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16 November 1994; accepted 21 March 1995

In Vivo Transfer of GPI-Linked Complement Restriction Factors from Erythrocytes to the Endothelium

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Many proteins are associated with the outer layer of the cell membrane through a posttranslationally added glycosyl phosphatidylinositol (GPI) anchor. The functional significance of this type of protein linkage is unclear, although it results in increased lateral mobility, sorting to the apical surface of the cell, reinsertion into cell membranes, and possibly cell signaling. Here evidence is presented that GPI-linked proteins can undergo intermembrane transfer in vivo. GPI-linked proteins expressed on the surface of transgenic mouse red blood cells were transferred in a functional form to endothelial cells in vivo. This feature of GPI linkage may be potentially useful for the delivery of therapeutic proteins to vascular endothelium.

Proteins can be anchored to cell membranes through the posttranslational attachment of a covalently linked glycosylated form of phosphatidylinositol. Proteins with GPI anchors exhibit a diversity of activities including complement regulation [for example, human decay accelerating factor (DAF) and human CD59], cell interaction (for example, lymphocyte functional antigen–3), and enzymatic activity (for example, alkaline phosphatase and acetylcholinesterase) (*1*). Proteins with a GPI tail are characterized by diffusion coefficients greater than most transmembrane proteins (*2*) and apical cell surface expression (*3*). Furthermore, DAF and GPI-linked T cell marker protein (Thy1), after isolation from a cell membrane, can reinsert into heterologous cell membranes in vitro and retain biological function (*4*).

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Because GPI-anchored proteins are broadly distributed and are easily extracted from and reinserted into cell membranes, we hypothesized that a GPI anchor might enable in vivo protein transfer from one cell to another under physiological conditions. To test this hypothesis we made transgenic mice that expressed two GPI-anchored complement restriction factors (CRFs), CD59 and DAF, in an erythroid-specific manner and then assayed for their presence on endothelial cells (ECs). Both of these glycoproteins regulate complement activation and are thought to protect against homologous lysis. DAF is a ~70-kD GPI-anchored glycoprotein that prevents the formation of C3 convertases [for example, activated C3 (C3b) associated with factor B and activated C4 (C4b) associated with C2] or dissociates the convertases once they are formed (*5*). CD59 is a ~19-kD GPI-anchored glycoprotein that binds to C8 and C9 of the assembling membrane attack complex (MAC), blocking both complement pathways at the terminal stage (*6*–*8*).

High level tissue-specific expression of human globin genes can be readily obtained in transgenic mice (*9*, *10*). We designed two expression constructs, #506 and #463 (*11*), that contain sequences encoding DAF and CD59 linked to the regulatory sequences of the globin gene (Fig. 1). Transgenic mice were produced from these constructs and bred to generate transgenic offspring for analysis.

The expression of human CD59 and DAF protein was detected on red blood cells (RBCs) of #506 transgenic mice by quantitative flow cytometry with appropriate monoclonal antibodies (*12*). We detected approximately 40,000 molecules of CD59 per RBC, about two times the amount found on human RBCs. DAF expression on the transgenic RBCs more closely resembled that observed on human RBCs (estimated to be about 3000 molecules per RBC). Similar amounts of expression were detected with both constructs. Transgenic RBCs were resistant to lysis by human complement, indicating that the proteins were functional. No deleterious effects of the CRF expression were detected in the transgenic mice even after multiple generations of breeding.

We examined the cellular distribution of these proteins in various tissues by immu-

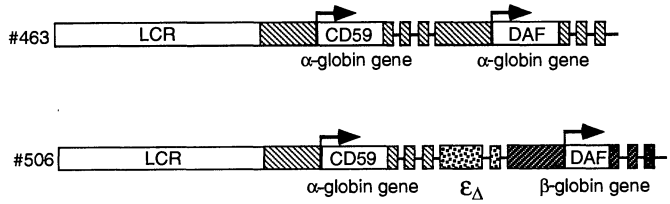


Fig. 1. Constructs used to make transgenic mice. In construct #463, the cDNAs for CD59 and DAF were inserted into the first exon of the human α-globin gene, and the ATG codon used for the synthesis of α-globin protein was simultaneously removed (*11*). For construct #506, the DAF cDNA was inserted into the ATG start site of the human β-globin gene. A deletion mutant (ε_Δ) of the human ε-globin gene was used in conjunction with the CRF:globin fusion genes and LCR to make a miniglobin locus (*11*).

