role for tyrosine phosphorylation in mediating human fertilization. The homology of *hu9* with PTKs provides a basis for evaluating tyrosine kinase signaling paradigms in ZP3-triggered acrosomal exocytosis, mechanisms that may have implications in fertility control strategies.

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21. Human sperm were obtained from fresh ejaculates, collected by centrifugation through a Percoll gradient [F. L. H. Ng, D. Y. Liu, H. W. G. Baker, *Hum. Reprod.* **7**, 261 (1992)], and capacitated by incubation in Ham's F-12 medium, supplemented with 20 mM $NaHCO_3$ and 7.5% serum, at a concentration of 5×10^6 cells per milliliter for 6 hours at 37°C in 5% CO_2. Sperm proteins were solubilized in SDS sample buffer, separated under reducing conditions on 8% polyacrylamide gels [U. K. Laemmli, *Nature* **227**, 680 (1970)], and transferred [H. Towbin, T. H. Staehelin, J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4359 (1979)] to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). After they were blocked for nonspecific reactivity, the blots were incubated with antibody to phosphotyrosine (PY20; ICN, Irvine, CA) (1 μg/ml). PY20 reactivity was visualized with peroxidase-conjugated goat antibody to mouse IgG (Kierkegaard and Perry Laboratories, Rockville, MD) followed by enhanced chemiluminescence detection (ECL Western Blot Kit, Amersham).
22. Sperm were extracted in RIPA buffer (*20*) in the presence of protease inhibitors, 4 mM dithiothreitol, 500 μM Na_3VO_4, and 10% glycerol. Extracted proteins were separated from insoluble material by centrifugation and incubated overnight at 4°C with mAb 97.25. Immune complexes were recovered with antibody to mouse IgG and IgM coupled to Sepharose (Sigma), washed three times in RIPA, and separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE).
23. An unamplified λgt11 human testis library (Clontech, Palo Alto, CA) was screened first with PY20 as described [M. Snyder, S. Elledge, D. Sweetser, R. A. Young, R. W. Davis, *Methods Enzymol.* **154**, 107 (1987); R. A. Lindberg and E. B. Pasquale, *ibid.* **200**, 557 (1991); K. Letwin, S.-P. Yee, T. Pawson, *Oncogene* **3**, 621 (1988)]. Positive clones were detected with an ^{125}I-labeled second antibody and then rescreened with mAb 97.25 and detected similarly. Clones were sequenced by the dideoxy method. DNA and amino acid sequences were analyzed with GCG [J. Devereux, P. Haeberli, O. Smithies, *Nucleic Acids Res.* **12**, 387 (1984)] and deposited in GenBank (accession number L08961).
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26. Total RNA (10 μg) [G. Cathala *et al.*, *DNA* **2**, 329 (1983)] was probed with ^{32}P-labeled *hu9* cDNA [L. G. Davis, M. D. Dibner, J. F. Battey, *Basic Methods in Molecular Biology* (Elsevier, New York, 1986)]. Ethidium bromide was used to ascertain equal loading and transfer of the RNA samples. Final washing of the blots was in 2× saline sodium citrate (SSC) [T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982)] and 0.1% SDS at 55°C.
27. The ^{35}S-labeled riboprobes were transcribed from *hu9* plasmid DNA with T3 (sense strand) and T7 (antisense strand) polymerase, subsequently hydrolyzed to a final length of 200 base pairs, and hybridized with tissue sections overnight at 55°C. After treatment with ribonuclease A, slides were washed stringently, including a final wash in 0.1× SSC and 10 mM EDTA at 65°C.
28. Excess unfertilized human eggs in Ham's F-12 medium containing 7.5 to 10% human serum, which had not been exposed to sperm and would normally have been discarded, were frozen and maintained at ≤−70°C. At the time of use, vials were thawed rapidly in a 37°C bath, and eggs were removed directly into phosphate-buffered saline containing 2 mM EGTA and 5% serum at 4°C. Oocytes were completely nonviable after this procedure. ZPs were bisected with a micro-scalpel, and matched ZP halves were used subsequently. After bisection, ZPs were washed (three times) and each half-ZP was placed pair-wise in matched drops (40 μl) of Ham's F-12 medium containing 7.5% serum. For each ZP used, one of the halves served as an internal control to which results from the other half were compared.
29. Sperm proteins were incubated for 2 hours at 4°C with anti-Hu9(539–553) (K16). Immune complexes were recovered with antibody to rabbit IgG coupled to Sepharose (Sigma) and washed three times in RIPA.
30. Antiserum to rat brain hexokinase was provided by J. E. Wilson, Michigan State University. The peptides used in this study were synthesized at the Salk Institute (under contract NO1-HD-0-2906 with NIH) and made available by the Contraceptive Development Branch, Center for Population Research, National Institute of Child Health and Human Development. J. Dean (National Institute of Diabetes and Digestive and Kidney Diseases) provided the human ZP3 cDNA. Human ZPs were donated by M. Gerrity and J. Rinehart, Reproductive Medical Associates, Glenview, IL. We appreciate the help of K. Burridge, University of North Carolina at Chapel Hill, and R. Alston and S. Dörre, Duke University, as well as all of the Duke University laboratory members during the writing of this manuscript. Supported by NIH (grants HD 29125 and HD 18201). D.J.B. is grateful for partial support, and R.C. for full support, from A. W. Mellon Foundation fellowships. H.D.M.M. was supported by the Wellcome Trust and the Medical Research Council, United Kingdom.

