. (**A**) Total protein extracted from *Aplysia* CNS was subjected to immunoblot analysis with a rabbit polyclonal antiserum that was prepared against a bacterially expressed GST fusion protein containing the COOH-terminal 190 amino acids of VAP-33. Molecular size standards are shown in kilodaltons on the left. (**B**) Specificity of the antiserum for VAP-33. Immunoreactivity was detected by immune serum (lane 2) or immune serum that had been preincubated with recombinant GST (lane 4), but not by preimmune serum (lane 1) or immune serum that had been preincubated with GST-13B (lane 3). (**C**) VAP-33 is an integral membrane protein. Adult *A. californica* CNS was fractionated as previously described (*26*), and the cell fractions were subjected to immunoblot analysis with antisera specific for VAP-33, VAMP, *A. californica* syntaxin, or *Aplysia* cell adhesion protein (ApCAM). P1, P2, P3, and S refer to the three pelleted fractions and the soluble fraction generated from two-step sucrose centrifugation as described (*26*).

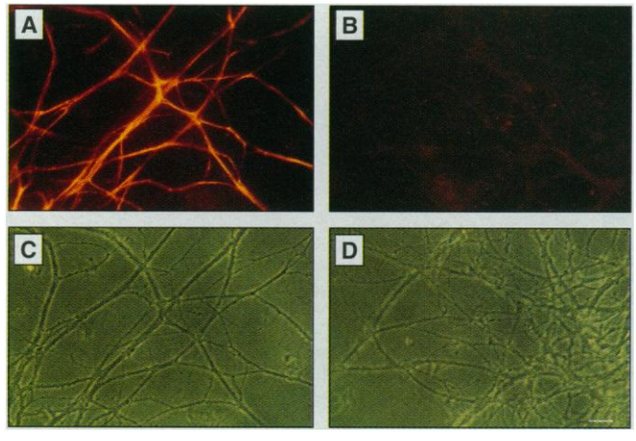

Fig. 3. VAP-33 is present in neurites. *Aplysia* sensory and motor cell cocultures were subjected to indirect immunofluorescence analysis with antiserum to VAP-33 (**A**) or preimmune rabbit antiserum (**B**) (*27*). Phase-contrast images for each culture are shown in (**C**) and (**D**), respectively. Bar, 5 μm.

Antiserum prepared against the bacterially expressed COOH-terminal 19-kD of VAP-33 recognized a single 33-kD protein on immunoblot analysis of protein extracts from *Aplysia* CNS (*13*) (Fig. 2A). This signal was not observed with preimmune serum or with immune serum that had been preincubated with excess recombinant antigen (Fig. 2B). Immunoreactivity copurified with membranes on cell fractionation, but showed a different profile to that of VAMP, suggesting that VAP-33 is associated with cellular membranes but is not enriched in synaptic vesicles (Fig. 2C). The fraction enriched in VAP-33 also contained the majority of immunoreactivity corresponding to ApCAM, an integral plasma membrane cell adhesion protein (*14*). Of the several isoforms of ApCAM, VAP-33 cofractionated with the transmembrane isoform. VAP-33 is an integral membrane protein; it remained associated with membrane in 1M KCl and was extracted entirely into the detergent phase by Triton-X114 (*15*, *16*).

Analysis of primary *Aplysia* neurons by indirect immunofluorescence revealed VAP-33 immunoreactivity throughout the neurites, some of which appeared to contain higher concentrations of VAP-33 than others (Fig. 3). This staining pattern differed markedly from those of VAMP and syntaxin (*17*).

To assess the physiological function of VAP-33, we examined its role in synaptic transmission at a well-characterized glutaminergic synapse between identified sensory and gill motor neurons in culture. We attempted to disrupt the activity of VAP-33 in vivo by intracellular injection of antibodies to this protein. Immunoglobulins from preimmune and immune sera were purified and injected into the presynaptic sensory neuron of cocultures of *Aplysia* sensory and motor neurons (*18*). The excitatory postsynaptic potentials (EPSPs) elicited in the motor neuron by extracellular stimulation of the presynaptic sensory neuron were recorded before and 15 to 20 hours after injection of immunoglobulin (*19*). Immunocytochemical analysis of injected cells confirmed that both immune and preimmune antibodies distributed throughout the processes of the sensory neuron (*17*). Although injection per se had a slightly depressive effect, cells injected with specific antibodies to VAP-33 showed a significant-

ly greater inhibition of evoked synaptic potentials (reduced by 55.4%) compared with those treated with preimmune immunoglobulins (reduced by 11.5%) (Fig. 4A). Transmissions at this synapse shows homosynaptic depression; the synapse depresses rapidly after repetitive presynaptic stimulation. In synapses blocked with antibodies to VAP-33, the residual transmission exhibited a normal rate of homosynaptic depression, indicating that the observed decrease in evoked EPSPs was not attributable to increased spontaneous activity (Fig. 4B). The residual transmission in inhibited synapses also remained proportionally responsive to the facilitative effects of serotonin (Fig. 4C), indicating that the structure of the synapses was not grossly perturbed.