nohistology with monoclonal antibodies (*13*). Both CD59 and DAF were detected on vascular endothelial and hematopoietic cells in all of the tissues examined: heart, kidney, liver, spleen, and lung (Table 1 and Fig. 2). Globin gene expression controlled by the human β-globin locus control region (LCR) in transgenic animals is restricted to the erythroid lineage (*9*, *10*, *14*). Northern analysis of RNA from various tissues confirmed this observation (*15*). However, to conclusively demonstrate that CRF:globin chimeric genes are not expressed in ECs and that the presence of CRF proteins on ECs resulted from intermembrane transfer, we conducted further experiments.

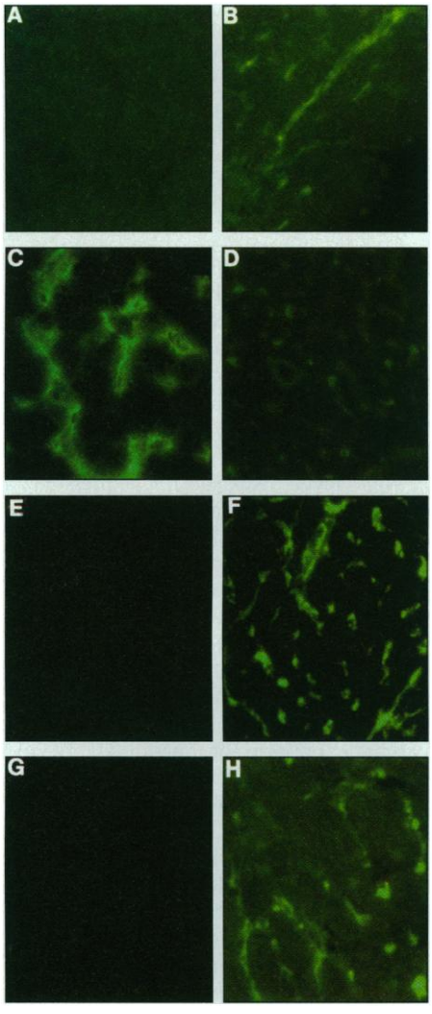


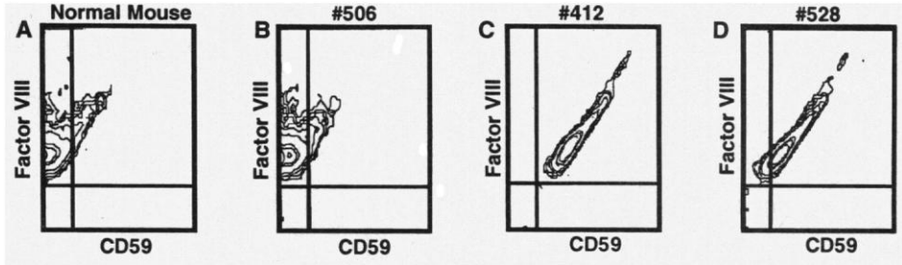
Fig. 2. Immunohistology from mouse heart. Samples were frozen, cryosectioned, and stained with monoclonal antibodies (*13*). Heart tissue from (**A**) nontransgenic and (**C**) #463 transgenic mice was stained for CD59. (**B**) Heart tissue stained for CD59 taken from a nontransgenic mouse containing reconstituted bone marrow from a transgenic mouse donor after a successful bone marrow transplant. (**D**) Heart tissue from #506 transgenic mice stained for DAF. (A) and (B) were photographed at low magnification (original magnification, ×200) to demonstrate overall staining patterns; (C) and (D), photographed at high magnification (original magnification, ×400), demonstrate CD59 and DAF staining associated with vascular endothelium. Transgenic and nontransgenic mouse heart tissues were stained for human iC3b and MAC after the hearts were perfused with 50% human plasma in a modified Langendorf perfusion system (*21*). The presence of iC3b and MAC was used as a marker to indicate complement activity (*26*). No human iC3b or MAC was observed on heart tissue from transgenic mice, (**E**) and (**G**), respectively, but both were detected on the vascular endothelium of nontransgenic mouse hearts after perfusion with human serum, (**F**) and (**H**), respectively. Original magnification for (E) to (H), ×400.