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Tissue- and Species-Specific Expression of sp56, a Mouse Sperm Fertilization Protein

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Mouse sperm recognize and bind to ZP3, one of three glycoproteins in the egg's zona pellucida. A mouse sperm protein, sp56, was identified that has the characteristics expected of the sperm protein responsible for recognition of ZP3. The complementary DNA encoding sp56 was isolated, and its primary sequence indicates that sp56 is a member of a superfamily of protein receptors. It was shown that sp56 expression is restricted to mouse spermatids and that the presence or absence of sp56 on sperm from different species accounts for species specificity of sperm-egg recognition in mice.

The mouse sperm protein sp56 was identified on the basis of its specific, high affinity for ZP3, the zona pellucida (ZP) protein recognized by sperm (*1*, *2*). sp56 is a homomultimeric peripheral membrane protein confined to the plasma membrane overlying the sperm's acrosome (*3*), and it has a specific affinity for ZP3's functional domain oligosaccharide (FD-oligo), the part of ZP3 recognized by sperm (*3*). Purified sp56, which binds to the ZP of unfertilized mouse eggs but not to fertilized embryos, blocks sperm-egg recognition (Fig. 1). Although a number of proteins have been proposed to function as the mouse sperm's ZP3 recognition protein (*2*–*4*), only sp56 has all of the characteristics required of that protein.

The complementary DNA (cDNA) encoding sp56 was isolated (Fig. 2). Nested polymerase chain reaction (PCR) of reverse-transcribed mouse testes polyadenylated [poly(A)$^+$] RNA was performed with degenerate primers derived from the sp56 NH_2-terminal amino acid sequence and with a primer derived from a fragment of the protein. Sequencing of a 1-kb PCR product confirmed that this polynucleotide encoded

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sp56, because it encoded all six, independently identified stretches of sp56 amino acid sequence (indicated in Fig. 2). The 1-kb PCR product was then used to screen a mouse testes cDNA library. The full-length cDNA clone that was isolated included the entire 1-kb PCR product along with upstream bases encoding the sp56 NH_2-terminal amino acids 1 through 10, confirmation that the clone encodes sp56 (Fig. 2).