The precise function of VAP-33 in the cycle of vesicle exo- and endocytosis is not yet clear. In culture, synapses are silent in the absence of stimulation; therefore, the inhibition of the evoked EPSPs by antibodies to VAP-33 is unlikely to result from a reduced rate of endocytosis. Reducing the activity of VAP-33 may therefore have reduced the number of vesicles available for release or inhibited the fusion event directly.

The membrane localization of VAP-33 and its interaction with VAMP suggest that VAP-33 may function in localizing vesicles, perhaps by anchoring them to the plasma membrane before release in a fashion similar to the docking role proposed for syntaxin and SNAP-25 (*5*, *20*). However, VAMP appears also to be required for release at a point subsequent to vesicle docking (*21*). Therefore, VAP-33 may participate at a step between docking and neurotransmitter release and, perhaps, function directly in the fusion of the vesicle and the plasma membrane.

VAMP recently has also been shown to interact with synaptophysin (*22*). Thus, at least four proteins interact directly with VAMP: syntaxin, SNAP-25, synaptophysin, and VAP-33. Two of these proteins, syntaxin and SNAP-25, form part of the 20S NSF-SNAP receptor complex thought to act directly in fusion (*20*, *22*, *23*). Although the significance of the interaction of VAMP with synaptophysin is not clear, the block of synaptic transmission that results from injection of specific antibodies to VAP-33 indicates that the interaction between synaptobrevin and VAP-33 is required, at some point, in the exocytosis of synaptic vesicles. The multiple interactions described for VAMP may reflect functionally distinct stages in the complex processes of docking, fusion, and exocytosis.