Table 1. Expression patterns of CD59 and DAF in ECs from transgenic animals. Tissue samples were frozen, cryosectioned, and stained for CD59 and DAF as described (*13*). Relative fluorescence intensity was assessed at the time of blinded microscopic examination and assessed by an arbitrary scale (JLPlatt) from 1 to 4 (background is 0). Under these conditions, equivalent human tissue expression is 2 for CD59 and 1 for DAF. Expression levels of CD59 were similar in #463 and #506 animals, and DAF expression was slightly higher in #506 animals.

Tissue	Fluorescence intensity	
	CD59	DAF
Construct #463		
Heart	2	1
Kidney	3	2
Lung	3	1
Spleen	3	2
Liver	1	1
Construct #506		
Heart	3	2
Kidney	3	3
Lung	3	2
Spleen	3	3
Liver	3	1

We examined ECs for expression of CD59 and factor VIII (an endothelial cell marker) by dual-label flow cytometry immediately on isolation and after culture in vitro (*16*) for five passages (~28 days). ECs were analyzed from control mice and transgenic mice expressing construct #506 and two other constructs, #412 and #528, which express CRFs directly in ECs (*17*). Factor VIII expression was observed in all ECs; however, only ECs from mice expressing constructs #412 and #528 had a population of cells that expressed both CD59 and factor VIII after culture (Fig. 3). Similar levels of CD59 expression were observed on #506 and #528 transgenic ECs at the time of isolation (*18*), consistent with a similar level of endothelial staining of the constructs by immunohistology. The absence of CD59 on endothelial cells after culture from animals expressing construct #506 indicates that LCR-globin–directed expression of CD59 was not occurring in ECs. Thus, complement regulatory antigens detected on vascular endothelium appeared to be transferred from RBCs to ECs.

The ECs were analyzed directly for RNA expression by reverse transcription and polymerase chain reaction amplification (RT-PCR) followed by Southern (DNA) blot analysis with a CD59 probe (*19*). ECs



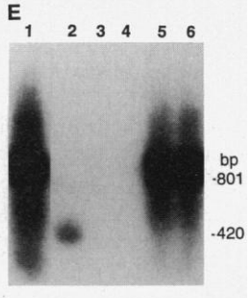


Fig. 3. Dual-color flow cytometry and RT-PCR analysis of cultured mouse ECs. Fat pad ECs were extracted from transgenic and nontransgenic mice (*16*) and cultured for five passages (~28 days) before analysis. Cells were analyzed by dual-color flow cytometry to assess the presence of CD59 and factor VIII on the cell surface. Factor VIII was used to confirm that the cells were endothelial. The ECs were obtained from transgenic mice that expressed CD59 either in an erythroid-specific manner (#506) or directly in ECs (#412 and #528). As indicated in (**A**) and (**B**), ECs derived from animals expressing construct #506 were essentially identical to those from nontransgenic animals. The ECs derived from animals expressing construct #412 (**C**) or #528 (**D**) were positive for both factor VIII and CD59. (**E**) A RT-PCR assay was used to analyze RNA expression in ECs from a nontransgenic mouse and transgenic mice from the same constructs used in dual-color flow cytometry. The sensitivity was maximized by transferring the RT-PCR gel by Southern blot and hybridizing it with a ^{32}P-labeled CD59 cDNA probe. An appropriately sized CD59 PCR product (*19*) was detected from the EC RNA of #412 and #528 transgenic mice (lanes 1 and 2, respectively). No CD59 PCR products were detected in RNA from #506 transgenic mice (lane 3) or nontransgenic mice (lane 4). Lanes 5 and 6 are RT-PCR results from whole-blood RNA extracted from a BMT donor line mouse (lane 5) and a nontransgenic BMT recipient mouse (lane 6).