The amino acid sequence encoded by this cDNA predicts the biochemical characteristics of the protein. The sp56 cDNA encodes a 547–amino acid open reading frame (Fig. 2). A presumptive 32–amino acid signal peptide is located between the first in-frame methionine and the experimentally determined NH_2-terminus of sp56, as one would predict from the extracellular location of sp56 (*3*). The protein sequence contains no obvious transmembrane-spanning domain, which is consistent with our finding that sp56 is a peripheral membrane protein (*3*). The polypeptide molecular mass of sp56 calculated from the cDNA sequence is 62 kD. The discrepancy between the molecular mass of sperm sp56 (56 kD) and a calculated molecular mass of 62 kD appears to be due to posttranslational truncation of sp56 polypeptide in spermatogenic cells. The sp56 amino acid sequence derived from the cDNA does not indicate the presence of regions having significant homology to carbohydrate recognition domains (CRDs) of previously characterized lectins. The presence of CRDs within the sp56 polypeptide sequence is expected, because sp56 is a lectin that has an affinity for α-galactose and a specific affinity for ZP3's FD-oligo (*2*, *3*).

Comparison of the sp56 amino acid sequence to the GenBank (version 82) and Protein Information Resources (version 40) databases indicates that sp56 is a member of a superfamily of protein receptors that contain multiple consensus repeats of approximately 60 amino acids in length, termed Sushi domains (*5*). The sp56 open reading frame contains six contiguous Sushi domains, followed by 44 amino acids specific to sp56, a seventh Sushi domain, and a highly basic COOH-terminal domain of 70 amino acids. The presence of seven Sushi repeats in sp56, each of which contains two disulfides, is consistent with protein electrophoretic studies that showed that sp56 contains numerous intramolecular disulfides (*3*). sp56 is most closely related to one member of the superfamily, the alpha subunit of complement 4B–binding protein (C4BPα) (*6*).

Nucleic acid and immunological probes were used to determine which tissues express sp56. A 2-kb sp56 mRNA was detected by Northern (RNA) blot analysis in testes, but not in other tissues (Fig. 3A). Protein immunoblot analysis with monoclonal antibody 7C5 to sp56 (*3*) revealed that sp56 polypeptide was present in testes and epididymus, but not in other mouse tissues (Fig. 3B). Note that 7C5 does not recognize reduced, denatured sp56 on protein immunoblots, but it does recognize the nonreduced 40-kD protein (*3*). 7C5, as expected, recognized nonreduced sp56 (40 kD) in epididymal proteins (*3*); however, it also recognized a series of nonreduced protein bands ranging in size from 40 to 44 kD in testes. Gel electrophoretic and NH_2-terminal amino acid sequencing studies demonstrated that this higher molecular mass testicular protein was as large as 62 kD under reducing conditions (the size predicted by translation of sp56 cDNA) and confirmed the identity of this protein as sp56 (*7*). It is likely that the decreasing size of sp56 (from 62 to 56 kD under reducing conditions) observed in testis represents posttranslation truncation of the protein.

In situ hybridization and immunohisto-

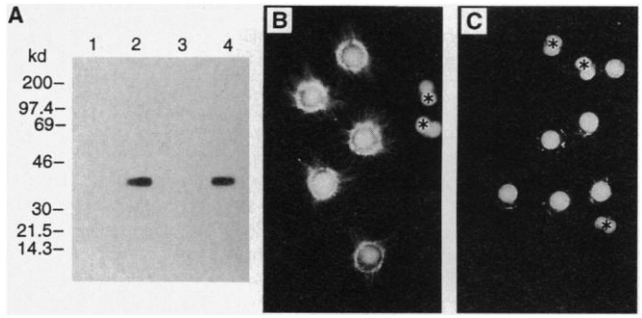

Fig. 1. Purified sp56 binds to mouse egg ZP and blocks sperm-egg binding. Unfertilized mouse eggs and two-cell embryos [internal controls, indicated by (*) in (B) and (C)] were isolated, fixed, and prepared for competition assay as previously described (*1*). Biochemically purified sp56 (*3*) was dialyzed extensively to remove detergent and incubated [at a concentration (6 nM) at which soluble ZP3 blocks 50% of sperm-egg binding (*1*)] for 8 hours with eggs and embryos in medium at 25°C. The eggs and embryos were then washed by serial transfer to remove unbound sp56. Protein immunoblot analysis (**A**) of SDS-extracted eggs or embryos revealed that purified sp56, like mouse sperm, bound to unfertilized eggs but not to fertilized embryos. Lane 1, 90 eggs; lane 2, 90 eggs exposed to purified sp56; lane 3, 90 embryos exposed to purified sp56; and lane 4, purified sp56 standard (10 ng). Whereas capacitated mouse sperm bound to the ZP of fixed, unfertilized mouse eggs (**B**) (76 ± 4 sperm bound per egg, where $n = 5$ eggs), sperm were blocked from binding to the ZP of eggs that had been preincubated with purified sp56 (**C**) (7 ± 5 sperm bound per egg, where $n = 6$ eggs). Cell culture and competition assays, with fixed eggs and embryos, were performed in the presence of quinuclidonol benzoate to inhibit ZP-induced acrosome reactions, as previously described (*1*). Protein immunoblots were developed with monoclonal antibody 7C5 to sp56 as previously described (*3*).

