Identification of a Stimulator of Steroid Hormone Synthesis Isolated from Testis

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Gonadal steroidogenesis is regulated by pituitary gonadotropins and a locally produced, unidentified factor. A 70-kilodalton (kD) protein complex secreted from rat Sertoli cells was isolated. The complex, composed of 28- and 38-kD proteins, stimulated steroidogenesis by Leydig cells and ovarian granulosa cells in a dose-dependent and adenosine 3′,5′-monophosphate–independent manner. The follicle-stimulating hormone–induced 28-kD protein appeared to be responsible for the bioactivity, but the 38-kD protein was indispensable for maximal activity. The 28- and 38-kD proteins were shown to be identical to the tissue inhibitor of metalloproteinase-1 (TIMP-1) and the proenzyme form of cathepsin L, respectively. Thus, a TIMP-1–procathepsin L complex is a potent activator of steroidogenesis and may regulate steroid concentrations and, thus, germ cell development in both males and females.

Sex steroids are required for development of the sexual morphotype and germ cell differentiation. The pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are the main regulators of gonadal steroid synthesis. However, locally produced factors also control gonadal function (*1*). The mammalian testis is divided into two compartments: the avascular seminiferous tubules, consisting of Sertoli and germ cells, and the vascularized interstitial area, which contains the Leydig cells (*2*). Testicular interstitial fluid (TIF) transfers nutrients and hormones from the bloodstream to the seminiferous tubules and between the seminiferous tubules and Leydig cells (*2*). Leydig cells, under the control of LH, produce androgens. Sertoli cells, under the control of serum FSH and locally produced testosterone, provide the necessary structural and functional environment for the initiation and maintenance of spermatogenesis, and hence for fertility (*2*). Because testosterone is critical for normal Sertoli cell function and spermatogenesis, a regulatory role for Sertoli cells in Leydig cell steroid synthesis has been suggested (*3*). Indeed, in all species examined (*3*), including humans (*4*), the seminiferous tubules, particularly the Sertoli cells, produce an FSH-responsive factor that regulates Leydig cell steroidogenesis. However, the nature and identity of this factor have remained unknown.

We undertook the isolation and characterization of this steroidogenesis-stimulating protein (STP) from medium conditioned by rat Sertoli cells cultured in the presence of FSH. Proteins from the medium were concentrated with hollow fiber cartridges and applied to a concanavalin A (Con A)–Sepharose column, which retained most of the bioactivity. Subsequent purification of STP was accomplished by gel-filtration chromatography (Fig. 1). We tested the bioactivity of collected fractions by measuring their effect on progesterone production by mouse MA-10 Leydig cells. Fractions 55 to 61, which correspond to a molecular size of 70 to 80 kD, stimulated steroidogenesis more than 200-fold (Fig. 1A). In the same experiment, saturating amounts of human chorionic gonadotropin (hCG) (50 ng/ml) stimulated progesterone production 150-fold. Two proteins with molecular sizes of 28 and 38 kD were detected when these fractions were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) and silver staining (Fig. 1B). Addition of the reducing agent β-mercaptoethanol did not affect the results obtained (*5*). The bioactivity closely correlated with the presence of the 28-kD protein. Fractions 62 to 65, which contained large amounts of the 38-kD protein, were inactive. In some experiments, proteins of 24 and 36 kD were also present in the bioac-