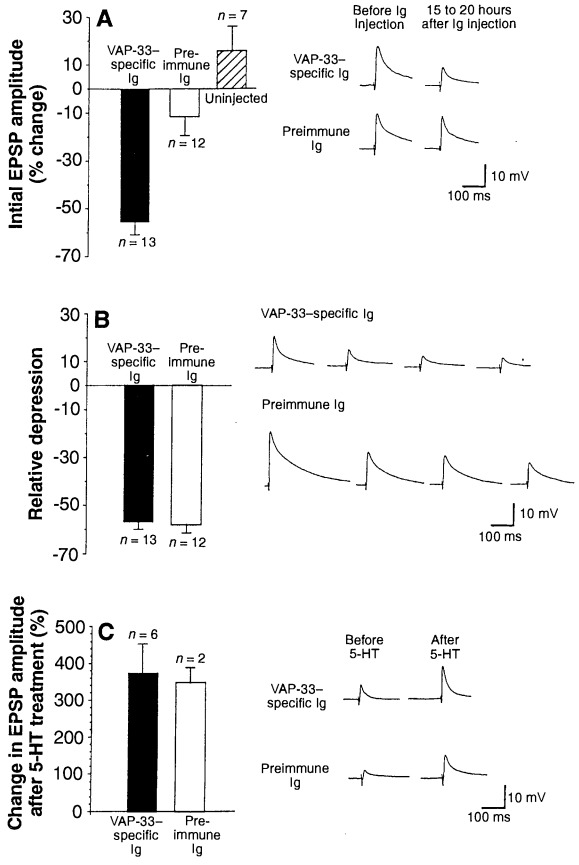

Fig. 4. Presynaptic intracellular injection of antibodies to VAP-33 inhibits synaptic transmission but does not affect homosynaptic depression or serotonin-induced dishabituation. (**A**) Initial evoked EPSPs recorded in the postsynaptic motor neuron after presynaptic injection of VAP-33–specific or preimmune immunoglobulin (Ig). Mean percentage changes (±SEM) in EPSP amplitude before and 15 to 20 hours after injection of the indicated Ig are indicated. Injection of VAP-33–specific Ig significantly decreased EPSP amplitude relative to preimmune Ig ($P < 0.01$, unpaired Student's t test). Representative recordings are shown on the right. (**B**) Homosynaptic depression is not affected by injection of VAP-33–specific Ig. EPSPs were recorded in the motor neuron after stimulation of the sensory cell at 0.05 Hz. The mean percentage changes (±SEM) between the mean of the second, third, and fourth EPSPs and the initial EPSP are indicated. Representative recordings are shown on the right. (**C**) Serotonin (5-HT)–induced dishabituation is not affected by injection of VAP-33–specific Ig. Sensory cells injected with either VAP-33–specific or preimmune Ig were stimulated extracellularly at 0.05 Hz for 80 s, and four evoked EPSPs were recorded in the motor cell. Serotonin was then added to 10 mM, and a further four EPSPs were recorded. The mean percentage change (±SEM) between the four EPSPs before and after serotonin treatment is indicated. Representative recordings of the EPSPs immediately before and after serotonin treatment are shown on the right.

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8. The entire coding sequence of *A. californica* VAMP (synaptobrevin) [S. Yamasaki *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 4688 (1994)] was subcloned into pMA424 (*7*) to generate the expression construct pMA-VAMP, which was then used to transform the *Saccharomyces cerevisiae* strain GGY1::171 with an *Aplysia* central nervous system–specific complementary DNA (cDNA) library generated in pGAD10. From an initial screen of 200,000 colonies, we isolated four individual clones containing different partial cDNAs corresponding to the same mRNA, of which pGAD13B contained the most complete coding sequence. The same screen also identified *A. californica* syntaxin and an additional unidentified gene.
9. Bacterially expressed proteins were purified and bound to glutathione-agarose beads (Sigma) as described (*24*). Full-length *A. californica* VAMP was translated and labeled with [^{35}S]methionine with the use of the T7-coupled TNT system (Promega) from a derivative of the expression plasmid pET17a (Novagen). The binding reaction mixture was incubated with gentle rotation for 30 min at room temperature in 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 0.05% Tween 20, after which the proteins were washed extensively in the same solution with the use of a vacuum manifold (Promega).
10. RNA was isolated as described [P. Chomczynski and N. Sacchi, *Anal. Biochem.* **162**, 156 (1987)], separated by agarose gel electrophoresis, immobilized on a Magnagraph nylon membrane (MSI), and hybridized to a digoxygenin-labeled DNA probe generated from the cDNA pGAD13B (Boehringer). Hybridization was performed at 42°C in a solution containing 5× standard saline citrate (SSC) and 50% formamide, and the membrane was subsequently washed in 0.2× SSC at 68°C.
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19. Immunoglobulins were purified from preimmune and VAP-33–specific antisera (E-Z-Sep; Pharmacia) and were pressure injected at a concentration of 3.5 mg/ml in 500 mM KCl and 10 mM Tris-HCl (pH 7.6) into the presynaptic cells of 4- to 5-day-old ex vivo cocultures of sensory and motor neurons from *A. cali-*

fornica (*18*). Electrophysiology was performed as previously described (*18*), with the exception that sensory cells were stimulated extracellularly.
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27. Five-day-old primary cocultures of *Aplysia* sensory and motor neurons were washed in phosphate-buffered saline (PBS) containing 30% (w/v) sucrose and then fixed with 4% (w/v) paraformaldehyde in the same solution. Cell membranes were permeabilized with 0.1% Triton X-100 in PBS and the fixative was quenched with 50 mM NH_4Cl. After blocking nonspecific interactions with 10% (v/v) goat serum in PBS containing 30% sucrose, the cells were incubated with either preimmune serum or VAP-33–specific antiserum prepared against the full-length polypeptide expressed in bacteria as a polyhistidine-tagged fusion protein by using the vector pET-30a (Novagen) (dilution of 1:400 in blocking solution). Immunoreactivity was visualized with Cy3-conjugated goat antibodies to rabbit immunoglobulin (Jackson Immunoresearch) at 1:200 in blocking solution.
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Requirement of MIP-1α for an Inflammatory Response to Viral Infection

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Macrophage inflammatory protein–1α (MIP-1α) is a chemokine that has pro-inflammatory and stem cell inhibitory activities in vitro. Its biologic role in vivo was examined in mice in which the gene encoding MIP-1α had been disrupted. Homozygous MIP-1α mutant (−/−) mice were resistant to Coxsackievirus-induced myocarditis seen in infected wild-type (+/+) mice. Influenza virus–infected −/− mice had reduced pneumonitis and delayed clearance of the virus compared with infected +/+ mice. The −/− mice had no overt hematopoietic abnormalities. These results demonstrate that MIP-1α is an important mediator of virus-induced inflammation in vivo.

