J. steroid Biochem. Vol. 36, No. 6, pp. 541–548, 1990
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0022-4731/90 $3.00 + 0.00
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GENETIC POLYMORPHISM OF THE HUMAN SEX HORMONE-BINDING GLOBULIN: EVIDENCE OF AN ISOELECTRIC FOCUSING VARIANT WITH NORMAL ANDROGEN-BINDING AFFINITIES

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(*Received* 29 *December* 1989)

Summary—Human sex hormone-binding globulin (hSHBG) is a plasma glycoprotein composed of two identical subunits. The protein, which has high affinity for testosterone and estradiol has been purified to homogeneity. In this study we have investigated, on neuraminidase-treated serum samples, the presence of genetic variations of hSHBG by polyacrylamide gel isoelectric focusing (IEF).

Based on IEF analyses of 110 serum samples from adult Mexican individuals we have identified two distinct IEF-patterns. The most frequent phenotype (95.45%) was characterized by two IEF-bands with pIs of 6.50 and 6.63, respectively. In five serum samples, a different 4-band pattern with pIs of 6.50, 6.63, 6.70 and 6.76 was identified. Family studies showed that this pattern was genetically determined. The frequency of this variant was 4.55%, and the observed phenotypes were consistent with the expression of an autosomal genetic system. The estimated gene frequencies for both alleles were shown to be in genetic equilibrium. Affinity constants, binding kinetics and serum concentrations of hSHBG from individuals having a 4-band pattern were similar to those obtained in individuals with a 2-band pattern, thus suggesting that the mechanism responsible for the generation of polymorphic variants of hSHBG reported herein did not involve the steroid binding site of the molecule. These findings may be of broad interest, as other serum binding proteins express genetic variants, which may permit their further structural and functional subclassification.

INTRODUCTION

Steroid hormones circulate in plasma bound either to albumin or to specific steroid-binding globulins. Human sex hormone-binding globulin (hSHBG) is a dimeric acidic glycoprotein with high affinity for testosterone and estradiol [1, 2]. This protein has been purified to homogeneity [3–7] and its aminoacid and gene sequences determined [8, 9]. Human SHBG consists of two identical subunits [6, 7, 10, 11] each with 373 aminoacids, 2 disulfide bonds and 3 oligosaccharide chains [8]. The estimated molecular weight of hSHBG based on the aminoacid sequence and carbohydrate content is 93 kDa. The site of synthesis is unclear although liver has been suggested as a strong possibility [12].

The biological significance of hSHBG still remains as an unsolved issue. However, inasmuch as it has been shown that hSHBG regulates the metabolic clearance rate of circulating testosterone [13, 14] and may also serve as a specific vector of testosterone to androgen dependent tissues [15, 16], its functions appear to be regulatory in nature.

Analyses of hSHGB by isoelectric focusing (IEF) have revealed the presence of microheterogeneity which in part is attributed to variability in sialic acid content or rearrangement in aminoacid composition [7, 8]. Treatment with neuraminidase reduces the number of bands detectable by IEF and results in a cathodic shift in the pI of desialylated hSHBG [6, 7, 17]. The residual microheterogeneity after desialylation with neuraminidase has also been previously reported for some other proteins [18]. In these studies it was clearly demonstrated that persistence of isoelectric variants even after treatment with mixed glycosidases, a technique known to remove more than 85% of carbohydrates and all sialic acid residues [19], reflects variations in the polypeptide chain. Evidence concerning the presence of electrophoretic variants of hSHBG have been reported previously [20–22]. In this study we decided to investigate, in a population survey, the presence of genetically determined polymorphism of hSHBG. We have taken advantage of the high resolution power of IEF

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and the availability of monospecific antibodies, which allowed us to identify hSHBG in serum even in the presence of other proteins.

MATERIALS AND METHODS

Materials

Nonradiactive steroids were purchased from Steraloids Inc. (Wilton, N.H.) and their chemical purity was established by paper chromatography and recrystallization. [1,2-^{3}H]testosterone ([^{3}H]T; 40–60 Ci/mmol) and [1, 2-^{3}H]dihydrotestosterone ([^{3}H]DHT; 40–60 Ci/mmol) were obtained from Du Pont NEN Research Products (Boston, Mass.). Radiolabeled steroids were purified prior to use on silica gel thin-layer plates (Silica gel 60F-254, E. Merck Darmstadt, West Germany) developed in chloroform:ethanol (25:2). Acrylamide, *N,N'*-diallyltartardiamide (DATD), *N,N'*-methylenebisacrylamide (bis), *N,N,N',N'*-tetramethylethylenediamine (TEMED), Coomassie brillant blue R-250, ammonium persulfate, sodium dodecyl sulfate (SDS), affi-gel blue, nitrocellulose paper, isoelectric focusing standards, and ampholytes were obtained from Bio-Rad Laboratories (Richmond, Calif.). Bovine serum albumin (BSA), Tris, glycine, glutamic acid, Tween-20, glycerol, neuraminidase (EC 3.2.1.18) from clostridium perfringes Type X and trypsin inhibitor type I-S were obtained from Sigma Chemical Co (St Louis, Mo.). Rabbit anti-human SHBG antiserum was a gift from Dr C. W. Bardin of the Population Council Center for Biomedical Research, New York. This reagent has been proved to be monospecific for hSHBG [23].

Blood samples were obtained from 110 adult (55 females and 55 males) Mexican individuals living in the Mexico City area. Sera were separated and kept frozen at -80°C until assayed.

Sample preparation

Serum albumin was first removed from serum samples before electrofocusing. This was accomplished by the use of Affi-gel Blue which effectively removed most of the albumin present in serum. Briefly: Affi-gel Blue was thoroughly washed and equilibrated with 0.02 M sodium phosphate buffer, pH 7.1 and dispensed into 12 × 75 mm polypropylene tubes (Falcon, Oxnard, Calif.) together with the serum sample in a final ratio of 2:1 (v/v), respectively. After 1 h of incubation at 4°C precipitates were removed by centrifugation and supernatants were saved and kept at -80°C for futher analysis.

Desialylation of serum samples was accomplished by incubation in the presence of neuraminidase at 37°C for 6 h as previously described by Van Baelen *et al.*[24]. Samples (0.2 ml) were adjusted to pH 5.8 with 1 N HCl and incubated with neuraminidase (0.05–0.5 U) in 0.05 M sodium acetate buffer, pH 5.8. After the incubation period, 0.1 ml of 0.02 M Tris, pH 7.4 and 10% glycerol (TG) containing 0.02% NaN_3 were added and samples centrifuged at 3000 rpm for 20 min at 4°C. Desialylation of hSHBG was evaluated by isoelectric focusing of samples before and after treatment with neuraminidase.

Isoelectric focusing

Isoelectric focusing in polyacrylamide gels (IEF–PAG) was performed, as described by Larrea *et al.*[25], in Gelamide-coated (Gelamide 250, Polysciences Inc., Penn.) 5 mm tubes, with a final acrylamide concentration of 5% T and 15% DATD. Ampholines (2%) in a pI range of 3–10 or 5–8 were used. The anolyte and catholyte electrode solutions consisted of 0.05 M Tris base and 0.05 M glutamic acid, respectively. Samples were focused for 20 h at 200 V at 4°C. The pH measurements were performed in 5 mm gel pieces previously immersed in 2 ml of distilled water. Electrofocusing also was performed on plastic-backed IEF–PAG (11 × 12 cm, LKB Uppsala, Sweden) with a pH range from 3 to 10 using an LKB Multiphor apparatus cooled at 4°C at a constant current of 0.05 A until a voltage of 1500 V was reached. Aliquots (20–40 μl) of diluted desialylated serum samples were applied on sample application pieces made from Whatman No. 3 mm paper at the cathodic side of the gel. To measure the pH gradient in these gels, 0.5-cm gel pieces were cut out and immersed in 2 ml of distilled water for 2 h. The pH of these solutions was measured at room temperature. The pI of the IEF–PAG standards and serum proteins was calculated by interpolation.

Identification of desialylated hSHBG

Immediately after isoelectric focusing, plastic-backed IEF–PAG were incubated in 100 ml of transfer solution (0.7% acetic acid) for 30 min at room temperature. Transfer of IEF–PAG proteins to nitrocellulose paper (NTC) was performed as follows: the blot consisted to three sheets of filter paper, two sheets of NTC membrane, all of them pre-wetted in transfer solution, the plastic-backed IEF–PAG (gel side down) and a piece of glass with 5 kg weight on the top. The blot was placed in a humid atmosphere overnight. After blotting, the NTC membrane was peeled away from the gel in one continuous motion and washed in 0.5 M Tris, pH 7.4, 0.15 M NaCl buffer (Tris–saline) three times at room temperature. Paper was blocked in Tris–saline containing 5% BSA for 8 hr at 4°C. After several washes, the NTC membrane was then incubated with a 1/100 dilution of rabbit anti-hSHBG antiserum in Tris–saline containing 0.1% Tween-20 for 24 hr at room temperature. Unbound antibodies were removed by several washes, and the NTC membrane was finally incubated with peroxidase-labeled anti-rabbit IgG (Sigma Chemical Co., St Louis, Mo.) for 2 h. The immunocomplexes were localized by staining for peroxidase in 0.3 mg/ml 3,3'-diaminobenzidine, 0.005% hydrogen peroxide. In some instances, IEF–PAG were removed from gel tubes, frozen at

−80°C, and sliced into 2 mm segments. Polyacrylamide gel slices were then eluted with continuous shaking in 0.5 ml TG buffer for 18 h at 4°C. Tubes were centrifuged at 3000 g and supernatants were saved for further analysis.

Binding parameters of hSHBG

The equilibrium binding characteristics of hSHBG were determined by saturation analysis using the dextran-coated charcoal (DCC) assay described by Larrea *et al.*[26]. Serum samples were previously treated with charcoal (1 mg/ml) for 1 h to remove endogenous steroids. Charcoal was removed by centrifugation at 3000 g at 4°C. Binding assay was performed in 0.2 ml of 10-fold diluted charcoal-stripped serum samples in the presence of [^{3}H]DHT (0.2 pmol) and increasing amounts of nonradioactive DHT to give a final concentration of 1–40 nM. A tube containing 200 ng DHT was used as an indicator of nonspecific binding. After 1 h of incubation at 0°C, 0.8 ml of a DCC suspension containing 250 mg Norit-A and 25 mg dextran T-70 in 100 ml TG buffer was added to each tube. Precipitates were sedimented, and the supernatants were decanted into counting vials using Instagel as a counting solution. The results were plotted according to the method of Scatchard[27]. Human SHBG which was eluted from IEF–PAG was measured by the one point assay method and expressed as specific cpm bound of [^{3}H]DHT. Briefly: aliquots (0.2 ml final volume) were incubated for 60 min with [^{3}H]DHT (1×10^{-9} M) with or without unlabeled DHT (1×10^{-7} M). Bound and free steroids were separated with DCC, as described above. Specific [^{3}H]DHT binding results when nonspecific binding is substracted from total binding.

Half-time of dissociation determinations

The dissociation rate and half-time of dissociation of the hSHBG–testosterone complex were studied either at 4 or 28°C on 10-fold diluted charcoal-stripped serum samples containing saturation amounts of [^{3}H]testosterone (1×10^{-9} M), as previously described [28]. Serum samples were incubated with [^{3}H]testosterone for 1 h either at 4 or 28°C to prelabel the binding sites. At this time [^{3}H]testosterone–hSHBG complex was incubated with an excess (1000 ×) of unlabeled testosterone. Aliquots were withdrawn at various times and the bound radioactivity determined by a 15 s incubation with charcoal followed by centrifugation and counting the charcoal free supernatant. The dissociation rate constant and dissociation half-time were calculated from the linear regression line.

RESULTS

Desialylation of serum samples

When a serum sample incubated in the presence or absence of neuraminidase was submitted to isoelectric focusing in polyacrylamide gel tubes and hSHBG analyzed by the one point method assay as described under material and methods, two isoelectic binding peaks were observed (Fig. 1). These areas of binding activity are thought to correspond to hSHBG, since incubations containing an excess of unlabeled DHT displaced the binding of radioactive DHT. The first peak focused in a broad pH area between 5.4 and 5.85, whereas the second, more basic peak of binding activity, focused in a well defined pH area of 6.9. These isoelectric binding areas are thought to correspond to native and desialylated hSHBG, respectively. Furthermore, isoelectric focusing patterns were indistinguishable when desialylation conditions were performed in the absence or presence of a protease inhibitor. These studies demonstrated the effectiveness of neuraminidase to change the charge properties of native hSHBG and suggested that neither desialylation nor isoelectric focusing conditions altered the binding site of hSHBG.

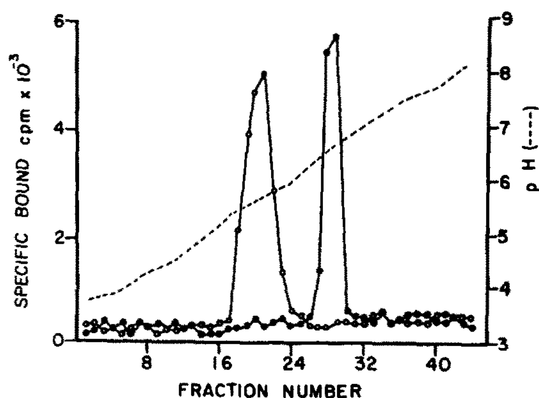

Fig. 1. Isoelectric focusing profiles of native (○——○) and desialylated (●——●) hSHBG. Sera were incubated in the presence or absence of neuraminidase and submitted to isoelectric focusing in polyacrylamide gels. After focusing, gels were frozen, sliced, and eluted in TG buffer. Human SHBG was identified in the eluates by the one point-binding assay as described in material and methods.

Population survey

As shown in Fig. 1, isoelectric focusing of serum samples in polyacrylamide gel tubes followed by elution of proteins from 2 mm gel pieces and incubation of eluates with tritiated DHT in the presence of absence of unlabeled steroid did not allow the identification of more than one isoelectric focusing species of hSHBG. We have investigated more than 250 serum samples using this method, and so far we have failed to demonstrate a single case of genetic variation of the pI of human desialylated SHBG. For this reason we decided to submit desialylated serum samples to isoelectric focusing in polyacrylamide slab gels followed by inmmunoblotting the proteins with monospecific hSHBG antiserum. With the use of this methodology, 110 serum samples of unrelated mexican individuals, corresponding to 55 married couples were investigated. In 105 cases studied (95.45%), a well defined pattern consisting of only two bands with

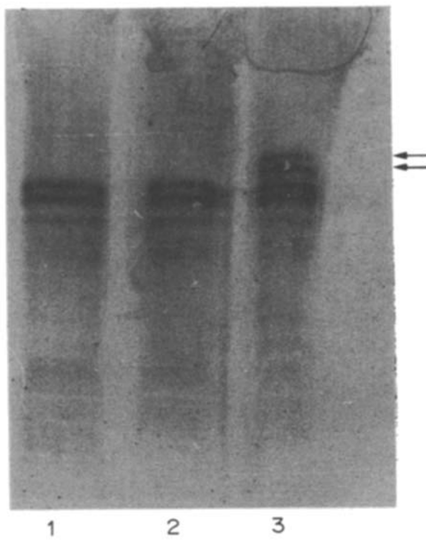

Fig. 2. Isoelectric focusing patterns of human desialylated SHBG. Neuraminidase-treated serum samples were focused on plastic-backed isoelectric focusing polyacrylamide gels. Human SHBG was identified by immunoblotting the proteins, after transfer them to nitrocellulose membranes, with specific antiserum. Lanes 1 and 2 represent the most frequent pattern of desialylated hSHBG in the subjects studied. Lane 2 shows hSHBG incubated in the presence of 10 times more neuraminidase (5 U) than hSHBG in lane 1. Lane 3 shows a representative desialylated hSHBG, treated identically as hSHBG in lane 2, in which two additional cathodic bands, identified by the arrows, were observed in 5 out of 110 individuals. Cathode and anode are at the top and the bottom of the figure, respectively. In all cases, a minor nonspecific anodic band was observed.

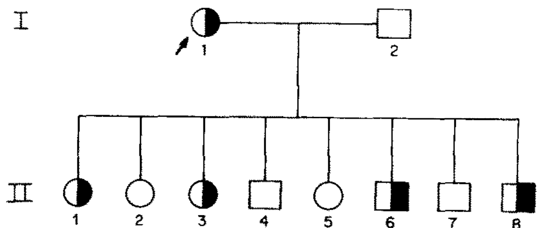

Fig. 3. Transmission of the hSHBG isoelectric focusing variant in family 1. The white color indicates normal hSHBG and the black color the variant. Squares and circles represent males and females, respectively.

pIs of 6.50 and 6.63, respectively was obtained (Fig. 2, lanes 1 and 2). However, in 5 (4.55%) serum samples a 4-band pattern was demonstrated. These five plasma samples were characterized by the presence of normal desialylated hSHBG plus two cathodic immunoprecipitated bands with a pI of 6.70 and 6.76, respectively (Fig. 2, lane 3). As shown in Fig. 2, the isoelectric focusing pattern obtained in these representative individuals was the same independent of the amount of neuraminidase present during desialylation of serum samples. In all cases the color intensity of desialylated hSHBG was stronger in individuals having a 2-band pattern than those showing a 4-band pattern. In addition, the isoelectric focusing patterns were the same notwithstanding we changed the experimental conditions such as the amount and time of incubation with neuraminidase, as well as increasing the focusing times. Repeated analyses of serum samples with a 4-band pattern,

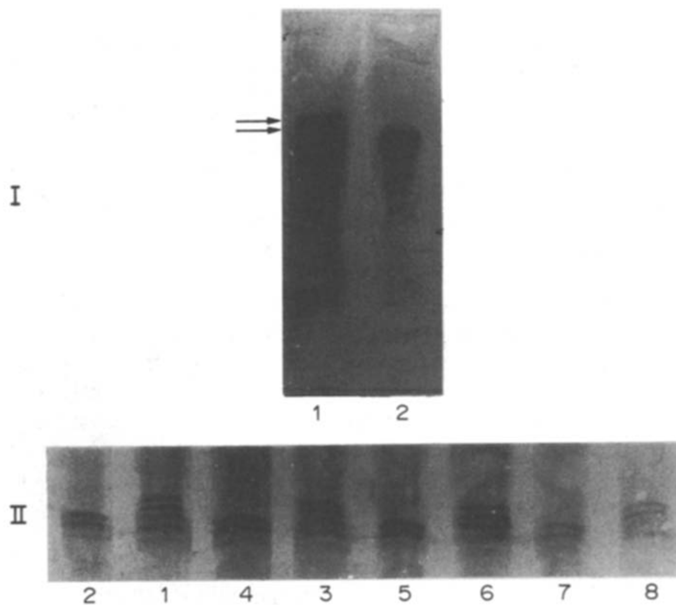

Fig. 4. Immunoblots of isoelectric focusing of serum samples from subjects belonging to family 1. The arrows indicate the two additional cathodic bands, present in one of the parents and transmitted to the offspring.

Table 1. Distribution of phenotypes and gene frequencies among the population studied

System	Phenotypes	No.	Gene	Frequency	χ^{2a}	P
	hSHBG 1-1	105	hSHBG 1	0.9773		
hSHBG	hSHBG 2-1	5	hSHBG 2	0.0227		
	hSHBG 2-2	0				
					0.053	<0.95

[a]χ^2-value for Hardy–Weinberg equilibrium test.

including several blood samples from the same individual, always resulted in an identical isoelectric focusing pattern. In no subjects studied was it possible to detect a single-band pattern and there was no sex predilection for the presence of a given isoelectric pattern of desialylated hSHBG. To evaluate whether the additional two cathodic bands in the five individuals represent a true hSHBG variant, we next examined the distribution of phenotypes among other members of their families.

Family studies

The study of the family members of two individuals in whom a 4-band pattern of hSHBG was demonstrated, showed the presence of the hSHBG variant in the offspring. In these families only one of the parents had hSHBG consisting of a 4-band pattern. Since the variant has both the normal and the two additional cathodic bands, on isoelectric focusing, we have called this condition as the heterozygous state. As shown in Fig. 3 (family 1), the 4-band focusing pattern of hSHBG, present in one of the parents (mother) (Fig. 4, I-1) was transmitted to half of her children (Fig. 4, II-1, 3, 6 and 8). In family 2 (data not shown) in a sibship of five members, only one showed the presence of hSHBG variant. The study of three families with parents showing a normal isoelectrofocusing pattern of hSHBG resulted, in all cases, in offspring (n = 12) with normal phenotype. Unfortunately, we have not found, among all the couples studied, a single family in which both parents possess the heterozygous (4-band pattern) variant of hSHBG.

At this point, and according to familial studies, segregation of hSHBG phenotypes appeared to fit in a polymorphic bi-allelic (hSHBG-1 and hSHBG-2) codominant autosomal system. On these bases, we have calculated the gene frequencies of hSHBG phenotpyes (hSHBG 1-1; hSHBG 2-1; hSHBG 2–2, which corresponded to homozygous for normal; heterozygous and homozygous for variant, respectively) and the χ^2 for Hardy–Weinberg proportion of our population [29]. The data presented in Table 1 show the estimated frequency for both alleles (hSHBG 1 and 2) and the χ^2-value which agreed that the population studied was in genetic equilibrium.

Binding parameters

The DHT-binding properties of hSHBG variant were obtained from Scatchard plot analyses (Fig. 5) and the results were compared with those of normal hSHBG. Table 2 shows the binding affinity constants of both normal and hSHBG variant for DHT at 4°C and 28°C, respectively. As depicted, the association constants and serum concentrations of hSHBG variant, measured at two different temperatures, were similar to those obtained for normal hSHBG under identical experimental conditions.

We next examined the dissociation rate (K-1) and the half-time of dissociation ($t_{1/2}$) for both normal and hSHBG variant. As shown in Fig. 6, both binding constants for normal (Fig. 6A and B) and variant (Fig. 6C and D) hSHBG, were studied at two different temperatures. Neither the dissociation rate constant nor the half-time of dissociation of both hSHBG's measured at 4 and at 28°C, were statistically different (Table 3) for the two forms.

DISCUSSION

It is well known that hSHBG displays microheterogeneity when analyzed by isoelectric focusing [6, 7, 17, 30, 31]. As with many other

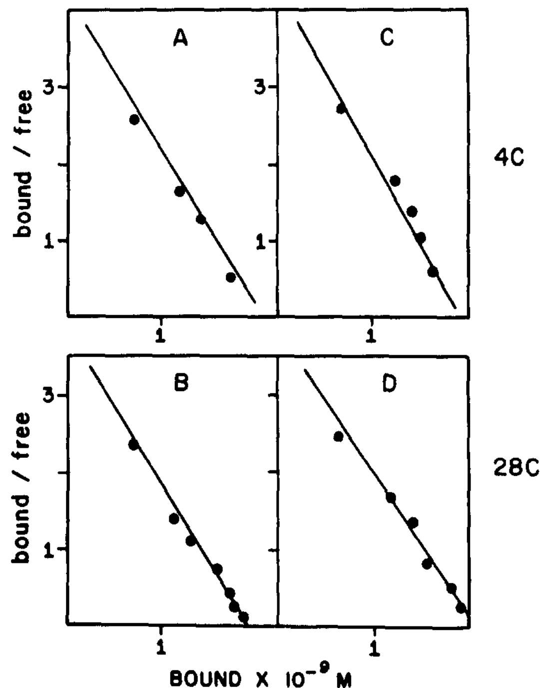

Fig. 5. Scatchard plot analyses of normal (A and B) and isoelectric focusing variant (C and D) hSHBG at 4°C and 28°C, respectively. Both serum samples were incubated in the presence of [^{3}H]DHT at various concentrations as indicated in materials and methods. Each point represents the mean of three determinations.

Table 2. Association constants and serum concentrations of normal and variant hSHBG

hSHBG		4°C M^{-1}	4°C pmol/ml	28°C M^{-1}	28°C pmol/ml
Normal					
1	M[b]	2.4×10^9	22	12×10^{8}[a]	15
2	F	1.0×10^9	43	15×10^8	48
3	F	2.0×10^9	40	20×10^8	38
4	M	1.0×10^9	16	10×10^8	26
Variant					
1	F	1.1×10^9	50	15×10^{8}[a]	54
2	F	2.0×10^9	38	17×10^8	42
3	M	2.0×10^9	14	14×10^8	16
4	M	1.0×10^9	9	13×10^8	10
5	M	1.7×10^9	17	15×10^8	15

[a]These figures were derived from Scatchard plot analyses as shown in Fig. 5.
[b]M = male; F = female.

glycoproteins, it may be related to their variable content to sialic acid, since treatment with neuraminidase reduces the number of isoelectric variants. Residual microheterogeneity after protein desialylation has been reported previously for hSHBG [6, 7, 17, 30], suggesting the presence of variations in aminoacid composition [8]. Therefore, one may conclude that variable content of sialic acid is responsible for only part of the protein microheterogeneity. The purpose of this investigation was to determine the presence of genetic polymorphism of hSHBG in individuals of both sexes living in the area of Mexico City.

The experimental strategy followed in this study was similar to that reported by Van Baelen *et al.*[24] for human corticosteroid-binding globulin. As indicated by these authors, pre-treatment of serum samples with neuraminidase greatly simplifies the isoelectric focusing data making feasible the comparison of patterns among different samples. Analysis of 110 serum samples with this technique enabled us to determine the most frequent hSHBG phenotype in our population. This was characterized by the presence of a 2-band pattern of equal color intensity which focused at pHs of 6.50 and 6.63. Moreover, it allowed us to identify, in five individuals, the existence of a distinct more complex focusing pattern. This consisted in the presence of two-additional cathodic bands, with a lower color intensity, when compared with samples having a 2-bands pattern, with pIs of 6.70 and 6.76, respectively. Initial evidence that the two additional cathodic bands were not the result of protein denaturation, was derived from the observation that the antiserum maintained

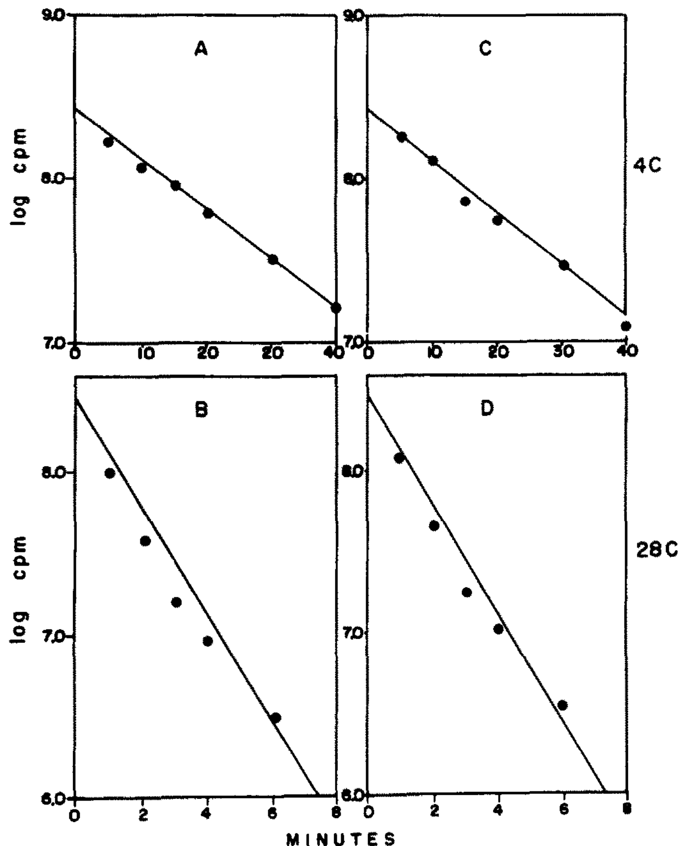

Fig. 6. Dissociation rates of testosterone–hSHBG complex at 4°C and 28°C. Serum samples corresponding to normal (A and B) and isoelectric focusing variant (C and D) hSHBG were incubated in the presence of [^{3}H]testosterone for 1 h at the indicated temperatures. At the end of this period, and excess of unlabeled testosterone (1000 ×) was added. Aliquots were withdrawn at several times, treated with charcoal and the bound radioactivity determined in the charcoal free supernatants. Each point represents the mean of three determinations.

Table 3. Dissociation rate constants (K-1) and dissociation half-times ($t_{1/2}$) for normal and for isoelectric focusing variant of hSHBG

hSHBG	4°C		28°C	
	K-1 (min^{-1})	$t_{1/2}$ (min)	K-1 (min^{-1})	$t_{1/2}$ (min)
Normal	0.0342 ± 0.0017	20.13 ± 1.029	0.250 ± 0.024	2.60 ± 0.26
Variant	0.0320 ± 0.0041*	20.58 ± 0.56*	0.250 ± 0.043*	2.90 ± 0.58*

*Nonsignificant K-1 and $t_{1/2}$ for normal vs variant at 4 and 28°C, respectively.

its specificity for both isoelectric focusing phenotypes (normal and variant). However, it could be argued that incomplete desialylation could be responsible for residual microheterogeneity. Indeed, in this communication we have not provided any evidence that hSHBG desialylation was complete and that variations in the polypeptide chains are responsible for the observed isoelectric focusing patterns. However, we performed desialylation under a variety of experimental conditions, including long incubation times and higher concentrations of neuraminidase. We also carried out repeated blood sampling of the same individual, conditions which always resulted in identical focusing patterns. In addition to these observations, hSHBG variant always focused at the cathodic side of normal hSHBG, which suggested that it could not be due to incomplete removal of sialic acid.

Furthermore, we studied whether the additional two cathodic bands present after desialylation were genetically determined. The results of segregation analyses of the two families characterized by an heterozygous hSHBG 2-1 parent, as well as of those families with both parents having the homozygous hSHBG 1-1 phenotype, suggest that both the usual 2-bands pattern and the two additional cathodic bands were genetically determined. Although the expected hSHBG 2-2 phenotype, which is most probably comprised only by the two cathodic bands, was not observed in our population, the estimated gene frequency for hSHBG 2 allele ($q = 0.0227$), gives the probability to find homozygous for hSHBG 2-2 phenotype ($q^2 = 0.0005$) of 1/2000 individuals. We do not consider this estimate as truly representative of the mexican population and therefore further studies should be carried out in this regard. Moreover, family studies showed that distribution of phenotypes among offspring was consistent with a simple Mendelian inheritance of an autosomal gene comprised most probably by two codominant-alleles.

The determination of binding affinities of hSHBGs, showed that the variant molecule had normal androgen-binding kinetics and affinity constants, as well as it was present in serum at concentrations within the normal range reported by others [23]. While the nature of this hSHBG polymorphism is not known, it most likely contained a variant polypeptide chain. Results from binding studies suggest that the mutation site, responsible for the generation of the polymorphism reported in this study, did not involve the steroid binding sites of the molecule. However, due to the inability to identify the homozygous variant of hSHBG (hSHBG 2-2) and therefore its binding properties, this later suggestion deserves to be further investigated.

In summary, this study confirms and extends previous reports [20–22] on the existence of structural variants of hSHBG. Herein, we have established the genetic nature of the polymorphism. This observation was derived from family studies which were consistent with a simple mendelian inheritance of an autosomal gene. The isoelectric focusing patterns are suggested to be the result of combinations of two codominant alleles, with gene frequencies in genetic equilibrium. Finally, affinity binding parameters for the hSHBG variant were indistinguishable from those obtained for normal hSHBG.

Acknowledgements—We wish to thank Dr C. Wayne Bardin (The Population Council Center for Biomedical Research, New York, N.Y.) for providing us with the human SHBG antiserum. The authors gratefully acknowledge Dr Raffaela Schiavon (National Institute of Pediatrics, SSA, Mexico City) for her valuable suggestions during the preparation of this manuscript. This work was supported in part by grants from the Population Council (New York, N.Y.) and the WHO Special Program of Research, Development and Research Training in Human Reproduction (Geneva, Switzerland).

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