were extracted from transgenic mice expressing constructs #506, #412, and #528 and a nontransgenic mouse, and then the ECs were cultured for five passages (~28 days) before RNA extraction. The CD59 PCR product was detected only in ECs from animals containing constructs #412 and #528 (Fig. 3). No CD59 PCR product was detected in ECs from transgenic animals expressing construct #506 or from nontransgenic mice.

We performed bone marrow transplants to conclusively show that the transgene-expressed CD59 could transfer from RBCs to ECs. Bone marrow–derived cells from a line of transgenic mice expressing CD59 from the globin promoter were transplanted into normal mice of similar genetic background (*20*). After myeloablation, 3 out of 20 nontransgenic mice were rescued with bone marrow from transgenic mice. Flow cytometric analysis of whole blood from bone marrow recipient mice demonstrated the presence of human CD59 on RBCs. Analysis of whole-blood mRNA from one bone marrow transplant (BMT) recipient and donor by RT-PCR confirmed the success of the transplant (Fig. 3). Immunohistological analysis of heart tissue in the BMT recipients demonstrated the presence of CD59 antigen on vascular endothelium. Specific staining for CD59 was observed in the heart of a BMT recipient, but not in the sample from a nontransgenic animal (Fig. 3).

We detected the transfer of these proteins by staining tissue sections with monoclonal antibodies. It is possible that staining could result from transfer of an epitope and not from transfer of the intact functional molecules. To rule out this possibility, we assayed for the function of these proteins in cardiac endothelium. We used a modified Langendorf heart preparation (*21*) and perfused the mouse hearts with human plasma. To facilitate perfusion of the organ, we initially perfused the hearts with oxygenated Iscove's modified Dulbecco's medium (IMDM) for 30 min before perfusion with IMDM supplemented with human plasma. The hearts were perfused with 50% human plasma for 60 min. Under these conditions antibodies present in human plasma bind to ECs of the mouse heart and activate complement. If CD59 and DAF are active, this process should be inhibited. These molecules function to inhibit complement intrinsically on the surface of the cell (*5*, *22*). DAF activity was assessed by monitoring the amount of iC3b (a product of C3 indicative of C3 convertase activity). We measured formation of the MAC to assess the biological activity of CD59. The MAC and iC3b were not detected in hearts taken from mice transgenic for both CD59 and DAF (Fig. 2). Heavy deposition of iC3b and MAC was observed in the hearts of nontransgenic mice. This demonstrates that both CD59 and DAF antigens are transferred in vivo to the vascular endothelium as functional complement-inhibiting proteins.

We have demonstrated that proteins containing a GPI linkage can transfer between membranes of cells in vivo. The functional significance of this process is unknown. However, in the case of GPI-anchored proteins such as DAF and CD59 that are involved in the inhibition of complement activation on host surfaces, transfer onto ECs may help maintain vascular integrity. For example, CD59 and DAF may be lost from the endothelial cell as a result of complement activation, as may occur during transient ischemia (*23*), after which these proteins could transfer from RBCs to the ECs. In this way the RBCs might serve as a reservoir. Transfer might ensure an even distribution of these proteins to confer complete protection of the vasculature. If transfer is a passive process, a gradient may actually occur favoring transfer from RBCs to ECs given the high concentration of RBCs in blood (approximately 1×10^9 RBCs per milliliter). Isolated CD59 will transfer to human ECs and rabbit RBCs in vitro using high-density lipoprotein as a carrier (*23*). Similar mechanisms of transfer may be involved in vivo. Variant surface glycoprotein, a GPI-anchored protein on trypanosomes, can transfer to RBCs in vitro (*24*), and human DAF can be incorporated into trypanosomes in vivo (*25*). It is not known if transfer is restricted to CD59 and DAF or a property of all GPI-linked proteins. The significance of CD59 and DAF transfer in understanding certain disease states, infections, and cell-cell interactions is yet to be elucidated as are the applications of such a system for delivering specific proteins to the endothelium of transgenic animals or in gene therapy.