The recruitment of leukocytes to sites of infection is crucial to the inflammatory clearance of pathogens. Various molecules may control leukocyte trafficking in vivo, including the chemokines, a family of low molecular weight proteins that induce leukocyte chemotaxis in vitro (*1*). MIP-1α is a member of the β chemokine subfamily, which also includes MIP-1β and RANTES. MIP-1α induces chemotaxis of monocytes, $CD8^+$ T cells (*2*), $CD4^+$ T cells, and B cells in vitro (*3*). However, the importance of MIP-1α in vivo has been unclear, because many of its activities are shared by other β chemokines, including an ability to bind to the same receptor (*4*). To determine its function in vivo, we used homologous recombination in mouse strain 129–derived embryonic stem (ES) cells to generate a deletion in the MIP-1α gene. The deletion included 300 nucleotides (nt) of DNA upstream of the mRNA start site, as well as the first exon and half of the second exon of the MIP-1α gene (Fig. 1). Injection of these cells into C57BL/6J blastocysts produced nine chimeras, five of which transmitted the mutant MIP-1α allele to the offspring. Matings between F_1 heterozygotes (+/−) yielded offspring of the MIP-1α genotypes +/+, +/−, and −/− in Mendelian proportions. The −/− mice had no gross abnormalities in development or in the histology of any major organ.

To examine MIP-1α expression in −/− mice, we isolated bone marrow macrophages, stimulated them with L929 cell–conditioned media (*5*), and prepared RNA. Northern (RNA) blot analysis with a MIP-1α complementary DNA (cDNA) probe revealed abundant MIP-1α mRNA (780 nt) in +/+ mice, less mRNA in +/− mice, and essentially no mRNA in the −/− mice (Fig. 1E). Because the deletion includes almost half of the MIP-1α coding region, any mRNAs transcribed from the mutant MIP-1α locus are unlikely to encode a functional protein.

MIP-1α inhibits the proliferation of early hematopoietic progenitor cells in vitro (*6*). Therefore, we compared the peripheral blood and bone marrow of +/+ mice to that of −/− mice to determine whether the mutation had affected hematopoiesis. No significant differences between the two genotypes were observed in peripheral blood (hematocrits, total white blood cell counts, and differential counts) or in bone marrow [total nucleated cells per femur and early hematopoietic progenitor cell number (*7*)] (two-tailed *t* test). Analysis of cell populations in lymph nodes and spleens revealed that both T and B cell numbers in the −/− mice were indistinguishable from those of +/+ mice. Thus, the −/− mice lack any overt hematopoietic abnormalities.

In light of evidence for a role of MIP-1α in autoimmune disease (*8*), we tested the response of the −/− mice to Coxsackievirus B3 (CVB3). CVB3 infection of humans is a major cause of myocarditis in young adults, and in neonates it is frequently lethal. CVB3 also induces myocarditis in various strains of mice, and the pathophysiology closely resembles that of humans; macrophages and neutrophils accumulate in the heart around day 5 postinfection (p.i.) and are gradually replaced by a T cell infiltrate by day 10 p.i. Substantial evidence suggests that the cardiac lesions result from an autoimmune mechanism mediated primarily by cytotoxic T lymphocytes, although direct viral-mediated cytopathology may also occur (*9*).

To determine the role of MIP-1α in CVB3-induced myocarditis, we killed mice at various times after infection and analyzed histologic sections of heart by light microscopy (*10*). Cardiac lesions were first observed in +/+ mice at day 6 p.i.; at days 10, 15, and 21 p.i., the majority of +/+ mice had moderate to severe myocarditis (Fig. 2). The lesions were characterized by a mononuclear cell infiltrate and evidence of cytolysis. In contrast, none of the −/− mice had myocarditis or evidence of cytolysis at any time.

To test whether some unidentified, natural, strain-specific differences between 129 and C57BL/6J mice at or near the β chemokine locus may account for the observed difference in inflammation between the two groups, we interbred +/+ mice having 129-derived DNA at the β chemokine locus (129+/129+) and infected them with CVB3 (*11*). Three of five infected 129+/129+ mice developed myocarditis, whereas none of an additional five −/− mice had inflammation. This result demonstrates

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