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Murine Sex-Limited Protein Expression Requires Androgens and Pituitary Hormones

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Abstract. Levels of the murine sex-limited protein (Slp) were measured by an enzyme-linked immunosorbent assay in normal and hypophysectomized female CDF1 (Slp^a) mice before and after a 15-day treatment with testosterone proprionate. Both groups of mice initially had undetectable levels of circulating Slp. After treatment, Slp serum levels of the nonhypophysectomized group had risen significantly above the Slp serum levels of the hypophysectomized group and the pretreatment controls. This indicates that the pituitary gland is necessary for the androgen-induced expression of Slp.

Introduction

The fourth component of complement (C4) (Meo et al. 1975) and the murine sex-limited protein (Slp) (Passmore and Shreffler 1970) are encoded by the *S* region of the mouse major histocompatibility complex (*H-2*). Although structurally homologous to C4, Slp has no hemolytic activity. The structural gene and regulatory elements for Slp synthesis map to the *S* region (Shreffler et al. 1981).

Three major types of S-region regulation of Slp serum levels are known: (1) mice homozygous for an Slp^o haplotype are genetically incapable of making Slp and have no detectable Slp in their serum; (2) mice with an Slp^a haplotype have Slp serum levels that are sexually dimorphic because the Slp serum levels are androgen-controlled; (3) mice with some exceptional wild-derived *H-2* haplotypes, i. e., $H\text{-}2^{w7}$, synthesize Slp constitutively and have high Slp serum levels in both male and female mice (Klein 1975).

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In Slp^a strains, testosterone was proven to regulate Slp serum levels by the fact that (1) normal mature male mice have 25–500 times the Slp serum levels of normal females (Ferreira et al. 1982); (2) Slp serum levels of castrated males fall to those of normal females (Passmore and Shreffler 1971); (3) Slp serum levels in testosterone-treated normal mature females rise to those of males (Passmore and Shreffler 1971); (4) Slp cannot be induced in Slp^a male mice with the *X*-linked mutation, testicular feminization (*tfm*) (Hansen and Shreffler 1976). The *tfm* mutation causes androgen insensitivity by altering or abolishing the androgen receptor. Since immature Slp^a mice (< 35 days) do not produce Slp even when treated with androgens (Passmore and Shreffler 1971), there must be an additional developmental aspect to the regulation of Slp expression.

It has been shown that some proteins whose synthesis is androgen-regulated require not only androgen for their expression but also peptide, steroid, or other hormones. Since pituitary hormones have been shown to influence the synthesis of other androgen-regulated proteins, the influence of the pituitary gland on androgen-regulated expression of Slp was investigated. Slp serum levels in androgen-treated normal and hypophysectomized female Slp^a mice were measured in order to assess the influence of pituitary hormones on induction of Slp synthesis by androgens.

Slp was initially detected and assayed by immunoprecipitation (Hansen et al. 1974). Recently, much more sensitive assays have been developed. Ferreira and coworkers (1982) developed an immunoradiometric assay (RIA) which utilizes monoclonal antibodies. Here, we describe an enzyme-linked immunosorbent assay (ELISA) that we have used to investigate the regulation of Slp in vivo. Unlike the RIA, which employs two monoclonal antibodies, this ELISA uses polyclonal antisera.

Materials and Methods

Reagents. Chemicals and their sources were as follows: polyoxyethylene sorbitan monolaurate (Tween 20) (J. T. Baker Co., Phillipsburg, New Jersey); ethylenediaminetetraacetate (EDTA) and Tris(hydroxymethyl)aminomethane (Mallinckrodt, Paris, Kentucky); bovine serum albumin (BSA), glycine, and p-nitrophenyl phosphate (PNPP) as Sigma 104 phosphatase tablets, goat antirabbit immunoglobulin G (IgG) conjugates to alkaline phosphatase (GARG-AP), and testosterone propionate (Sigma Chemical Co., St. Louis, Missouri); diethylaminoethyl (DEAE)-cellulose (DE-52 cellulose, Whatman Biochemical Ltd., Kent, United Kingdom); Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, New Jersey).

Mice. Normal and hypophysectomized female CDF1 (BALB/c × DBA/2) mice of the same age were purchased from the Charles River Breeding Laboratory (Wilmington, Massachusetts). The hypophysectomies were performed at 7 weeks of age by the supplier. Drinking water of both normal and hypophysectomized mice was supplemented with 5% glucose. Experiments were initiated when the mice were 9 weeks of age. All other mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) and drank plain water.

Hormonal treatment. Mice were injected subcutaneously on alternate days for 15 days with 0.1 mg testosterone propionate in 0.1 ml sesame oil as a carrier.