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11. To prepare DNA for microinjection we amplified the complementary DNA (cDNA) of human CD59 by PCR using the plasmid YTH53.1 #1 (*7*) as a template. PCR primers were used that cloned the entire coding sequence with convenient restriction sites (5′ primer: 5′-GGATCCCCATGGGAATCCAAGGAGG-GTC-3′; 3′ primer: 5′-CGACCCATGGTTAGGGAT-GAAGGCTCC-3′). The cDNA of human DAF was cloned from human placental mRNA (Clonetech, Palo Alto, CA) by PCR. PCR primers were designed to clone the coding sequence with convenient restriction sites (5′ primer: 5′GGAGCTCTAGATCAT-GACCGTCGCGCGGC-3′; 3′ primer: 5′-GGATCCT-GCAGCTAAGAAACTAGGAACAGTCTG-3′). The cDNAs for CD59 and DAF were each inserted into the start codon of a genomic human α1-globin gene [D. Greaves *et al.*, *Nature* **343**, 183 (1990)] in the case of construct #463. In construct #506, the cDNA for DAF was inserted into the start codon of the human β-globin gene. In addition, a Bgl II–Bst X1 deletion mutant of the human ε-globin gene was placed between the CRF:α-globin and CRF:β-globin fusion genes. A 6.5-kb fragment of the human locus control region (LCR) was ligated on the 5′ end of the globin genes containing CD59 and DAF. Thus, CD59 and DAF are expressed in a hematopoietic tissue–specific manner (*9*). Transgenic mice were produced by pronuclear microinjection of the constructs according to established procedures [B. Hogan, F. Costantini, F. E. Lacy, *Manipulating the Mouse Embryo* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1986)].
12. RBCs were immunostained and analyzed on a Coulter Profile II flow cytometer (Coulter Electronics, Miami, FL) according to standard protocol (*7*). The relative number of molecules per RBC were quantitated with the Quantum Simply Cellular System (Flow Cytometry Standards, San Juan, Puerto Rico) according to the manufacturer's protocol.
13. Samples for immunofluorescence studies were snap frozen in isopentane and stored at −80°C until they were sectioned. Frozen tissue sections (4 μm) were prepared in a cryostat (Leica, Heidelberg, Germany). The sections were air-dried, fixed with acetone, and washed with phosphate-buffered saline (PBS) as described [J. Platt *et al.*, *J. Exp. Med.* **158**, 1227 (1983)]. Each section was incubated with monoclonal antibodies (mAbs). The mAbs were detected with a double fluorochrome layer consisting of fluorescein isothiocyanate (FITC)–conjugated affinity-isolated $F(ab)'_2$ goat antibody to the appropriate monoclonal immunoglobulin G (IgG) and FITC-conjugated affinity-isolated $F(ab)'_2$ rabbit antibody to goat IgG. Tissue sections were washed with PBS after incubations and mounted with a *p*-phenylenediamine and glycerol solution. Background immunofluorescence was evaluated by preparing sections as described above, but with the omission of the primary antibody. The murine mAb to human C5b neoantigen was obtained from Quidel (San Diego, CA). A mouse mAb to the neoantigen of the MAC (MBM5) was acquired from A. F. Michael, University of Minnesota [R. J. Falk, A. P. Dalmasso, Y. Kim, *J. Clin. Invest.* **72**, 560 (1983)]. Murine mAb to human DAF was obtained from Harland Bioproducts for Science (Indianapolis, IN). A rat mAb (YTH 53.1) was used to detect human CD59 (*7*).
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15. For Northern (RNA) analysis, total cellular RNA was extracted from heart, lung, kidney, and liver tissues of transgenic mice expressing constructs #463 or #506 and control mice according to established procedures [P. Chomczynski and N. Sacchi, *Anal. Biochem.* **162**, 156 (1987)]. After whole-body perfusion (*9*) to remove peripheral blood, total cellular RNA was extracted from tissue samples of #463, #506, and control mice. Northern blot analysis of tissues with and without whole-body perfusion were compared with a ^{32}P-labeled RNA probe generated with a CD59 cDNA riboprobe (Promega Madison, WI). An appropriate-size hybridization band (~1 kb) was observed in all tissues derived from transgenic mice without whole-body perfusion. No hybridization signal was observed in tissues derived from control and perfused transgenic mice except for a faint signal from transgenic kidney which was attributed to residual blood contamination.