Fig. 2. Sequence of the sp56 cDNA clone. Biochemically purified sp56, which is identical to sp56 purified by ZP3 affinity chromatography (*3*), was subjected to amino acid sequencing. The NH_2-terminus was determined as previously described (*3*). The internal amino acid sequence was obtained by sequencing proteolytic fragments of the protein. Endo Arg C, tryptic, and chymotryptic fragments of sp56 were subjected to solid-phase automated Edman-degradation peptide sequencing. Quantitation of the starting material and the amino acids released confirmed that only sp56 sequences were obtained. Degenerate PCR primers (*10*) designed from peptide sequences were used to amplify a fragment of sp56 cDNA. Nested PCR was performed on a single-stranded cDNA template made from mouse testes poly(A)$^+$ RNA by using oligo(dT)-primed paramagnetic particles (*10*), with degenerate primers N4 and F5W, followed by another round of PCR of 1/100 of the original amplification product with primers N5 and F5W. The 1-kb amplicon that was generated contained a sequence encoding part of the sp56 NH_2-terminus (amino acids 11 to 18, encoded by PCR primer N5), Endo Arg C peptide 1 (amino acids 271 to 294), Endo Arg C peptide 2 (amino acids 291 to 303), tryptic peptide 1 (amino acids 199 to 205), chymotryptic peptide 1 (amino acids 12 to 18), and chymotryptic peptide 2 (amino acids 342 to 349, encoded by PCR primer F5W). The full-length sp56 cDNA clone was obtained by screening an oligo(dT)-primed mouse testes Lambda-Zap cDNA library with ^{32}P-labeled, random-primed 1-kb PCR product and ^{32}P-labeled primers specific to the 1-kb PCR product. The seven underlined sequences are Sushi domains, which begin and end with C. The GenBank accession number for the entire cDNA sequence is U17108. Abbreviations for the amino acid residues are as follows: 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.

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-32 MITWSFIDLWRTSHSTLFQMTLATVLMAPVLGDCGPPPLLPFASPTNQLYESTTFPSGTVLK
 31 YTCHHGFKRVNSSHLSCDENGSWVYSTFCARKRCKNPGELVNGKVEIPSDLLVGSIIEFSCS
 93 KGYLLIGSATSRCEVQGKGVDWSDSLPECVIATCEPPPPISNGKHSGRDDDLYTFGSVVIYN
155 CDPTFTLLGNASIVCTVVNRTVGVWRPHPPACQKIVCHRPQIPKGYLAPGFRQFYAYRDALE
217 IRCKKGFILRGSSVIHCEANGEWFPSIPTCEPNGCTNIPDISYASWEGYKFPLRNFEVFEIG
279 AKLKYQCKPGYRASLNDPQTVTCQENLTWSSTNGCERICCPTPDMEKIKIVSERRDFTGTCI
341 YAYGDYVFYICNEGSYPMSTDGRSSCQADGKWDPAIPSCQADSGLQNRLALFTFPNISETNV
403 TNKTYLFGHEENSTEHAMKGVCLKPMVINGNLSVERVIYAELENITIQCDPGYTIVGSPNII
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525 WIQLHRKPQSMKINRSFRLCN
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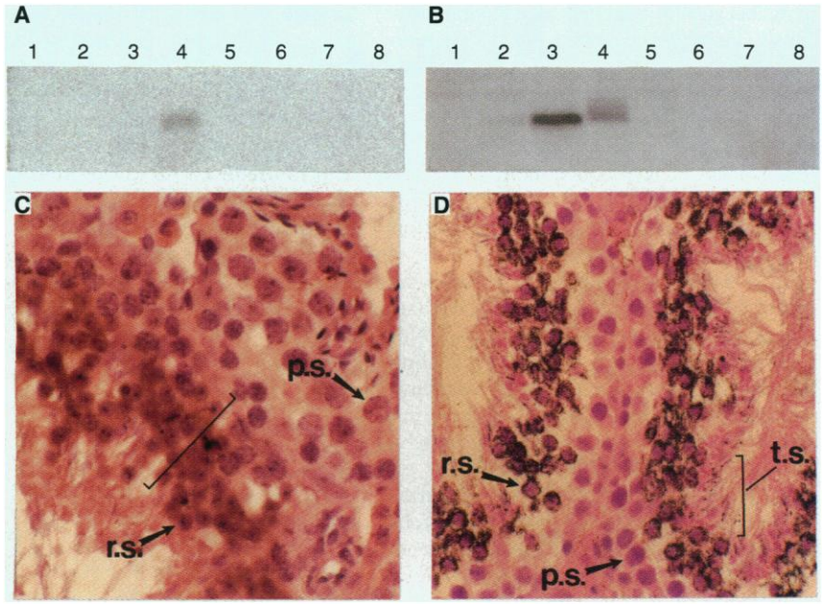

Fig. 3. Detection of sp56 and its mRNA in mouse tissues and spermatogenic cells. (**A**) Northern blot analysis of mouse tissues. Mouse poly(A)$^+$ RNA (1 μg from heart, liver, total epididymus, testes, abdominal muscle, lung, brain, and kidney, lanes 1 to 8, respectively) was electrophoresed, transferred and cross-linked to nylon transfer membrane, and probed with a ^{32}P hybridization probe prepared from a 104-base pair (bp) Sph I–Stu I fragment from the sp56 clone, corresponding to amino acids 385 through 418 (*10*). The blot was prehybridized, hybridized, and developed according to the specifications of the membrane manufacturer. A ~2.1-kb band was detected only from mouse testes. Mouse ovaries contained no hybridizable band (*7*). Reprobing with a 780-bp ^{32}P-labeled polynucleotide encoding human glyceraldehyde-3-phosphate dehydrogenase (American Type Culture Collection 57091) confirmed the presence of RNA in each lane. (**B**) Protein immunoblot analysis of mouse tissues. Mouse tissues [same lanes as in (A)] were homogenized, and protein was extracted with SDS and subjected to protein immunoblotting with monoclonal antibody 7C5, as previously described (*3*). sp56 was detected as a nonreduced "doublet" of 40 kD in blotted epipidymal proteins. A series of nonreduced protein bands, ranging from 40 to 44 kD, were detected from blotted testes proteins. (**C**) Detection of sp56 mRNA by in situ hybridization. Testes were fixed overnight in Z-fix (Anatech, Battle Creek, Michigan), processed, and embedded with paraffin. Dried sections (5 μm) were prepared, blocked, hybridized with either digoxigenin-labeled sp56 sense or antisense RNA probes [full-length probes were fragmented to ~300 bp as recommended by the manufacturer (Boehringer Mannheim)], and detected as previously described (*11*). Slides were counterstained with eosin and haematoxylin. Antisense probe staining is apparent in round spermatids (r.s.). Serial sections hybridized with sense RNA probes demonstrated no binding (*7*). (**D**) Detection of sp56 polypeptide by immunohistochemistry. Testes sections (3 μm), from the same tissue blocks used in (C), were blocked and probed with monoclonal antibody 7C5, and the bound antibody was detected by silver-enhanced colloidal gold staining as described (*3*). Sections were counterstained with hematoxylin and eosin. sp56 was localized in round spermatids (r.s.) and testicular spermatozoa (t.s.). Pachytene spermatocytes (p.s.) did not contain detectable sp56. Serial sections probed with normal mouse immunoglobulin demonstrated no binding (*7*).

Table 1. Correlation between species specificity of sperm-egg binding and species specificity of sp56.

Sperm source	Binding of sperm to the mouse egg*	Biochemical identification of sp56†	Immunological identification of sp56‡	Identification of sp56-like transcripts§
Mouse	Yes	2.3 pg per sperm	40 kD (−DTT) 56 kD (+DTT)	2-kb band
Hamster	Yes	>2.3 pg per sperm	40 kD (−DTT) 56 kD (+DTT)	2-kb band
Guinea pig	No	Not detected	Not detected	Not detected
Human	No	Not detected	Not detected	Not detected

*The binding assay was performed as previously described (*1*). These results confirmed previous studies (*9*). †Two-dimensional SDS–polyacrylamide gel electrophoresis (SDS-PAGE) of proteins from 2 × 10^7 sperm, performed as previously described (*3*). ‡Biochemically purified sp56, prepared as described (*3*), was used to raise antiserum against the protein. Protein immunoblotting and immunoadsorption of proteins from 10^7 sperm were performed as described (*3*). –DTT and +DTT refer to SDS-PAGE performed in the absence or presence of dithiothreitol, respectively. §Northern blot analysis of RNA from mouse, hamster, guinea pig, and human testes. Methods are described in Fig. 3. Blots were probed with ^{32}P-labeled, random-primed DNA developed from full-length sp56 cDNA. Fresh hamster and guinea pig testes were provided by the Scripps Research Institute Department of Animal Resources. A human testis section, frozen in liquid nitrogen, was provided by the Scripps Clinic Department of Urology, as an orchiectomy product. Reprobing with the glyceraldehyde-3-phosphate dehydrogenase probe confirmed presence of RNA in each lane.

chemistry of serial testes sections were used to identify cells containing sp56 mRNA and polypeptide, respectively. The sp56 mRNA was detected with an sp56-specific antisense RNA probe in the nuclei and cytoplasm of round spermatids, and in no other cells (Fig. 3C). The detection of sp56 mRNA in round spermatids may indicate that transcription activation of the sp56 gene occurs in a haploid cell. sp56 polypeptide was detected in round and elongating spermatids and in testicular spermatozoa, but it was not detected in earlier stages of spermatogenesis (Fig. 3D). A number of previously identified proteins are expressed during late stages of spermatogenesis (*8*). Of these proteins, only sp56 and protamines are restricted to sperm.

Sperm-egg recognition and binding in mammals is largely species-specific (*9*), suggesting that molecular recognition between the sperm head plasma membrane and the ZP surface involves different molecules in different species. Protein and nucleic acid probes for sp56 were used to determine whether the presence or absence of sp56 correlated with an ability of sperm from other species to recognize and bind to the mouse egg. Mouse and hamster sperm, which bind to the ZP of mouse eggs, contained sp56. However, sp56 was not detected in guinea pig sperm or human sperm, which do not bind to mouse egg ZP (Table 1). These results were confirmed by Northern blot analysis of testes, with an sp56 cDNA probe. Because no antigen cross-reactive with the polyclonal antiserum to sp56 was identified in guinea pig or human sperm (confirmed at the nucleic acid level by Northern blotting of testicular RNA), we conclude that these sperm either contain no sp56 or a protein distantly related to sp56. Therefore, if sp56 mediates sperm-egg recognition in mouse, its presence or absence accounts for the species specificity of that event.

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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-

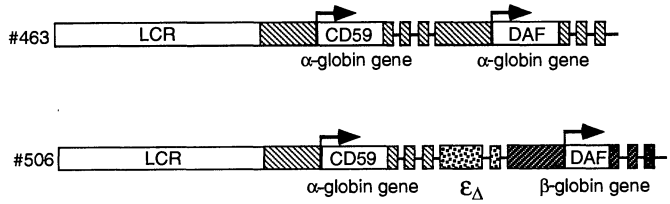

Fig. 1. Constructs used to make transgenic mice. In construct #463, the cDNAs for CD59 and DAF were inserted into the first exon of the human α-globin gene, and the ATG codon used for the synthesis of α-globin protein was simultaneously removed (*11*). For construct #506, the DAF cDNA was inserted into 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*).