function, possibly through direct interaction with IQGAP1.

The amount of α-catenin, but not that of β-catenin, associated with E-cadherin was reduced when IQGAP1 (but not IQGAP1-ΔC) was overexpressed in EL cells (Fig. 4C) (*20*). Overexpression of IQGAP1 or IQGAP1-ΔC did not affect the amounts of E-cadherin, α-catenin, or β-catenin expressed in these cells, and the amounts of recombinant IQGAP1 and IQGAP1-ΔC were similar (*13*). Thus, overexpression of IQGAP1 appeared to induce dissociation of α-catenin from the cadherin-catenin complex. The dissociation of α-catenin from β-catenin may be responsible for the in vivo action of IQGAP1.

Treatment of cells with pervanadate results in the dissociation of α-catenin from the cadherin-catenin complex, and the dissociation of α-catenin from the complex correlates with the decrease in cadherin activity (*21*). Dissociation of α-catenin from the cadherin-catenin complex also occurs during the passage of human breast epithelial cells (*22*). Our data suggest that IQGAP1 might participate in these processes.

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10. L cells, EL cells, and nEαCL cells were cultured as described (*3*). Confluent cells were fixed and stained with polyclonal antibodies to IQGAP1 and with a monoclonal antibody to E-cadherin (ECCD-2) or a monoclonal antibody to β-catenin (Transduction Laboratories, Lexington, KY). Antibodies to IQGAP1 were generated against the NH_2-terminal domain of human IQGAP1 (amino acids 1 to 216).
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12. Cross-linking experiments were performed essentially as described (*11*). Cells were suspended in phosphate-buffered saline in the absence or presence of 0.75 mM DSP, and incubated for 20 min at room temperature. DSP activity was quenched by addition of 50 mM glycine in phosphate-buffered saline. The cells were then lysed in a solution containing 50 mM tris-HCl (pH 7.5), 1 mM EDTA, 50 mM NaCl, 10 μM (*p*-amidinophenyl)methanesulfonyl fluoride, leupeptin (10 μg/ml), 0.25% (w/v) Triton X-100, and 1 mM $CaCl_2$, and the lysates were incubated for 2 hours at 4°C in the absence or presence of the indicated antibodies. The resulting immunoprecipitates were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis with the indicated antibodies. About 50% of IQGAP1 was immunoprecipitated from EL cells incubated in the absence or presence of DSP (*13*).
13. S. Kuroda *et al.*, unpublished data.
14. Complementary DNAs encoding mouse α-catenin and mouse β-catenin, as well as a cDNA fragment encoding the cytoplasmic domain of mouse E-cadherin, were inserted individually into the Bam HI site of pMAL C-2. The encoded MBP fusion proteins were expressed in and purified from *Escherichia coli*. GST-IQGAP1 was purified from overexpressing insect cells as described (*23*). Various concentrations of MBP fusion proteins were mixed with affinity beads coated with GST or GST-IQGAP1. The beads were washed, and bound MBP fusion proteins were co-eluted with GST or GST-IQGAP1 by the addition of reduced glutathione. The eluates were subjected to SDS-PAGE and immunoblot analysis with antibodies to MBP (New England Biolabs, Beverly, MA).
15. Various concentrations of MBP–α-catenin and MBP–β-catenin were mixed and incubated for 1 hour at 4°C. The mixture was then incubated with beads coated with GST, GST-IQGAP1, or GST–E-cadherin (the cytoplasmic domain). The interaction of MBP fusion proteins with GST fusion proteins was examined (*14*).
16. GST–IQGAP1-N and GST–IQGAP1-C were produced and purified as described (*23*). GST–IQGAP1-ΔC was purified from overexpressing insect cells. MBP–β-catenin (50 nM) or MBP–E-cadherin (250 nM) was mixed with affinity beads coated with the indicated GST fusion proteins (40 pmol each). The beads were washed, and the interaction of MBP fusion proteins with GST fusion proteins was examined (*14*).
17. The plasmids pEF-BOS-Myc-IQGAP1, pEF-BOS-Myc–IQGAP1-N, pEF-BOS-Myc–IQGAP1-C, and pEF-BOS-Myc–IQGAP1-ΔC were constructed (*9*, *13*) and microinjected (0.01 mg/ml) into nuclei of subconfluent EL cells. After 5 hours, the cells were fixed and doubly stained with antibodies to Myc and to β-catenin. Similar results were obtained by transfection (*13*).
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19. EL cells or nEαCL cells were seeded into 10-cm dishes and transiently transfected with the indicated plasmids with the use of Lipofectamine (Gibco-BRL, Rockville, MD). After incubation for 20 hours, the cells were scraped from 5 to 10 dishes and transferred to a plastic dish coated with antibodies to Aic2 (HC) (*18*). The adherent cells (5×10^5) were then transferred to 48-well culture dishes and incubated for 24 hours. These various immunoisolated cells showed the same confluency. Recombinant IQGAP1 and Cdc42 were expressed in ~80% of the collected cells. The dissociation assay was performed in the presence of either Ca^{2+} or EGTA as described (*3*).
20. Immunoisolated cells (5×10^6) were transferred to six-well culture dishes (*19*), and, after incubation for 24 hours, they were treated with DSP (*12*) and lysed. The lysates were centrifuged at 100,000*g* for 30 min, and the resulting supernatant was mixed with antibodies to E-cadherin (Transduction Laboratories). After incubation at 4°C for 1 hour, the immunoprecipitate was separated by centrifugation and subjected to SDS-PAGE and immunoblot analysis with the indicated antibodies.
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24. We thank A. Nagafuchi and S. Tsukita for providing L, EL, and nEαCL cells; cDNAs encoding E-cadherin, α-catenin, or β-catenin; and antibodies to E-cadherin (ECCD-2) or to α-catenin. We also thank M. Takeichi for providing ECCD-2 and Kazusa DNA Research Institute for support of a cDNA Research Program. Supported by grants-in-aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (1997), by the Japan Society for the Promotion of Science Research for the Future, by the Human Frontier Science Program, and by grants from Kirin Brewery Co. Ltd.

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Positive Selection Through a Motif in the αβ T Cell Receptor

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The two lineages of T cells, αβ and γδ, differ in their developmental requirements: only αβ T cells require major histocompatibility complex recognition, a process known as positive selection. The αβ T cell receptor (TCR), but not its γδ counterpart, contains a motif within the α-chain connecting peptide domain (α-CPM) that has been conserved over the last 500 million years. In transgenic mice expressing an αβ TCR lacking the α-CPM, thymocytes were blocked in positive selection but could undergo negative selection. Thus, the α-CPM seems to participate in the generation of signals required for positive selection.

Positive selection of αβ thymocytes generates a T cell repertoire that is self–major histocompatibility complex (MHC) restricted (*1–4*), whereas negative selection ensures that the immune system is self-tolerant (*5–7*). Although the αβ TCR mediates both forms of thymic selection, the distinction between positive and negative selection signals has been difficult to define. The TCR α-chain has an amino acid motif in its connecting peptide domain (α-CPM) that is conserved in all α-chains from bony fish to humans (*8*). Although the α-CPM has been conserved over the last 500 million years (*9*), it is not found within the γδ TCR. αβ TCRs containing a defective α-CPM are unresponsive to antigens and are aberrantly associated to the CD3 complex (*8*). To test whether the α-CPM

imparts a specific signaling function to αβ T cells during thymic selection, we generated transgenic mice expressing a wild-type αβ TCR or a TCR that lacked the α-CPM and followed the selective events during T cell ontogeny. These experiments showed that thymocytes expressing TCRs without a complete α-CPM were not efficiently positively selected, but nevertheless could be negatively selected.

The wild-type 3BBM74 TCR (*10*) is positively selected in mice expressing I-A^b and negatively selected in mice expressing I-A^{bm12}. Transgenic mice expressing a Vα2.1/Vβ8.1 wild-type 3BBM74 receptor (encoded by the α wild-type and β wild-type cDNAs) or an α-CPM defective 3BBM74 TCR [encoded by the αIII and βIII cDNAs; described in (*11*)] were generated by injecting DNA into C57BL/6 (B6) zygotes. Recombination deficient, B6.RAG-$2^{-/-}$ mice expressing either the wild-type or mutant TCR transgenes were used in all experiments.

The mutant and wild-type TCRs were comparably expressed on the surface of thymocytes (Fig. 1A); however, the mutant mice contained fewer TCR^{hi} thymocytes. The mean of TCR expression on mutant peripheral T cells was slightly decreased (~30%), but the range of TCR expression on mutant and wild-type T cells was similar (Fig. 1A). Wild-type or mutant TCRs were immunoprecipitated from thymocytes or lymph node T cells, and the presence of the CD3 γ, δ, and ε subunits and ζ chains was detected by protein immunoblotting (Fig. 1B). The CD3δ subunit was absent from immunoprecipitates of mutant TCRs, even in the presence of the mild detergent digitonin. Furthermore, the ζ chain did not coprecipitate with the mutant TCR expressed by peripheral T cells. In contrast, all CD3 subunits and the ζ chain were coimmunoprecipitated with the wild-type TCR (Fig. 1B).

Analysis of the $CD4^-8^-$ (double-negative; DN), $CD4^+8^+$ (double-positive; DP), and $CD4^+8^-$ or $CD4^-8^+$ (single-positive; SP) thymocyte populations showed a reduction in the percentage of $CD4^+8^-$ SP cells in the mutant animals (Fig. 2A). The percentage (Fig. 2B) and total number (*12*) of $CD4^+8^-$ SP thymocytes could differ by a factor of 30. The thymi of mice expressing the mutant TCR maintained a low number of $CD4^+8^-$ thymocytes for >10 weeks (Fig. 2B); thus, the developmental defect was not relieved with time. Thymi from mutant TCR mice contained twice the number of cells as thymi from transgenic mice expressing the wild-type TCR (*12*), and the percentage of DP thymocytes was increased in the mutant mice (Fig. 2A). To study the efficiency of allelic

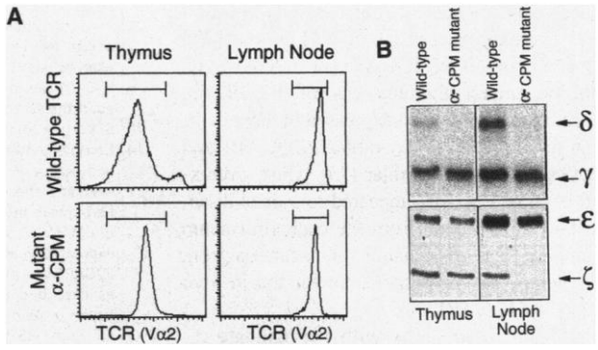

Fig. 1. Surface expression and composition of wild-type and mutant TCR/CD3 complexes. (**A**) Thymocytes and lymph node cells from wild-type and mutant B6.Rag-$2^{-/-}$ transgenic mice were harvested and stained with the mAb to Vα2, B20.1 (*34*), which is specific for the transgenic α-chain. (**B**) Thymocytes and lymph node cells from wild-type and mutant transgenic mice were harvested and lysed in a buffer containing 1% digitonin. The TCR complex was immunoprecipitated with B20.1 mAb and resolved by SDS–polyacrylamide gel electrophoresis. The CD3 γ, δ, ε, and ζ proteins were identified as previously described (*35*).

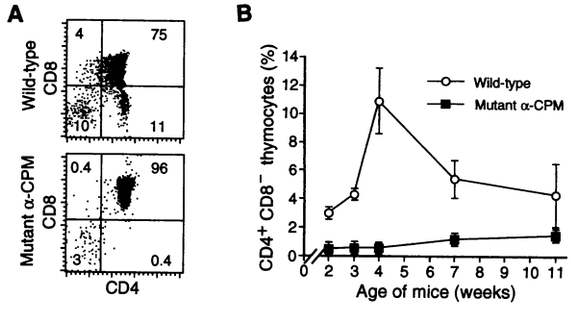

Fig. 2. Analysis of positive selection in the thymus. B6.Rag-$2^{-/-}$ mice expressing either the wild-type or the mutant TCR were used for these experiments. (**A**) Thymocytes were collected, stained with mAbs to CD4 and CD8 (*34*), and analyzed by flow cytometry. The numbers indicate the percentage of cells in each quadrant. (**B**) After staining with mAbs to CD4 and CD8 (*34*) and flow cytometric analysis, the percentage of $CD4^+8^-$ SP thymocytes was calculated. Each data point represents the mean percentage of $CD4^+8^-$ SP thymocytes from two or three mice. Wild type (open circles); α-CPM mutant (filled squares).

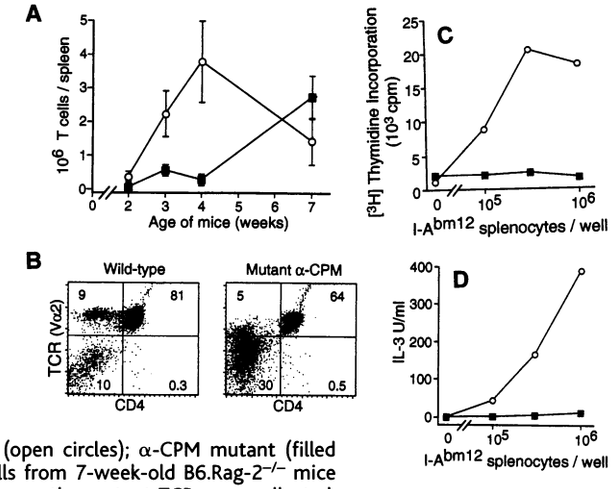

Fig. 3. Analysis of peripheral cells in mice bearing a positive selection ligand (I-A^b). (**A**) Splenocytes were collected, counted, stained with mAbs to Vα2 and Vβ8 (*34*), and analyzed by flow cytometry. After determining the percentage of TCR^+ cells and the total number of splenocytes, the total number of T cells per spleen was calculated. Each data point represents the mean number of T cells per spleen from two or three mice. Splenocytes were used because it is difficult to obtain lymph nodes from extremely young Rag-$2^{-/-}$ animals. Wild type (open circles); α-CPM mutant (filled squares). (**B**) Lymph node cells from 7-week-old B6.Rag-$2^{-/-}$ mice expressing either the wild-type or the mutant TCR were collected, stained with mAbs to CD4 and Vα2 (*34*), and analyzed by flow cytometry. The numbers indicate the percentage of cells in each quadrant. (**C**) Mixed leukocyte cultures were initiated between 2 × 10^5 responder lymph node cells from 7-week-old mice expressing either the wild-type (open circles) or the mutant (filled squares) TCR and titrated numbers of irradiated stimulator spleen cells from B6.C.H-2-bm12 (I-A^{bm12}) mice. After 4 days, the cultures were pulsed with 0.5 μCi of [^{3}H]thymidine overnight, and the incorporation was determined. There was no response to stimulator cells from B6 (I-A^b) mice (*12*). (**D**) Mixed leukocyte cultures were carried out as described in (C), and the concentration of interleukin-3 was determined as described (*8*). Wild type (open circles); α-CPM mutant (filled squares). There was no response to stimulator cells from B6 (I-A^b) mice (*12*).

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exclusion, a parameter of positive selection (*13*), wild-type and mutant animals were crossed to B6 mice to restore recombination. Expression of the wild-type TCR excluded the rearrangement of endogenous α-chains, whereas expression of the mutant receptor did not (*12*). Taken together, these data suggest a severe block in positive selection.

The appearance of transgenic T cells in the periphery was analyzed in recombination-defective (RAG-$2^{-/-}$) mice (Fig. 3A). Within the first 4 weeks, T cells expressing the mutant receptor increased slowly, reflecting the inefficient positive selection in the thymus (Fig. 2). By 7 weeks, the number of splenic T cells in wild-type and mutant TCR transgenic mice were roughly equivalent (Fig. 3A). These mutant lymphocytes were thymus-derived $CD4^+8^-$ T cells (Fig. 3B) (*14*). Even though $CD4^+$ T cells expressing the mutant receptor accumulated slowly in the periphery, they were unresponsive to the I-A^{bm12} alloantigen (Fig. 3, C and D), probably because of inadequate ζ chain coupling to the mutant TCR (Fig. 1B). Thus, peripheral T cells in the mutant mice may have been selected by an escape or a default pathway (*15*). Alternatively, the mutant T cells may have been refractory to antigen stimulation subsequent to an expansion in the periphery.

Mice expressing the wild-type or the mutant TCR were crossed to B6.C.H-2-bm12, Rag-$2^{-/-}$ animals to introduce a ligand (I-A^{bm12}) that induces negative selection. Thymocytes and lymph node cells from offspring expressing I-A^{bm12} and the transgenic TCR were analyzed. There were few $CD4^+8^-$ SP thymocytes in mice expressing either the wild-type TCR (Fig. 4A) or the mutant TCR, and the expression of I-A^{bm12} reduced thymocyte number by ~30% in both strains (*12*). In the periphery of both wild-type and mutant mice, there were few TCR^+CD4^+ cells (Fig. 4A), indicating that both the wild-type and mutant T cells were negatively selected.

To directly examine the susceptibility of DP thymocytes to negative selection, an in vitro assay was used (*16–19*). DP thymocytes respond to negative selection ligands by down-regulating CD4 and CD8. Therefore, thymocytes from B6.Rag-$2^{-/-}$ (I-A^b) mice expressing either the wild-type or the mutant TCR were cultured with antigen presenting cells (APCs) from B6.C.H-2-bm12 (I-A^{bm12}) mice in the presence of varying amounts of an I-A^{bm12} blocking antibody (Fig. 4B). In this way, the I-A^{bm12} alloantigen available on the APC surface could be titrated. Both wild-type and mutant DP thymocytes responded similarly to the I-A^{bm12} antigen over an equivalent concentration range of blocking antibody by down-regulating CD4 and CD8 (Fig. 4B). Thus, DP thymocytes bearing the α-CPM mutant TCR were as responsive to their ligand as wild-type DP thymocytes. Staining the thymocytes in these cultures with Hoechst 33342 revealed that both wild-type and mutant thymocytes became apoptotic as a consequence of antigen (I-A^{bm12}) recognition (Fig. 4C). Thus, DP thymocytes bearing a TCR lacking the α-CPM were able to undergo negative selection that resulted in apoptosis.

We show that positive selection is more profoundly affected than negative selection in mice expressing a TCR with a mutant α-CPM, which suggests that positive selection is regulated by a distinct structure within the αβ heterodimer. Although the mutant TCR contains alterations in its connecting peptide, transmembrane, and cytoplasmic domains (*11*), the α-CPM is likely to be the critical element. Positive selection is normal in transgenic animals coexpressing a wild-type α chain and the βIII chain (*12*). Furthermore, only TCRs with a mutant α-CPM exhibit a poor association to CD3δ (*20*). That positive selection is defective in CD3$\delta^{-/-}$ mice (*21*) is consistent with our observation that the α-CPM mutant TCR is aberrantly associated to the CD3δ chain (Fig. 1B).

These data support a molecular affinity model for thymic selection, which proposes that the TCR/CD3 complex decides between positive and negative selection by initiating qualitatively distinct signaling pathways. A low-affinity ligand would initiate positive selection through the α-CPM and CD3δ. A high-affinity ligand would activate additional elements of the TCR/CD3 complex, generating distinct negative selection signals. That independent signals mediate positive and negative selection has been postulated (*22*), and other experimental evidence is consistent with this idea (*23–26*).

The conserved structural differences between αβ and γδ TCRs may be related to the differences in the ontogeny of αβ and γδ T cells (*21, 27–29*). Unlike γδ cells, the development of αβ T cells depends on an MHC-driven, intrathymic, positive selection and the presence of the α-CPM and CD3δ (*21*) within the TCR complex. Given that the α-CPM has been conserved in a T cell lineage for which positive selection is obligatory, we suggest that the α-CPM evolved to facilitate the type of signals specifically required for positive selection.

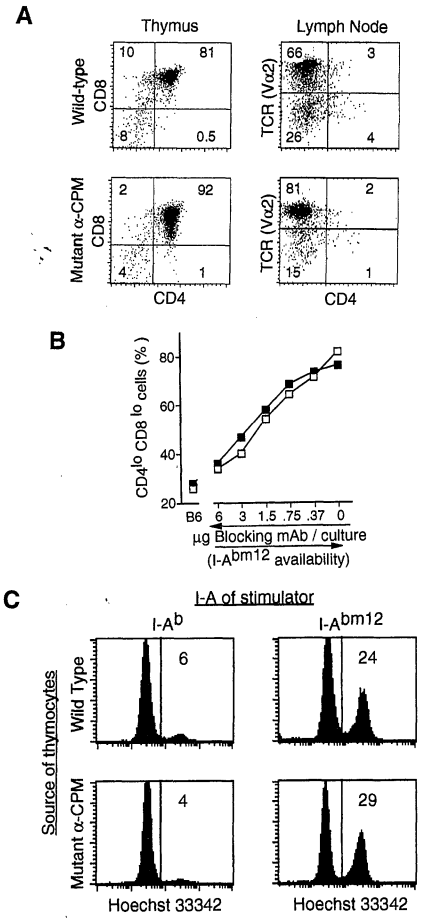

Fig. 4. Analysis of negative selection induced by the alloantigen I-A^{bm12}. (**A**) Seven-week-old B6.Rag-$2^{-/-}$ I-$A^{b/bm12}$ mice expressing either the wild-type or the mutant TCR were used for these experiments. Thymocytes were stained with mAbs to CD4 and CD8 (*34*) and analyzed by flow cytometry. Lymph node cells were stained with mAbs to CD4 and Vα2 (*34*) and analyzed by flow cytometry. The numbers indicate the percentage of cells in each quadrant. (**B**) B6.Rag-$2^{-/-}$ (I-A^b) mice expressing either the wild-type (open squares) or mutant (filled squares) TCR were used in an in vitro assay (*16–19*) to determine the responsiveness of DP thymocytes to I-A^{bm12}. Thymocytes were cocultured for 16 hours with splenocytes from B6 (I-A^b) or B6.C.H-2-bm12 (I-A^{bm12}) animals (*36*) in the presence of titrated amounts of the I-A^{bm12} blocking mAb, 3JP (*34*). Cells were then stained with mAbs to Vα2, CD4, and CD8 (*34*). DP thymocytes were gated and analyzed for intensity of CD4 and CD8 staining. (**C**) Thymocytes were cocultured for 12 to 16 hours with I-A^{bm12} splenocytes (*36*). Cells were harvested, stained with Hoechst 33342, and subsequently stained with mAbs to Vα2, CD4, and CD8 (*34*). DP thymocytes were gated and analyzed for intensity of Hoechst 33342 staining. Thymocytes undergoing apoptosis stain more brightly with Hoechst 33342; the percentage of these cells is indicated in the figure.

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34. The monoclonal antibodies (mAbs) to Vα2.1 (B20.1), Vβ8 (MR5-2), CD3ε (145-2c11), CD4 (H129.19), and CD8 (53-6.7) were purchased from PharMingen (San Diego, CA). The ζ chain mAb H146-968 (*32*) and the I-A^{bm12} mAb 3JP (*33*) were purified from culture supernatants using protein G Sepharose beads (Pharmacia). Cells were analyzed on a FACScan or a FACStar Plus (Becton Dickinson) using the CellQuest software (Becton Dickinson).
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36. The stimulation of DP thymocytes and Hoechst staining to detect apoptotic cells was carried out as previously described (*16–19*). Briefly, stimulator splenocytes were prepared from B6 (I-A^b) or B6.C.H-2-bm12 (I-A^{bm12}) mice. We cultured 2 × 10^6 stimulators (in 2 ml) for 12 to 16 hours in the presence or absence of titrated amounts of the I-A^{bm12} blocking mAb 3JP (*34*) in 24-well plates with 1 × 10^6 thymocytes from B6.Rag-$2^{-/-}$ mice expressing the wild-type or the α-CPM mutant TCR. Cells were then harvested and stained with Hoechst 33342 (1 μg/ml) followed by staining with CD4 mAb, CD8 mAb, and propidium iodide (2.5 μg/ml) and analyzed by flow cytometry.
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DARPP-32: Regulator of the Efficacy of Dopaminergic Neurotransmission

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Dopaminergic neurons exert a major modulatory effect on the forebrain. Dopamine and adenosine 3′,5′-monophosphate–regulated phosphoprotein (32 kilodaltons) (DARPP-32), which is enriched in all neurons that receive a dopaminergic input, is converted in response to dopamine into a potent protein phosphatase inhibitor. Mice generated to contain a targeted disruption of the DARPP-32 gene showed profound deficits in their molecular, electrophysiological, and behavioral responses to dopamine, drugs of abuse, and antipsychotic medication. The results show that DARPP-32 plays a central role in regulating the efficacy of dopaminergic neurotransmission.

Midbrain dopaminergic neurons play a critical role in multiple brain functions (*1–3*). Abnormal signaling through dopaminergic pathways has been implicated in several major neurological and psychiatric disorders, including Parkinsonism, schizophrenia, and drug abuse (*4*). The physiological and clinical importance of dopamine pathways in the brain makes it imperative to elucidate the mechanisms by which dopamine, acting on its receptors, produces its biological effects on target neurons.

One well-studied molecular target for the actions of dopamine is DARPP-32 (*5*), which is highly enriched in virtually all medium spiny neurons in the striatum (*6*). Dopamine, acting on D1-like receptors, causes activation of protein kinase A (PKA) and phosphorylation of DARPP-32 on threonine-34 (*7*). Conversely, dopamine, acting on D2-like receptors, through both inhibition of PKA and activation of calcium/calmodulin–dependent protein phosphatase (protein phosphatase 2B/calcineurin), causes the dephosphorylation of DARPP-32 (*8*). Several other neurotransmitters that interact with the dopamine system also stimulate either phosphorylation or dephosphorylation of DARPP-32 through various direct and indirect mechanisms (*9*). DARPP-32, in its phosphorylated but not its dephosphorylated form, acts as a potent inhibitor of protein phosphatase-1 (PP-1) (*10*). PP-1 controls the state of phosphorylation and the physiological activity of a wide array of neuronal phosphoproteins, including neurotransmitter receptors, ion channels, ion pumps, and transcription factors (*11*).

That numerous pathways regulate, or are regulated by, the DARPP-32/PP-1 signaling cascade suggests the central importance of DARPP-32 in mediating the biological effects of dopamine. To evaluate this hypothesis, given the absence of any specific pharmacological antagonists for DARPP-32, we generated mice that lack this protein (*12*). The absence of DARPP-32 protein from mice homozygous for the mutated DARPP-32 gene was demonstrated by immunoblotting striatal extracts. Immunocytochemistry confirmed that the DARPP-32 protein was absent from mutant mouse brain (*13*), although the brains of the DARPP-32 mutant mice appeared normal structurally (*14, 15*).

Phosphorylated DARPP-32 inhibits dephosphorylation of numerous other proteins by PP-1. Therefore, we examined the possi-