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17. Construct #412 is similar to #463 with two notable exceptions: There is only one α-globin gene in which CD59 is inserted at the start codon, and the α-globin promoter and LCR are replaced with a chick β-actin promoter. In the line of mice used in this report, expression was characterized on heart ECs but not RBCs. As for construct #528, it is a 70-kb genomic fragment that includes the complete CD59 gene, promoter, and flanking sequences. Expression was detected on ECs and RBCs. Expression on RBCs was lower than that observed in #463 and #506 mice.
18. Mouse hearts were removed and dissected into quarters to expose the linings of the chambers and rinsed in PBS. The heart fragments from two mice were digested in 5 ml of dispase [50 U/ml (Collaborative Biomedical Products, Bedford, MA)] for 30 min at 37°C in a shaking water bath. The resulting cell suspension was pelleted and washed three times in PBS. Cells (1×10^8) were stained for factor VIII (with FITC) (FL1) and for CD59 (with phycoerythrin) (FL2). Appropriate single-color and matched-isotype controls were also stained. Cells were analyzed on a Coulter Profile II cytometer by using a dual plot of forward scatter versus FL1 to gate the population of ECs. It was estimated that this population comprised approximately 5% of the cells analyzed (calculated without discrimination of the forward scatter). Gated cells were then analyzed on a dual-color plot of FL1 versus FL2.
19. Blood (~200 μl) was collected from transgenic and nontransgenic mice. Immediately after collection, a fraction enriched in polyadenylated RNA was isolated by Quick Prep (Pharmacia, Piscataway, NJ) according to the manufacture's protocol. Reverse transcriptase reactions were performed with the First-Strand cDNA Synthesis Kit (Pharmacia) according to the manufacture's protocol before PCR. Primers were used to amplify an 801–base pair (bp) fragment (after splicing) which extended from the first exon of CD59 to the second exon of α-globin from a nontransgenic bone marrow recipient, a representative bone marrow donor, and transgenic mice expressing constructs #412 and #506. The sequence of the CD59 primer was 5′-ATGGGAATCCAAGGAGGGT-3′, and the sequence of the α-globin primer was 5′-GCAAAGGTGCCCTTGAGG-3′. Primers were used that would amplify a 420-bp (after splicing) product extending from exon 1 to exon 4 from construct #528 mRNA. The sequences of these primers were (5′ primer) 5′-AGGCTGGAAGAGGATCTTGG-3′ and (3′ primer) 5′-TCTCCTGGTGTTGACT-TAGG-3′.
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21. Mice were anesthetized with isoflurane or pentobarbital (50 mg per kilogram of body mass, administered intraperitoneally) and the chest opened. The heart was cooled with ice slush and explanted. The tip of a 22-guage angiocatheter was secured in the aortic root while the heart was maintained at 4°C. The Langendorff circuit consisted of a reservoir, a roller pump, and a column that provided 25 cm of perfusion pressure to the heart. Before suspending the heart from the circuit, the circuit was filled with IMDM with 50 mM Hepes (Gibco, Grand Island, NY) and allowed to equilibrate thermally and to a pH of 7.4. The perfusion fluid was oxygenated by bubbling O_2 (0.5 liter/min) into the reservoir and maintained at 38°C. The heart was suspended from the apparatus and the heart rate monitored. After 30 min of stabilization, 7 ml of medium was removed from the reservoir and 10 ml of 60% human plasma, diluted in IMDM, was added to yield a final concentration of 50% human plasma. The heart rate was monitored for 1 hour. Biopsies of the heart obtained at the conclusion of the procedure were prepared and stained as previously described (*13*).
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1 February 1995; accepted 26 April 1995