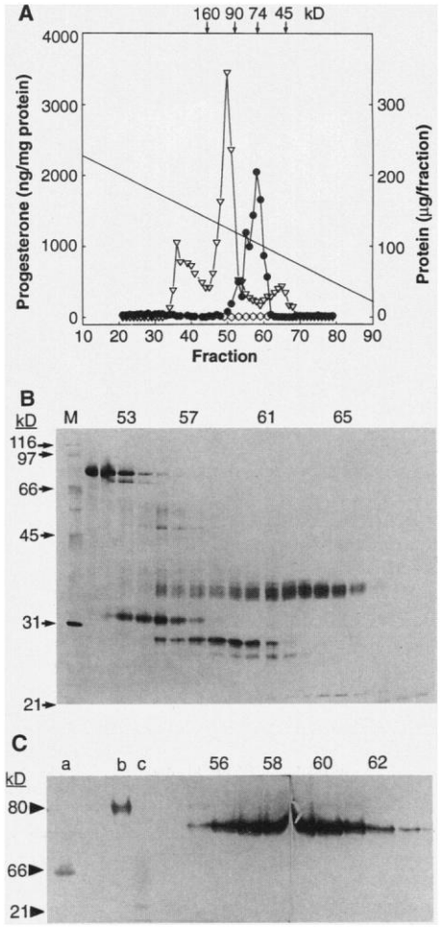

Fig. 1. Isolation of STP. (**A**) Elution profile of protein (▽) and bioactivity (●, ◇) from the gel-filtration column. Sertoli cells were isolated from 10- to 15-day-old Sprague-Dawley rats and cultured (*19*) in Ham's F12–Dulbecco's modified Eagle's medium (50:50, v/v) supplemented with insulin, transferrin, and sodium selenite, with or without FSH (100 ng/ml) (USDA-oFSH-19-SIAFP). The medium was changed on day 2, collected at 1- or 2-day intervals up to day 8, centrifuged, and stored at −20°C. Thawed medium was concentrated (10-fold) in a hollow fiber membrane cartridge (cutoff, 10 kD) and applied in buffer A [50 mM tris-HCl (pH 7.4), 50 mM NaCl] to a Con A–Sepharose 4B (Pharmacia) column (1.5 by 8 cm) that had been equilibrated with the same buffer. Bound proteins were eluted with the same buffer containing 0.5 M α-methyl-D-glucoside, and were concentrated with Centriprep-10 concentrators (Amicon; cutoff, 10 kD). The concentrated glycoproteins were applied to a Sephadex G100 Superfine (Pharmacia) column (1.5 by 115 cm) that had been equilibrated with buffer A. Proteins were eluted with buffer A and 0.5-ml fractions were collected (flow rate, 6 ml/hour). The protein content of the fractions was determined and bioactivity was assessed by measuring the effect on progesterone synthesis by MA-10 cells; the amount of progesterone released into the culture medium was determined by radioimmunoassay (*4*). (●) Medium from Sertoli cells cultured in the presence of FSH; (◇) medium from Sertoli cells cultured in the absence of FSH, or control (unconditioned) medium supplemented with FSH. The elution of molecular size standards (in kilodaltons) is indicated (arrows) and represented by the diagonal line. (**B**) SDS-PAGE in the absence of β-mercaptoethanol and silver staining (*4*) of fractions of the gel-filtration column shown in (A). Fraction numbers are shown above the lanes. M, molecular size standards. (**C**) Silver-stained gel after native gel electrophoresis of the bioactive fractions (*20*). Standards: a, bovine serum albumin; b, human transferrin; c, soybean trypsin inhibitor.

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tive preparations and appear to be less glycosylated forms of the 28- and 38-kD proteins, respectively. Medium alone, supplemented with FSH and chromatographed on the same columns, was without effect on Leydig cell steroidogenesis (Fig. 1A). Thus, the active factor was not LH, which may be present in the FSH preparations used. Further experimentation with antiserum to LH as well as studies with β-mercaptoethanol, which dissociates LH into its component subunits, also indicated that the active factor was not LH (5). Native gel electrophoresis of the bioactive fractions showed that the molecular size of STP was 70 kD (Fig. 1C). Medium from Sertoli cells cultured in the absence of FSH was also collected; proteins purified under identical conditions had no effect on steroidogenesis in MA-10 cells (Fig. 1A). Analysis of these fractions by SDS-PAGE demonstrated the presence of the 38-kD protein but not the 28-kD protein (5). These results further suggested that the FSH-induced 28-kD protein is responsible for the bioactivity.

Dose-response studies with crude, Con A–purified, and Sephadex G100–resolved bioactive preparations showed that bioactivity per microgram of total protein increased 1000-fold during the purification procedure (Fig. 2A). The bioactivity of purified STP was labile; whereas freshly isolated STP stimulated progesterone synthesis more than 200-fold, half of this activity was lost after storage for 3 days at 4°C. Freezing of the protein at −20°C resulted in a further decrease in bioactivity.

STP also induced a 12-fold increase in pregnenolone synthesis by Leydig cells from immature rats (Fig. 2B). The extent of stimulation of steroid synthesis obtained with STP was identical to that obtained with saturating amounts of hCG. Unlike hCG, STP did not affect the intracellular concentration of adenosine 3′,5′-monophosphate (cAMP) (Fig. 2C). These results suggest that STP acts through a cAMP-independent mechanism. STP (100 ng/ml) also induced a 10-fold increase in progesterone production by isolated rat granulosa cells (6) (STP, 42.08 ± 6.3; basal, 4.3 ± 0.8 ng per milligram of protein) and stimulated 20α-hydroxyprogesterone synthesis in Y-1 mouse adrenocortical cells (5), thus indicating that its effects are not confined to Leydig cells. The maximal effect of STP on progesterone production by Leydig cells was not additive with the effect of saturating

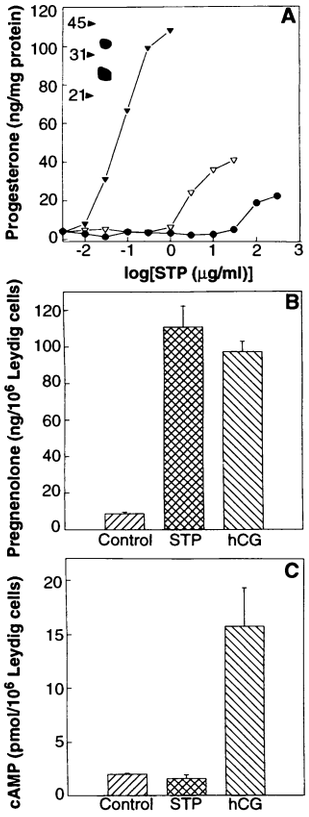

Fig. 2. Effect of STP on steroidogenesis. (**A**) Dose-response relations for the effects of concentrated Sertoli cell medium (●), Con A–purified proteins (▽), and STP isolated from the gel-filtration column (▼) on MA-10 Leydig cell steroidogenesis. Cells were exposed to the various protein fractions for 2 hours. Results are means of triplicates from a single experiment; qualitatively similar data were obtained in three independent experiments. (**Inset**) SDS-PAGE of Sephadex G100–purified STP preparation. The positions of molecular size standards are shown in kilodaltons. (**B**) Effect of purified STP (100 ng/ml) and hCG (50 ng/ml) on steroidogenesis in Leydig cells isolated from immature, 20-day-old rats (*21*). Because Leydig cells from immature rats synthesize several steroids in addition to testosterone, we measured the first steroid product formed, pregnenolone, as an index of steroid formation. Pregnenolone metabolism was blocked by trilostane and SU-10603, and pregnenolone concentrations were determined by radioimmunoassay. Data are means ± SD (n = 6). (**C**) Effect of STP on intracellular cAMP concentration. Leydig cells from immature rats were incubated in the absence or presence of hCG (50 ng/ml) or STP (100 ng/ml), after which cAMP was extracted and measured (*19*). Results are means ± SD (n = 6).

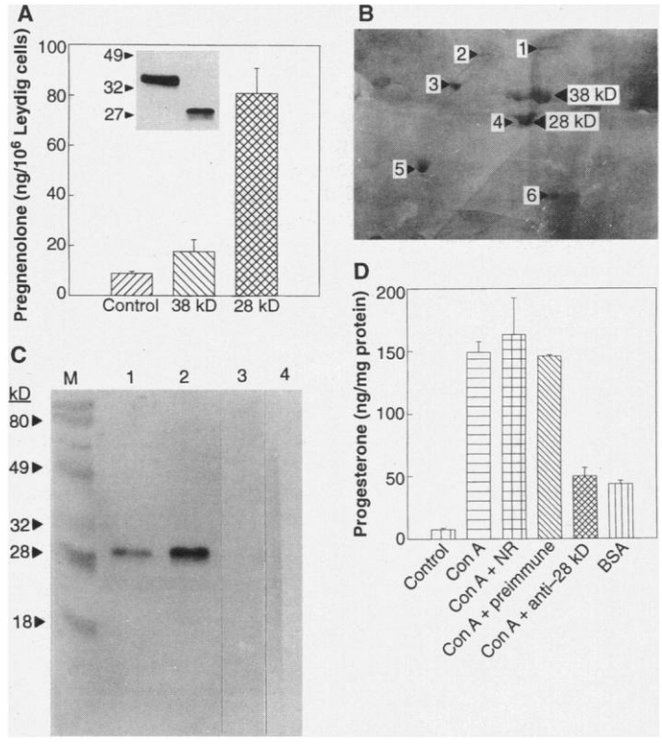

Fig. 3. Steroidogenesis in response to the 28-kD protein. (**A**) Effects of the 28- and 38-kD proteins on steroidogenesis in Leydig cells. Both proteins were isolated by electroelution. The purity of the electroeluted proteins was assessed by SDS-PAGE (**inset**) and their bioactivity was examined with Leydig cells from immature rats as described (Fig. 2B). Results are means ± SD (n = 6). (**B**) Two-dimensional PAGE of STP (*22*). The 28- and 38-kD proteins are shown, as are standard proteins applied with STP: 1, conalbumin [76 kD; isoelectric point (pI), 6.0 to 6.6]; 2, albumin (66.3 kD; pI, 4.98 to 5.18); 3, actin (43 kD; pI, 5.45 to 6.53); 4, carbonic anhydrase (31 kD; pI, 5.9 to 6.0); 5, tryspin inhibitor (21.5 kD; pI, 4.5); 6, myoglobin (17.5 kD; pI, 6.6 to 7.0). (**C**) Immunoblot analysis of a crude, 10-fold concentrated preparation of proteins secreted by rat Sertoli cells (lane 1) and Con A–purified proteins (lanes 2 to 4) with an antiserum to the 28-kD protein (lanes 1 and 2), preimmune serum (lane 3), or normal rabbit serum (lane 4). Samples were subjected to SDS-PAGE and transferred to Immobilon-P membranes, which were then incubated sequentially with the antiserum, alkaline phosphatase–conjugated secondary antibodies, and appropriate substrate. The antiserum to the 28-kD protein was generated in rabbits (*23*). Lane M, molecular size standards. (**D**) Immunoneutralization studies performed as described (*4*) with Con A–purified Sertoli cell proteins and anti–28-kD protein (anti–28 kD), preimmune, and nonrelated (NR) antisera. Supernatants from the immunoprecipitations were tested for bioactivity with MA-10 cells. BSA, 250 μg of bovine serum albumin were used as a substitute for the Con A–purified proteins; the increased extent of progesterone synthesis relative to control may be attributable to the effect of albumin on steroidogenesis (*4, 24*). Data are means ± SD (n = 4).

amounts of hCG (STP, 216 ± 15; STP plus hCG, 236 ± 19 ng of progesterone per milligram of cell protein).

Separation of the 28- and 38-kD proteins resulted in a decrease in bioactivity, suggesting that the 38-kD protein is important in stabilizing or facilitating the effect of the 28-kD protein. The isolated 28-kD protein induced a 10-fold increase in pregnenolone synthesis by Leydig cells, whereas the isolated 38-kD protein had no effect (Fig. 3A); in the same experiment, hCG induced a 15-fold increase in steroid production. These data on the potencies of the isolated proteins should be interpreted with caution, however, given that the proteins were isolated by SDS-PAGE and electroelution, procedures that may affect their activity.

Two-dimensional PAGE analysis of STP indicated that both the 28- and 38-kD proteins are heterogeneous, with isoelectric points of 5.6 to 6.0 and 5.4 to 6.4, respectively, suggesting that they may be glycosylated (Fig. 3B). Deglycosylation studies demonstrated that carbohydrate moieties constituted at least 20% of the mass of the proteins, with sialic acid being the primary component (5). The isolated 28-kD protein was used to generate a rabbit antiserum. The antiserum specifically recognized the 28-kD protein (Fig. 3C) and neutralized the bioactivity of the Con A–purified fraction (Fig. 3D).

The NH_2-terminal sequences of the 28- and 38-kD proteins were determined. Because, in some experiments, we also observed 24- and 36-kD proteins, all four proteins were sequenced from three different preparations (Fig. 4A). The sequence obtained for the 28-kD protein was identical to that of a 25–amino acid NH_2-terminal sequence of the rat tissue inhibitor of metalloproteinase-1 (TIMP-1) (7). The sequence obtained with the 38-kD protein was identical to that of the NH_2-terminal portion of the proenzyme form of cathepsin L (8), which is also identical to the Sertoli cell cyclic protein–2 (CP-2) (9). The 24- and 36-kD proteins had identical sequences to those of the 28- and 38-kD proteins, respectively.

Both the antiserum to the 28-kD protein and an antiserum to TIMP-1 recognized the 28-kD protein in STP, human fibroblast TIMP-1, and a 28-kD protein in TIF (Fig. 4B). Furthermore, the antiserum to TIMP-1 also neutralized the bioactivity of the Con A–purified fraction (5). Partial tryptic digestion of both the 28-kD protein and TIMP-1 generated similar peptide patterns (5). We prepared RNA from FSH-treated rat Sertoli cells and isolated a complementary DNA (cDNA) fragment by reverse transcription and the polymerase chain reaction (RT-PCR) (10) with primers based on the mouse TIMP-1 nucleotide sequence (11). This fragment was partially sequenced and showed 92 and 76% sequence identity with the cDNAs encoding mouse and human TIMP-1, respectively (12). The identity of the 38-kD protein was also confirmed by immunoblot analysis with an antiserum to cathepsin L (Fig. 4C).

Treatment of rat Sertoli cells with FSH induced the expression of the 28-kD protein (TIMP-1) mRNA but had no effect on the abundance of the mRNA encoding the 38-kD protein (procathepsin L) (Fig. 5A). To confirm that the 28-kD protein is responsible for STP activity, we transfected a full-length human fibroblast TIMP-1 cDNA into cultured rat Sertoli cells. Conditioned medium from Sertoli cells transiently transfected with the TIMP-1 cDNA contained the 28-kD protein and STP bioactivity, whereas medium from cells transfected with the vector alone was inactive and contained very low amounts of the 28-kD protein that were induced by the serum used for the transfection (Fig. 5B).

Rat TIMP-1 was initially isolated from medium conditioned by the clonal rat osteosarcoma cell line UMR-106 (7). Conditioned medium from UMR-106 cells was therefore used to isolate STP bioactivity. Fractions with STP bioactivity also contained immunoreactive 28-kD (TIMP-1) and 38-kD (procathepsin L) proteins (5).

TIMP-1, a well-characterized member of the TIMP family, is present in many tissues (13) and binds to members of the matrix metalloproteinase family of proteins (the interstitial collagenases). Metalloproteinases are secreted as zymogens and their activity is controlled, at least in part, by the co-secretion of TIMPs. The interaction of metalloproteinases with TIMPs affects various biological processes, including angiogenesis, embryo implantation, cell migration, tumor cell metastasis, and ovulation. In the ovary, TIMP-1 is synthesized by both luteal and granulosa cells and its synthesis and secretion are regulated by gonado-

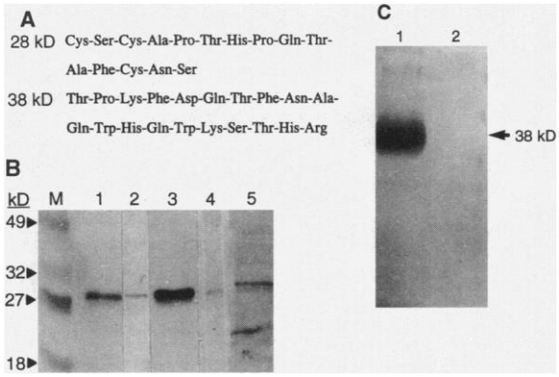

Fig. 4. Identification of the 28- and 38-kD proteins. (**A**) Amino acid sequence analysis of the 28- and 38-kD proteins. Isolated STP was subjected to electrophoresis and transferred to an Immobilon-P (Millipore) membrane (25). The NH_2-terminal sequence of the purified proteins was determined by Edman degradation with an Applied Biosystems model 470A protein sequencer and an on-line phenylthiohydantoin analyzer. (**B**) Immunoreactivity of the 28-kD protein in STP (lanes 1 and 2), human fibroblast TIMP-1 (lanes 3 and 4), and TIF (lane 5) with antiserum to TIMP-1 (lanes 1, 3, and 5) or to the 28-kD protein (lanes 2 and 4). Lane M, molecular size standards. (**C**) Immunoblot analysis of STP with an antiserum to cathepsin L (lane 1) and normal rabbit serum (lane 2).

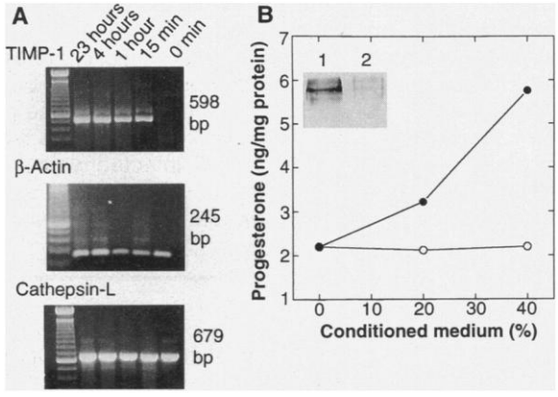

Fig. 5. (**A**) Regulation of the abundance of mRNAs encoding the 28- and 38-kD proteins by FSH. Sertoli cells from immature rats were cultured for 4 days in Ham's F12–Dulbecco's modified Eagle's medium and then incubated for the indicated times with FSH (100 ng/ml). RNA was isolated from the cells and RT-PCR was performed with primers specific for the 28-kD protein (TIMP-1) (11, 12), the 38-kD protein (procathepsin L) (9), and β-actin (Stratagene). The sizes (base pairs) of the amplified products are shown on the right. (**B**) Biological activity of recombinant human TIMP-1 expressed in cultured rat Sertoli cells. Sertoli cells from immature rats were cultured in Ham's F12–Dulbecco's modified Eagle's medium for 4 days and then transfected with the pCMV-5 vector containing full-length human TIMP-1 cDNA (●) or the vector alone (○) with the use of lipofectamine (Gibco BRL) (10). Forty-eight hours after transfection, medium was collected and concentrated 10-fold, and various concentrations were examined for bioactivity with MA-10 cells. (**Inset**) Immunoblot analysis with the antiserum to the 28-kD protein of the concentrated conditioned medium from cells transfected with TIMP-1 cDNA (lane 1) or the vector alone (lane 2).

tropins (*14*). Although TIMP-1 binds to interstitial collagenase with high (subnanomolar) affinity (*15*), the metalloproteinase-inhibiting activity is apparent only at TIMP-1 concentrations of 1 to 3 μg/ml (*7*). STP activity of the 28-kD (TIMP-1)–38-kD (procathepsin L) complex is apparent at concentrations of 30 to 100 ng/ml. TIMP-1 was originally described as an erythroid potentiating factor (*16*) and exhibits growth factor activity at concentrations of 30 to 100 ng/ml (*17*). Thus, TIMP-1 appears to be a multifunctional protein with tissue-specific activities.

Cathepsin L is a lysosomal cysteine protease that is present in many tissues and functions in the metabolism and turnover of intracellular proteins (*8*). It is synthesized as a proenzyme (39 kD), which is processed and targeted to lysosomes by the mannose-6-phosphate receptor (*8*). Procathepsin L is the major protein released by malignantly transformed mouse fibroblasts (*8*). Cathepsin L cleaves various intracellular and extracellular proteins, including serum proteins and components of the extracellular matrix (*8*), and has been implicated in biological processes such as tumor invasion and metastasis, bone resorption, prohormone activation, and sperm maturation (*8, 9*). Procathepsin L is also identical to CP-2, a Sertoli cell product secreted by the seminiferous tubules during a specific period of spermatogenesis; maximal synthesis and secretion occur at the time of initiation of meiosis and spermatid release (*9*). Medium from cultured seminiferous tubules at this specific stage shows the greatest stimulatory effect on androgen production by Leydig cells (*18*). Thus, it appears that STP secretion in the adult is under the control of specific germ cell types (*3*). The precise role of procathepsin L in STP is not clear. Because TIMP-1 has 12 conserved cysteine residues paired into six disulfide bonds, cleavage of these bonds by a cysteine protease might contribute to the expression of maximal STP bioactivity.

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Natural Protection Against HIV-1 Infection Provided by HIV-2

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Significant differences have been observed in the rates of transmission and disease development in human immunodeficiency virus (HIV) types 1 and 2. Because many HIV-2–infected people remain asymptomatic for prolonged periods, the hypothesis that HIV-2 might protect against subsequent infection by HIV-1 was considered. During a 9-year period in Dakar, Senegal, the seroincidence of both HIV types was measured in a cohort of commercial sex workers. Despite a higher incidence of other sexually transmitted diseases (STDs), HIV-2–infected women had a lower incidence of HIV-1 than did HIV-seronegative women, with a relative risk of 0.32 ($P = 0.008$). An understanding of the cross-protective mechanisms involved may be directly relevant to HIV-1 vaccine development.

HIV-1 and HIV-2 share substantial genetic relatedness, partial antigenic cross-reactivity, and a common cellular receptor. Although HIV-1, the prototype acquired immunodeficiency syndrome (AIDS) virus, has reached epidemic proportions throughout the world, HIV-2 is largely confined to West Africa, where varying rates of HIV-1 infection have been reported (*1*). Studies conducted in West Africa have shown that although both viruses are transmitted by similar routes, the rates of sexual and perinatal transmission of HIV-2 are significantly lower than those of HIV-1 in the same populations (*2–4*). Initial cross-sectional reports of HIV-2 suggested an association of HIV-2 infection with AIDS (*5*) that appeared to be weaker than the association of