Serum. Mice were bled from the retroorbital sinus. The blood was allowed to clot for 15–30 min at 4 °C. Serum was collected after centrifugation at 4 °C and was frozen at −70 °C until assayed. Samples were taken before the first injection and after 15 days of hormonal treatment.

Buffers. Tris-buffered saline with sodium azide (TBSN3) was made with 10 m*M* NaCl, 10 m*M* NaN_3, and 10 m*M* Tris, pH 8.5. Phosphate-buffered saline with Tween 20 and sodium azide (PBSTN3) was made with 20 m*M* NaH_2PO_4 and Na_2HPO_4, pH 7.2, in 0.15 *M* NaCl, 0.05% Tween 20, and 3 m*M* NaN_3.Tris-BSA buffer (TBSAN3) was made with 50 m*M* Tris, pH 8.0, containing 1.0% BSA and 3 m*M* NaN_3. Glycine-buffered saline (GBS) consisted of 55 m*M* glycine, 55 m*M* NaCl, 1.5 m*M* $MgCl_2$, and 3 m*M* NaN_3, pH 10.5. Substrate solution consisted of 8 mg/ml PNPP in 1.5 m*M* $MgCl_2$ and 3 m*M* NaN_3.

Antisera. Mouse Slp-specific antibody was prepared by immunizing SWR/J (Ss^h, Slp^o, $Ig\text{-}1^c$) female mice with serum from mature male DBA/2J (Ss^h, Slp^a, $Ig\text{-}1^c$) mice. Freshly collected DBA/2J serum was emulsified with an equal volume of complete Freund's adjuvant. This mixture (0.025 ml) was injected into each footpad of SWR/J recipients. Booster injections with serum mixed with incomplete Freund's adjuvant were given every 10–14 days until high titer antisera were obtained. The IgG fraction of the hyperimmune serum was purified by chromatography on DEAE-cellulose using a linear gradient of 0.075–0.3 *M* NaCl in 0.02 *M* Tris, pH 7.5. The anti-Slp-positive fractions were determined by double immunodiffusion versus Slp-positive serum and were pooled and concentrated to the initial serum volume by positive pressure ultrafiltration (Amicon UM10 membrane, Amicon Corp., Lexington, Massachusetts).

Rabbit C4-specific antibody was prepared as described (Goldman et al. 1978). In brief, rabbits were immunized with precipitin arcs from an immunoelectrophoresis of B10.D2 new serum developed with a standard C4-specific antibody (Anti-Ss serum was kindly supplied by Dr. D. C. Shreffler). Antibodies to mouse serum components other than C4 were removed by passage of the rabbit serum over a Sepharose 4B affinity column to which was coupled (Cuatrecasas and Anfinsen 1971) mouse serum of low C4 content, i. e., from a mouse of the $H\text{-}2^k$ haplotype.

ELISA. Anti-Slp was diluted in TBSN3 to a final concentration of 20 μg/ml. Fifty microliters were added to each well of a 96-well flat-bottomed polyvinyl chloride microtiter plate (Dynatech, Alexandria, Virginia 1-220-2a). This quantity of antibody approached saturation of the plate (Parsons 1981). After a 1 h incubation at 37 °C in a humidified chamber, the wells were washed three times with PBSTN3. Unknowns and standards were diluted in PBSTN3 containing 30 m*M* EDTA, and 100 μl aliquots were added to the wells of the microtiter plate. The samples were further incubated overnight at 4 °C. After three washes with PBSTN3, 50 μl of anti-C4 serum at a 1 : 75 dilution in PBSTN3 was added to each well, the samples were incubated for 3 h at 37 °C, and the wells were washed three times with PBSTN3. Fifty microliters of GARG-AP at a 1 : 500 dilution in TBSAN3 buffer were added to each well and incubated for 1 h at 37 °C. After three final washes with PBSTN3, 50 μl of GBS was added to each well, followed by 50 μl of freshly prepared substrate solution. After incubation at 37 °C for 15 min to develop color, the reaction was terminated by adding 50 μl of 1 *M* NaOH. Optical density at 405 nm was read on an ELISA plate reader (Dynatech).

Results

Figure 1 depicts a standard curve that was established with a pool of mature male DBA/2J (Slp^a) sera. DBA/1J (Slp^o) serum is shown as a negative control. Using doubling dilutions of the DBA/2 sera, good linearity was obtained between dilutions of 1 : 250 and 1 : 16 000 with an apparent upper limit of usable sensitivity between dilutions of 1 : 8 000 and 1 : 16 000. Tests on individual sera from several other male Slp^a (i. e., DBA/2, B10.D2, BALB/c, and PL/J) and Slp^o (i. e., DBA/1, B10.AKM, B10.BR, and SWR/J) strain mice verified both the level of sensitivity and the specificity of the assay (data not shown). In no case did Slp^o mice give false positives or Slp^a mice give false negatives.

Table 1 presents the Slp serum levels in normal and hypophysectomized female mice before and after a 15-

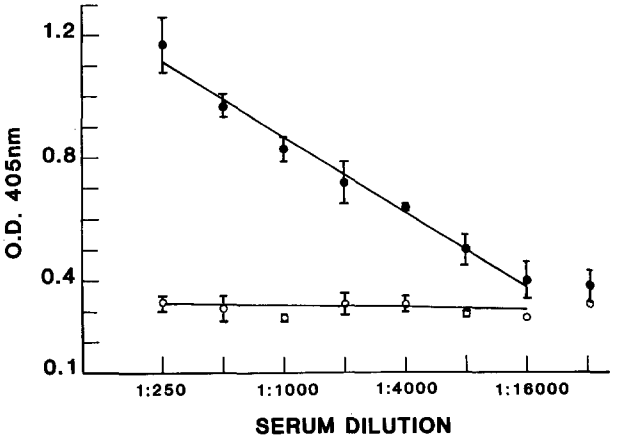

Fig. 1. Standard curve for Slp ELISA. *Solid circles* (●——●) represent serial dilutions of a reference pool of a mature DBA/2 male serum (Slp^a), and *open circles* (○——○) are serial dilutions of a pool of mature DBA/1 male serum (Slp^o). Each point represents an average of triplicate measurements and the error bars are 2 standard error of mean (SEM).

Table 1. Slp serum levels in androgen treated and hypophysectomized female CDF1 mice

	No.	Weight (g)	Slp serum level	
			Day 0	Day 15
Normal	11	22.4 (0.3)*	$<0.6^{\dagger}$	14.8 (0.9)
Hypophysectomy	29	16.6 (0.3)	<0.6	1.5 (0.6)
P value‡		<0.001		<0.001

* Average (standard error of mean)
† Average relative to a pool of mature DBA/2 (*Slp*^{a}) serum (SEM)
‡ Student's T-test

day course of testosterone propionate treatment. The Slp levels were measured using the ELISA, and the values are relative to the standard pool of male DBA/2 mouse serum. Both day 0 groups had Slp serum levels which were less than 0.6% of the standard pool (lower limit of detection). By day 15 of treatment, the hypophysectomy group had risen to 1.5% of the standard, whereas the normal group had risen to 14.8% of the standard. The treated normal group's Slp serum levels were significantly different from the pretreatment groups ($P<0.001$) and from the treated hypophysectomy group ($P<0.001$).

The hypophysectomized mice failed to gain weight in comparison with the age-matched normal controls. Of the 29 hypophysectomized mice, 27 had body weights (mean of 16.2 g) that were significantly less than the other two (mean of 21.5 g, $P<0.01$). None of these 27 stunted mice had Slp serum levels that rose above 0.6% of the standard, whereas the two normal sized mice from the hypophysectomy group had significant Slp serum levels (17.0% and 9.0% of the standard). In preliminary experiments using six DBA/2 mice, weights were measured and histological examination of the hypophysis-hypothalmic region was performed. In this experiment, hypophysectomized mice that failed to gain weight had no detectable pituitary tissue at autopsy, whereas hypophysectomized mice that had normal weights did have pituitary tissue at autopsy (data not shown). Since this was consistent with other reports in the literature which indicate that increase in body weight is an accurate indicator of the completeness of hypophysectomy, postmortem examinations were not done on the larger experimental group presented here. If body weight 4 weeks postsurgery is used as a measure of completeness of hypophysectomy, no fully hypophysectomized mouse made detectable quantities of Slp.

Discussion

Slp provides an unusual opportunity for examining the interactions of genetic and hormonal control on the phenotypic expression of a serum protein. In Slp^a strain mice it was initially believed that only male mice had Slp in their sera, whereas female mice had none. Using a sensitive RIA, Ferreira and co-workers (1982) showed that untreated females of Slp^a strains had low circulating levels of Slp. It is not known whether this low level of Slp expression is due to the low level of androgens in the female's circulation or to the expression of the *Slp* gene in the absence of androgen stimulation.

The ELISA was used to investigate hormonal factors other than androgens that affect Slp expression. Since highly specific anti-Slp sera can easily be raised, we used polyclonal antisera instead of monoclonal antibodies to achieve specificity. Anti-C4 (anti-Ss) sera recognize the C4 determinants on both C4 and Slp. Anti-Slp derived from immunizing Slp^o strain mice with serum from male Slp^a strain mice only recognizes Slp determinants and therefore does not react with C4. Our sandwich ELISA places Slp between an anti-Slp alloantiserum that recognizes the Slp determinants on Slp and an anti-C4 xenonantiserum that recognizes the C4 determinants on Slp. Samples were diluted in a buffer containing EDTA to prevent C4 from binding to the plate by activation of the classical complement pathway by the antibody on the plate. The presence of EDTA during this step eliminated the nonspecific binding of C4, as evidenced by the fact that the optical densities obtained with sera from Slp^o strains were the same at all dilutions only when EDTA was present.

As shown in Table 1, normal CDF1 females that are androgen-treated for 15 days have a significant increase in their serum Slp levels, whereas hypophysectomized CDF1 females do not. This indicates that Slp expression in vivo is dependent not only on androgens but also on pituitary factors. These may be either directly or indirectly permissive to androgen action, or pituitary factors may directly or indirectly cause Slp expression with androgens modulating the pituitary factor expression. There is ample precedent evidence for pituitary hormones being permissive to androgen action in other systems. Alpha-2U-globulin, an androgen-regulated rat urinary protein, exhibits androgenic and ontogenic regulation in vivo similar to Slp. Its androgen regulation is also dependent on pituitary factors with growth hormone acting directly, and ACTH and TSH acting indirectly via cortisol and T_3, respectively (Roy and Chatterjee 1983). In the rat prostate, an androgen-regulated organ, prolactin is synergistic with testosterone in causing an increase in weight and functional activity of the prostate (Yamanaka et al. 1975).

The requirement for hormones other than androgens for the in vivo expression of Slp might explain why in vitro androgen-regulated expression has not been successful. This laboratory has shown that hepa-

tocytes from male Slp[a] mice synthesize Slp in vitro for several days after initiation of culture, but then cease making Slp although they continue to synthesize other proteins (manuscript submitted and unpublished data). Adding androgens to these cultures did not prolong the duration of Slp synthesis (T. M. Danoff, M. B. Goldman, and J. N. Goldman, manuscript submitted and unpublished data), nor did it induce Slp synthesis in vitro in hepatocytes from female mice. The in vitro unresponsiveness to androgens compared to the in vivo androgen responsiveness can be explained by humoral factors not present in the in vitro culture that are permissive to androgen action in vivo.

Further experiments are necessary to determine which pituitary factors are permissive to androgen action. Once so determined, in vitro androgen-regulated Slp synthesis may be possible which would open the way to understanding androgen regulation at the molecular level.

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References

Cuatrecasas, P. and Anfinsen, C. B.: Affinity chromatography. *In* W. B. Jakoby (ed.): *Methods in Enzymology, Vol. 22*, pp. 345–378, Academic Press, New York, 1971

Ferreira, A., Eichinger, D., and Nussenzweig, V.: The murine sex-limited protein (Slp): Reassessment of its sex limitation. *J. Immunol. 129:* 1506–1508, 1982

Goldman, J. N., Gleason, K. R., and Goldman, M. B.: Immunochemical properties of the murine Ss protein. *J. Immunol. 120:* 225–232, 1978

Hansen, T. H. and Shreffler, D. C.: Characterization of a constitutive variant of the murine serum protein allotype, Slp. *J. Immunol. 117:* 1507–1513, 1976

Hansen, T. H., Krasteff, T. N., and Shreffler, D. C.: Quantitative variations in the expression of the mouse serum antigen Ss and its sex-limited allotype Slp. *Biochem. Genet. 12:* 281–293, 1974

Klein, J.: A case of no sex-limitation of Slp in the murine *H-2* complex. *Immunogenetics 2:* 297–299, 1975

Meo, T., Krasteff, T., and Shreffler, D. C.: Immunochemical characterization of murine H-2 controlled Ss (serum substance) protein through identification of its human homologue as the fourth component of complement. *Proc. Natl. Acad. Sci. U.S.A. 72:* 4536–4540, 1975

Parsons, G. H.: Antibody-coated plastic tubes in radioimmunoassay. *In* J. J. Langone and H. van Vunakis (eds.): *Methods in Enzymology, Vol. 73*, pp. 224–239, Academic Press, New York, 1981

Passmore, H. C. and Shreffler, D. C.: A sex-limited serum protein variant in the mouse: Inheritance and association with the H-2 region. *Biochem. Genet. 4:* 351–365, 1970

Passmore, H. C. and Shreffler, D. C.: A sex-limited serum protein variant in the mouse: Hormonal control of phenotypic expression. *Biochem. