β_2-Microglobulin–Independent MHC Class Ib Molecule Expressed by Human Intestinal Epithelium

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A major histocompatibility complex class Ib protein, CD1d, is expressed by human intestinal epithelial cells (IECs) and is a ligand for CD8$^+$ T cells. CD1d was found to be expressed on the surface of human IECs as a 37-kilodalton protein that was β_2-microglobulin (β_2M) independent with no N-linked carbohydrate. Transfection into a β_2M$^-$ cell line confirmed that CD1d could be expressed at the cell surface in the absence of β_2M. These data indicate that IECs use a specialized pathway for CD1d synthesis and that a β_2M-independent class Ib protein may be the normal ligand for some intestinal T cells.

The major histocompatibility complex (MHC) has a family of β_2M-associated membrane glycoproteins that includes MHC class Ia and Ib proteins. The MHC class Ia proteins are polymorphic molecules that are ubiquitously expressed and play a central role in the immune system by binding peptide antigens for presentation to CD8$^+$ T cells (*1*). The MHC class Ib proteins are relatively nonpolymorphic and may be specialized for binding specific antigens for presentation to particular subpopulations of T cells (*2*). Although some MHC class I proteins can be expressed on the cell surface independently of β_2M (*3*), these appear to be nonfunctional byproducts of the dominant pathway that generates β_2M-associated proteins for antigen binding (*4*). Mice deficient in β_2M (β_2M$^{-/-}$) express low or undetectable amounts of cell surface MHC class I proteins and lack CD8$^+$ T cells in most sites except the intestinal mucosa (*5*). The intestinal mucosa of β_2M$^{-/-}$ mice contains normal numbers of CD8$^+$ T cells with the $\gamma\delta$ T cell antigen receptor (TCR), which suggests the local expression of a β_2M-independent ligand for CD8$^+$ T cells.

In addition to MHC class Ia proteins, IECs in the murine and human intestinal mucosa express MHC class Ib proteins encoded by the CD1 locus (CD1d in humans) (*6*) and, in mouse, by the TL locus (*7*). To determine the structure of CD1d on the cell surface of human IECs, we radiolabeled normal colonic IECs with ^{125}I and immunoprecipitated their proteins with two monoclonal antibodies (mAbs) to CD1d, 3C11 and 1H1 (Fig. 1A) (*8*). Both mAbs were generated in rat to the murine homolog of CD1d, but they also recognized human CD1d (*6*, *9*). Under nonreducing (NR) conditions, both mAbs immunoprecipitated single, major bands of ~100 kD from IEC lysates. In contrast, immunoprecipitation of MHC class Ia proteins from these lysates resulted in a heavy chain of ~44 kD in association with β_2M. Under reducing (R) conditions, the 1H1 and 3C11 immunoprecipitates resolved as single bands of ~37 kD, suggesting that CD1d was expressed on the cell surface as a disulfide-linked multimer.

Other immunoprecipitations have indicated that CD1d was expressed as a 48- to 54-kD protein, consistent with its predicted size and four sites for N-linked glycosylation (*6*, *10*). To determine whether the smaller size of CD1d on the surface of fresh IECs may be caused by proteolysis occurring in the intestine, we immunoprecipitated CD1d from two intestinal epithelial cell lines, HT29 and T84. A band of 37 kD was observed with the 1H1 and 3C11 mAbs under R conditions from both surface-labeled HT29 (Fig. 1A) and T84 cells (*11*), so the 37-kD size was not the result of proteolysis in the intestine.

To confirm that the 37-kD molecule detected on the surface of IECs was CD1d, we performed immunoprecipitations with mAb NOR3.2, a murine mAb generated to human CD1d (*10*, *12*). The results were similar to those obtained with the 1H1 and 3C11 mAbs: the NOR3.2 immunoprecipi-

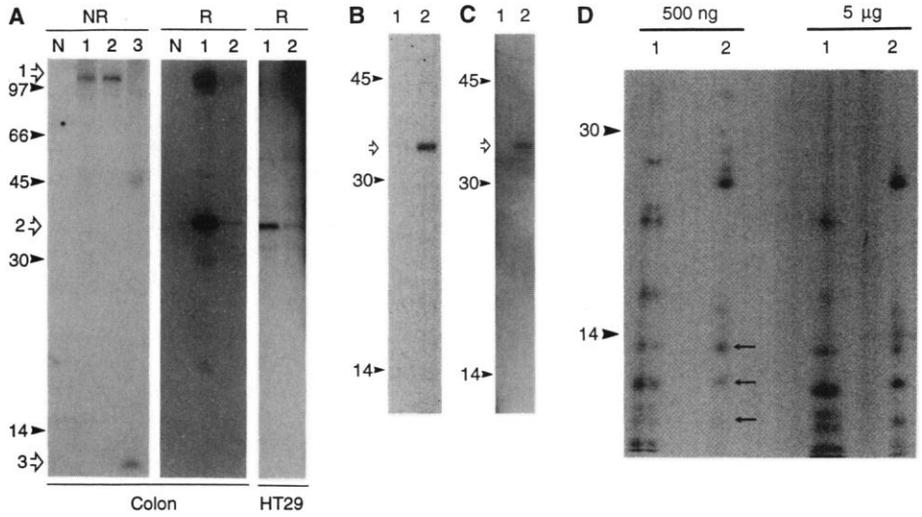

Fig. 1. Identification of CD1d on the surface of human IECs. (**A**) Immunoprecipitates of cell surface proteins from normal human IECs or the human IEC line, HT29, with the mAbs to CD1d, 3C11 (lanes 1) and 1H1 (lanes 2); the MHC class I framework mAb, W6/32 (lane 3); and nonimmune normal rat serum (lanes N), resolved by SDS-PAGE under NR or R conditions. CD1d under NR conditions (arrow 1) and R conditions (arrow 2) and β_2M (arrow 3) are designated. Autoradiography is shown after a 5-day exposure. (**B**) Immunoprecipitates of radioiodinated proteins from normal human IECs with the mAb to CD1d, NOR3.2 (lane 2), or an isotype-matched control mAb (immunoglobulin G1), Leu4 (lane 1), resolved by SDS-PAGE under R conditions. CD1d is designated by an arrow. (**C**) Reimmunoprecipitation of the 37-kD band in (A) (arrow) with normal rabbit serum (lane 1) or a rabbit antipeptide antiserum (lane 2). (**D**) *Staphylococcus aureus* V8 protease digestion of bacterially expressed CD1d (lanes 1) and the 37-kD protein (lanes 2) identified in (A) (arrow 2) at the indicated concentrations of V8 protease under R conditions. Bands identical in the two different preparations, indicated by arrows at 500 ng of V8 protease, are shown. The same bands are also observed with 5 µg of V8 protease. The bands were detected by a phosphorimager (Molecular Dynamics) after a 3-day exposure. Molecular size markers are indicated on the left in kilodaltons.

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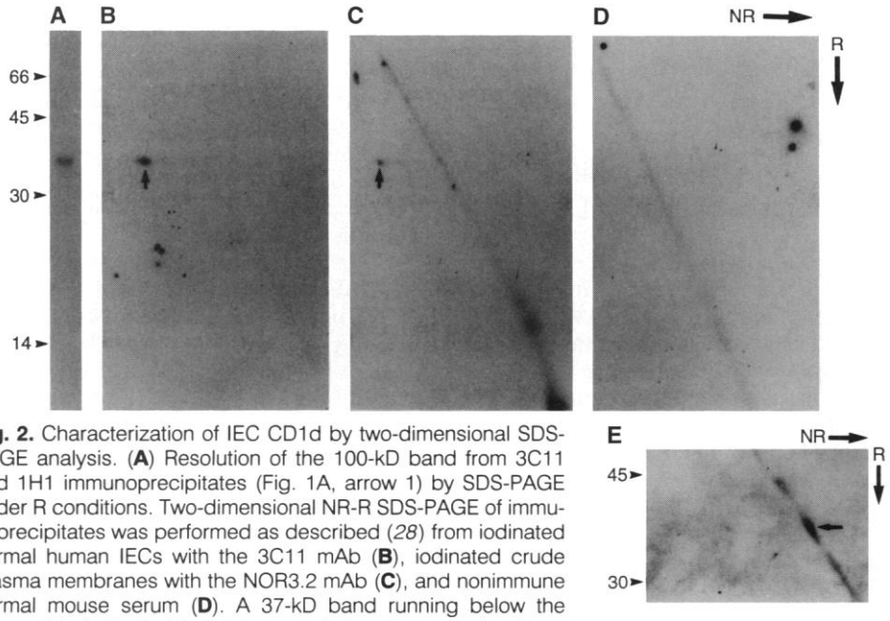

Fig. 2. Characterization of IEC CD1d by two-dimensional SDS-PAGE analysis. (**A**) Resolution of the 100-kD band from 3C11 and 1H1 immunoprecipitates (Fig. 1A, arrow 1) by SDS-PAGE under R conditions. Two-dimensional NR-R SDS-PAGE of immunoprecipitates was performed as described (*28*) from iodinated normal human IECs with the 3C11 mAb (**B**), iodinated crude plasma membranes with the NOR3.2 mAb (**C**), and nonimmune normal mouse serum (**D**). A 37-kD band running below the diagonal is indicated by the arrows. Autoradiography is shown after a 6-day exposure. (**E**) Two-dimensional NR-R protein immunoblot of a 3C11 immunoprecipitate from unlabeled normal colonic IEC plasma membranes with a rabbit antipeptide antiserum detected by enhanced chemiluminescence (Amersham) after a 1-min exposure. A major 37-kD band on the diagonal, consistent with a monomer, is indicated by the arrow. Molecular size markers are indicated on the left in kilodaltons.

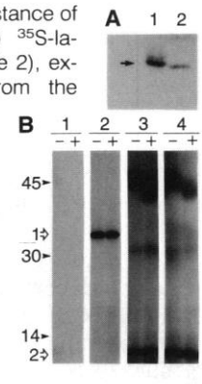

Fig. 3. *N*-Glycanase resistance of cell surface CD1d. (**A**) ^{35}S-labeled CD1d protein (lane 2), expressed in bacteria from the CD1d cDNA as described for Fig. 1D (*14*), was co-resolved with the 3C11 immunoprecipitate of iodinated normal human IECs (lane 1) under R conditions on a 12.5% polyacrylamide gel. The resultant bands were detected by a phosphorimager after a 3-day exposure. The 37-kD protein from a 3C11 immunoprecipitate of iodinated normal human IECs is indicated by the arrow. (**B**) Immunoprecipitates of iodinated normal human IECs with nonimmune normal rat serum (lanes 1), mAb 3C11 (lanes 2), mAb W6/32 (lanes 3), and the mAb to β_2M, A88 (lanes 4), resolved by SDS-PAGE on a 12.5% gel under R conditions before (−) or after (+) digestion with *N*-glycanase. The location of CD1d (arrow 1) and β_2M (arrow 2) are indicated. Molecular size markers are indicated on the left in kilodaltons. Autoradiography is shown after a 3-day exposure.

tated a single, major band of 37 kD under R conditions (Fig. 1B). Further confirmation that the 37-kD protein was CD1d was obtained by reimmunoprecipitation with an antipeptide antiserum raised in rabbits to the α1 and α2 domains of CD1d (Fig. 1C) (*13*) and by peptide mapping (Fig. 1D) (*14*). At least three identical peptides were observed when both the 37-kD band and metabolically labeled CD1d expressed in bacteria were subjected to V8 protease digestion, indicating that the bacterially expressed CD1d and the 37-kD protein are closely related or identical.

To address the possible multimeric nature of CD1d, we extracted the 100-kD band precipitated by the 1H1 and 3C11 mAbs under NR conditions and subjected it to electrophoresis under R conditions (Fig. 2) (*15*). A single molecular species of 37 kD was observed (Fig. 2A). Similarly, immunoprecipitates from iodinated normal colonic IECs, when resolved by two-dimensional, NR-R polyacrylamide gel electrophoresis (PAGE), had identical patterns, each with a dominant 37-kD band below the diagonal (Fig. 2B) (*11*). Immunoprecipitations from radiolabeled crude plasma membranes and two-dimensional gel analysis did not reveal any additional major bands running off the diagonal with CD1d, suggesting that CD1d was not linked to another protein exposed only at the cytoplasmic face of the membrane (Fig. 2C) (*16*).

These results suggested that CD1d was expressed as a disulfide-linked homotrimer on the cell surface of IECs. However, despite our use of iodoacetamide in the lysis buffer, we could not entirely rule out artifactual cross-linking during the labeling or immunoprecipitation steps. Therefore, a two-dimensional NR-R protein immunoblot of a 3C11 immunoprecipitate from unlabeled, crude, normal colonic IEC plasma membranes was performed with a rabbit antipeptide antiserum (*17*). In contrast to the results obtained with the iodinated material, the dominant CD1d band was observed on the diagonal, consistent with a monomer (Fig. 2E). Minor molecular species below the diagonal, consistent with a dimer and trimer, were seen only after a long exposure (*11*). Therefore, although CD1d may form a noncovalently associated multimer on the cell surface, the disulfide-linked multimeric form of CD1d observed here is largely due to artifactual cross-linking induced by the oxidative conditions present during labeling.

The apparent molecular size of CD1d on IECs was only slightly larger than the predicted molecular size of 35,500 for a mature, nonglycosylated form of CD1d (6). This small difference in molecular size could be observed when the mobility of CD1d isolated from IECs was compared directly with that of bacterially expressed CD1d, although distinct labeling methods could account for some differences in mobility (Fig. 3A). This suggested that underglycosylation could account for the smaller size of CD1d. To determine whether any of the four potential sites for N-linked glycosylation were used, we treated 3C11 immunoprecipitates from normal colonic IECs with *N*-glycanase (*18*). The results indicated that the 37-kD cell surface form of CD1d does not contain any N-linked carbohydrate (Fig. 3B). As a control, MHC class Ia proteins, containing one N-linked carbohydrate side chain, were immunoprecipitated and digested with *N*-glycanase (Fig. 3B). As we expected, removal of the single N-linked carbohydrate chain from the MHC class Ia proteins was observed.

Another feature of the immunoprecipitates with the CD1d-specific mAbs was the absence of β_2M, suggesting that the 37-kD form of CD1d was not associated with β_2M. We investigated the possibility of an association with β_2M that could be disrupted by the CD1d-specific mAbs by immunoprecipitations with a mAb to human β_2M, A88. This antibody immunoprecipitated β_2M and the *N*-glycanase–sensitive MHC class Ia heavy chains, but not CD1d, from the cell surface of radiolabeled colonic IECs (Fig. 3B). These results indicate that the 37-kD form of CD1d was expressed on the cell surface of normal IECs without β_2M, although a weak association with β_2M could not be entirely ruled out.

To confirm that CD1d can be expressed at the cell surface in the absence of β_2M, we transfected the CD1d complementary DNA (cDNA) into a human melanoma cell line, FO-1, that does not express β_2M (*19*). After transfection, Geneticin sulfate–resistant clones were analyzed by flow cytometry with the 1H1 mAb. Expression of CD1d was detected on the cell surface of each of the seven clones analyzed (Fig. 4); thus, CD1d can be expressed on the cell surface independently of β_2M. The murine homolog of CD1d, CD1.1,

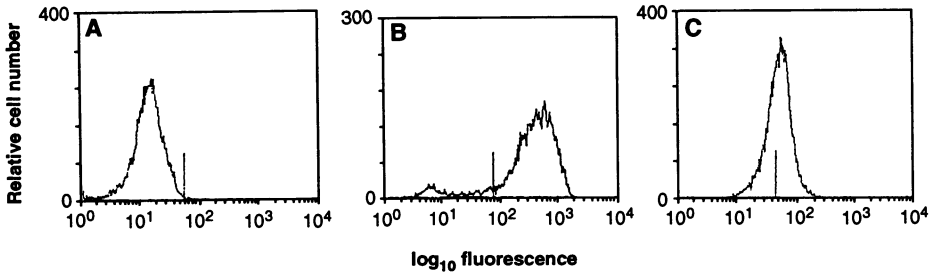

Fig. 4. The β_2M independence of cell surface CD1d. Indirect immunofluorescence of the β_2M-negative cell line, FO-1, transfected with the human CD1d cDNA and the murine CD1.1 cDNA. Staining with the 1H1 mAb is shown for the nontransfected FO-1 cell line (**A**) and a representative CD1d (**B**) and murine CD1.1 transfectant (**C**).

can also be expressed at the cell surface in the absence of β_2M (Fig. 4).

The data presented here show that CD1d on the surface of IECs has a structure that is distinct from other class Ia or class Ib proteins. Surface expression of MHC class I proteins in β_2M-negative cell lines and $\beta_2M^{-/-}$ mice has been demonstrated, but this expression is at low amounts with only some class I alleles and does not appear to be a functional pathway for antigen presentation (*1*, *3*–*5*). Some class I proteins can also be expressed at the cell surface in association with β_2M in the presence of specific inhibitors of N-linked glycosylation and may be functional (*20*). However, there is no evidence that these forms are normally expressed. There is evidence that other CD1 proteins may associate weakly with β_2M (*21*), but all other studies of CD1a through CD1d and of murine CD1.1 expressed in a variety of cell types have demonstrated β_2M-associated membrane glycoproteins (*6*, *10*, *22*). These observations indicate that synthesis of the CD1d isoform described here may be due to tissue-specific processing of CD1d.

Tissue-specific differences in glycosylation have been reported for a number of glycoproteins, but these generally reflect differences in processing of the high-mannose core by glycosyltransferases, rather than a complete failure to add this core (*23*). The data presented here suggest that IECs have a mechanism to specifically block the addition of the high-mannose core, presumably through cotranslational association of CD1d with proteins in the endoplasmic reticulum. The lack of association with β_2M may be dictated by the conformation of nonglycosylated CD1d and indicates that a conventional site for binding peptide antigens may not be present. This suggests that CD1d expressed on the surface of IECs does not function as a conventional antigen-presenting molecule and may have a different immunological role in the intestinal mucosa.

Studies have demonstrated that CD1d can be recognized by $CD8^+$ T lymphocytes (*24*, *25*). IECs stimulate the proliferation of $CD8^+$ T cells, and this proliferation can be blocked by the mAb to CD1d, 3C11 (*24*). Specific recognition of CD1-transfected target cells by $CD8^+$ T cells from the intestinal mucosa has also been demonstrated (*25*). The intestinal mucosa is a specialized immunological compartment composed largely of $CD8^+$ cytolytic T cells (*26*) with an oligoclonal TCR repertoire (*25*, *27*). The target antigens recognized by these mucosal T cells and their biological function have not yet been determined. The results reported here indicate that alternative isoforms of CD1d or CD1d that present a limited array of nonconventional antigens may be the target ligands for these T cells.

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8. Normal human IECs, isolated nonenzymatically from resected surgical specimens in the presence of EDTA and dithiothreitol (DTT) (*6*), and the HT29 cell line were radiolabeled with ^{125}I by the lactoperoxidase-catalyzed method as described [R. S. Blumberg *et al.*, *J. Biol. Chem.* **265**, 14036 (1990)], and a lysate of proteins was prepared in immunoprecipitation buffer containing 20 mM tris, pH 7.8; 150 mM NaCl; 1 mM EDTA; 10 mM iodoacetamide; 1% NP-40; and protease inhibitors [phenylmethylsulfonyl fluoride (100 μg/ml) and aprotinin, chymostatin, pepstatin A, antipain, and leupeptin (each 1 μg/ml)]. Immunoprecipitates were prepared and resolved by SDS-PAGE in the absence (NR) or presence (R) of 5% β-mercaptoethanol on a 12.5% gel.
9. The specificity of the 1H1 mAb has been tested by immunofluorescence and immunoprecipitation (*6*). The 3C11 mAb specifically recognizes CD1d on stable transfectants as shown by immunofluorescence and immunoprecipitation (*11*).
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12. Radiolabeling, solubilization of proteins, immunoprecipitation procedures, and SDS-PAGE of proteins from normal colonic IECs were performed as described (*8*), except that the lysate was boiled in 1% SDS and diluted to 0.1% SDS before immunoprecipitation.
13. After autoradiography, the 37-kD bands from the 1H1 and 3C11 immunoprecipitates in Fig. 1A were identified (arrow 2); eluted from the gel in 100 mM NH_4HCO_3, 0.1% SDS, 1.0% NP-40, and myoglobin (50 μg/ml) with protease inhibitors; precipitated with 20% trichloroacetic acid; washed with acetone; and resuspended in immunoprecipitation buffer as described (*8*) with 1.0% SDS. After boiling and dilution to 0.1% SDS, immunoprecipitates were prepared with a rabbit antipeptide antiserum to CD1d (*6*) and the immunoprecipitates resolved by SDS-PAGE under R conditions.
14. A CD1d cDNA (*6*) with the leader sequence deleted and an initiation methionine inserted as the first codon of the α1 domain was cloned into the pT7-7 plasmid and expressed in *Escherichia coli* [S. Tabor and C. Richardson, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1074 (1985)]. We induced T7 RNA polymerase in the presence of rifampicin and ^{35}S-labeled cysteine-methionine to specifically label CD1d. Bands from the ^{35}S-labeled bacterially expressed CD1d and the 37-kD protein resolved from iodinated 3C11 immunoprecipitates of normal human IECs were removed from a gel and digested with either 5 μg or 500 ng of *Staphylococcus aureus* V8 protease (ICN) per lane after electroelution into a stacking gel as described [E. Harlow and D. Lane, *Antibodies* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988), pp. 641–642]. After 30 min of digestion at room temperature, the peptides were resolved by SDS-PAGE under R conditions into a 12.5% gel.
15. After autoradiography, the 100-kD band from the 1H1 and 3C11 immunoprecipitates in Fig. 1A were identified (arrow 1), eluted from the gel as described (*13*), resuspended in Laemmli buffer [J. K. Laemmli, *Nature* **227**, 680 (1970)] containing reducing agents, and resolved by SDS-PAGE.
16. Plasma cell membranes were prepared from normal colonic IECs by cavitation with high-pressure nitrogen by the method of Katzoumi and coworkers [P. Katzoumi, C. A. Parkos, C. Delp-Archer, *Am. J. Physiol.* **264**, C1327 (1993)]. The whole-cell membranes were resuspended in relax buffer [10 mM tris (pH 7.4), 10 mM NaCl; 100 mM KCl, 3.5 mM $MgCl_2$, and 1 mM adenosine triphosphate (ATP)] and pelleted at 100,000*g* in a TL-100 ultracentrifuge (Beckman). Radiolabeling and solubilization of proteins were performed as described in Fig. 1 (*8*), except that the iodination was performed in relax buffer and the lysate was boiled in 1% SDS with dilution to a final concentration of 0.1% SDS before immunoprecipitation with the NOR3.2 mAb. Immunoprecipitates were resuspended in Laemmli buffer [J. K. Laemmli, *Nature* **227**, 680 (1970)] in the absence of reducing agents and resolved by two-dimensional NR-R SDS-PAGE on a 12.5% slab gel.
17. An unlabeled lysate of proteins from normal colonic IEC plasma cell membranes, prepared as described (*16*), was immunoprecipitated with the 3C11 mAb. The immunoprecipitate was resolved by two-dimensional NR-R SDS-PAGE, transferred to nitrocellulose, and protein immunoblotted with a rabbit antipeptide antiserum to CD1d. Immunodetection was performed with horseradish peroxidase–conjugated goat antibody to rabbit immunoglobulin and enhanced chemiluminescence (Amersham).
18. Iodination and immunoprecipitation of cell surface proteins from normal human IECs was as described in Fig. 1 (*8*). Immunoprecipitates were boiled in 0.8% β-mercaptoethanol and 0.5% SDS. After being boiled, the immunoprecipitates were cooled to room temperature and two volumes of

0.25 M $NaPO_4$ and 1 mM EDTA containing 1.0% NP-40 and 1 μl of *N*-glycanase (specific activity 0.227 U/μl; Genzyme) were added, and then the mixtures were incubated for 24 hours at 37°C. The treated and untreated immunoprecipitates were resuspended in Laemmli buffer with reducing agents and resolved by SDS-PAGE.
19. The FO-1 cell line, a human β_2M-negative cell line [C. M. D'Urso *et al.*, *J. Clin. Invest.* **87**, 284 (1991)], was transfected with 20 μg of circular human CD1d or murine CD1.1 (*6*) cDNA in the pSRα Neo expression vector [Y. Takebe *et al.*, *Mol. Cell. Biol.* **8**, 466 (1988)] by electroporation with a gene pulsar unit (Bethesda Research Lab) at 400 V and a capacitance of 1600 μF. After 48 hours, transfectants were selected at 2×10^5 cells per well in 96-well flat-bottomed plates with Geneticin sulfate (G418; 3.0 mg/ml; specific activity 500 μg/mg; Gibco). Geneticin sulfate–resistant wells were screened by flow cytometry with the 1H1 mAb.
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Induction of an Olfactory Memory by the Activation of a Metabotropic Glutamate Receptor

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Female mice form an olfactory memory of male pheromones at mating; exposure to the pheromones of a strange male after that mating will block pregnancy. The formation of this memory is mediated by the accessory olfactory system, in which an increase in norepinephrine after mating reduces inhibitory transmission of γ-aminobutyric acid from the granule cells to the mitral cells. This study shows that the activation of mGluR2, a metabotropic glutamate receptor that suppresses the γ-aminobutyric acid inhibition of the mitral cells, permits the formation of a specific olfactory memory without the occurrence of mating by infusion of mGluR2 agonists into the female's accessory olfactory bulb. This memory faithfully reflects the memory formed at mating.

Female mice form an olfactory memory of the pheromones of the male with which they mate. Subsequent exposure to the pheromones of a strange male will block a female's pregnancy, but exposure to the pheromones of a male of the same strain as the original male does not block pregnancy (*1*, *2*). The synaptic changes underlying this memory formation occur in the accessory olfactory bulb (AOB) (*2*–*6*). In the AOB, the mitral cells, when activated by vomeronasal nerve inputs, depolarize granule cells by means of glutamate released at dendrodendritic synapses (*2*) (Fig. 1). This depolarization in turn releases γ-aminobutyric acid (GABA) from granule cells and hyperpolarizes mitral cells (*2*).

Norepinephrine, the turnover of which is enhanced after mating, reduces the GABA-mediated feedback inhibition and induces an olfactory memory of pheromones present at mating (*4*). A metabotropic glutamate receptor (mGluR), mGluR2, is expressed predominantly at the dendrites of granule cells and, when activated by its potent agonist, (2S,1′R,2′R,3′R)-2-(2,3-dicarboxycyclopropyl)glycine (DCG-IV), suppresses the GABA-mediated inhibition of the mitral cells (*7*, *8*). Because DCG-IV and norepinephrine both reduce GABA transmission from granule cells to mitral cells, blockage of the GABA-mediated feedback inhibition by DCG-IV could, in theory, permit the formation of a specific pheromonal memory without the occurrence of mating.

We investigated the role of mGluR2 in olfactory memory formation by designing the following three-stage behavioral protocol (*3*, *9*) (Fig. 2). Drugs were infused into the AOB of estrous females 0 and 1.5 hours after the beginning of a 6-hour exposure to BALB/c male pheromones without mating. At the next estrus, the females were mated with a male from a CBA strain and then reexposed to the pheromones of the BALB/c male to test for formation of a pheromonal memory. Under this protocol, it can be determined that memory formation occurs at the time of drug infusion if the drug treatment prevents pregnancy block by the test pheromonal exposure. The results of a series of experiments are summarized in Fig. 2.

Because DCG-IV, though less effectively than mGluR2, activates the *N*-methyl-D-aspartate (NMDA) receptor (*8*), we first tested *trans*-1-aminocyclopentane-1,3-di-

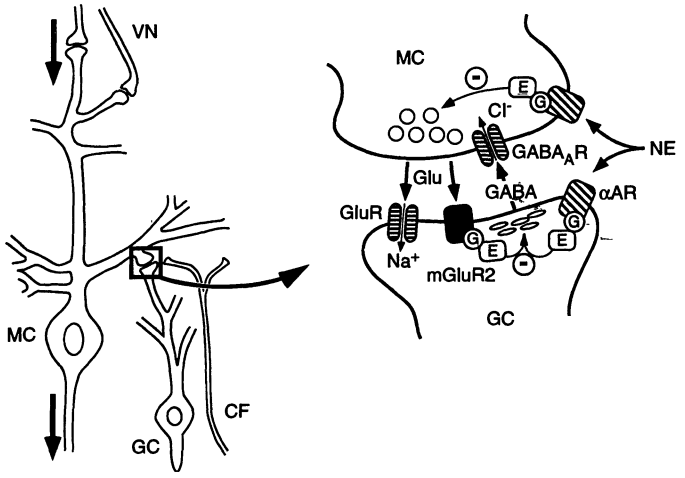

Fig. 1. Synaptic relations in the AOB and a model for the microcircuitry in dendrodendritic synapses between a granule cell and a mitral cell. VN, vomeronasal nerve; MC, mitral cell; GC, granule cell; CF, centrifugal fiber of norepinephrine projection from locus ceruleus; Glu, glutamate; GluR, ionotropic glutamate receptor; $GABA_AR$, $GABA_A$ receptor; αAR, α-adrenergic receptor; G, G protein; E, intracellular effector; NE, norepinephrine. The signaling pathways that inhibit GABA release are marked by minus symbols. As is the case in the synaptic circuitry of the main olfactory bulb (*17*), the reduction of GABA transmission by norepinephrine may be caused by the inhibition of mitral cell excitation. A direct presynaptic action of norepinephrine on the granule cell, however, is also possible; and two pathways for norepinephrine inhibition of GABA transmission are indicated.

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