HERG, a Human Inward Rectifier in the Voltage-Gated Potassium Channel Family

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In contrast to other members of the Eag family of voltage-gated, outwardly rectifying potassium channels, the human *eag*-related gene (*HERG*) has now been shown to encode an inwardly rectifying potassium channel. The properties of HERG channels are consistent with the gating properties of Eag-related and other outwardly rectifying, S4-containing potassium channels, but with the addition of an inactivation mechanism that attenuates potassium efflux during depolarization. Because mutations in *HERG* cause a form of long-QT syndrome, these properties of HERG channel function may be critical to the maintenance of normal cardiac rhythmicity.

The *ether-à-go-go* (*eag*) gene was originally identified in *Drosophila* on the basis of its leg-shaking mutant phenotype (*1*), attributed to an increase in neuronal excitability and transmitter release at the neuromuscular junction (*2*). The polypeptide predicted by the sequence of the cloned gene exhibits the characteristic features of K^+ channels (*3*), and expression studies in frog oocytes confirm that *eag* encodes a functional K^+ channel (*4, 5*). The *eag* locus defines an extended gene family, with members falling into subfamilies on the basis of their sequence similarity: Eag, named for the original isolate from *Drosophila*; Elk, for *eag*-like K^+ channel; and Erg, for *eag*-related gene (*6*). Both *eag* and *M-EAG*, its mouse counterpart, encode voltage-gated, outwardly rectifying K^+ channels (*4, 5*). We report here that *HERG*, a gene in the Erg subfamily isolated from human hippocampus (*6*), encodes a K^+ channel with inwardly rectifying properties in spite of the extensive homology with the outwardly rectifying members of the Eag family (49% amino acid identity) (*7*).

Inward rectifiers are a large class of K^+-selective ion channels that preferentially conduct inward K^+ currents at voltages negative to the K^+ equilibrium potential (E_K). Their physiological roles are best understood in the heart, where their small outward conductances regulate the resting potential and contribute to the terminal phase of repolarization (*8*). At positive voltages they close, thus helping maintain the plateau of the action potential. In addition to the cardiac inward rectifier I_{K1}, the "delayed rectifier" I_{Kr}, which contributes substantially to the repolarization of the action potential, also exhibits profound inward rectification (*9*). Recent molecular characterization of inward rectifiers has focused on a family of channels sharing a hydropathy profile that predicts two transmembrane domains (*10*), in contrast to the six putative transmembrane domains in S4-containing channels of the Shaker (Sh) (*11*), Slowpoke (Slo) (*12*), and Eag (*6*) families.

We generated an expression construct for heterologous expression of *HERG* in frog oocytes from overlapping complementary DNAs (cDNAs) and a genomic fragment previously isolated from human DNA libraries (*13, 14*). A small inward current was sometimes seen after repolarization from a series of voltage steps (Fig. 1A). By comparison, oocytes expressing *M-EAG* have large outward currents under the same conditions (Fig. 1C). When oocytes injected with *HERG* cRNA were presented with hyperpolarizing voltage steps from 60 mV in elevated external K^+ concentration ($[K]_o$), large inward currents were observed (Fig. 1, D and E). HERG currents rapidly turned on and then declined with rates that were voltage-dependent. Currents evoked by stepping to −105 mV had a time-to-peak of 27 ± 4.6 ms (mean ± SD; $n = 7$). Current deactivation could be fitted with a biexponential function with time constants of 68.2 ± 6.6 ms and 292 ± 20.4 ms (mean ± SD; $n = 6$). The voltage range of activation of the inward HERG current shifted as $[K]_o$ was altered (Fig. 2A), as for inward rectifiers, with a Nernstian relation indicating K^+ selectivity (Fig. 2B).

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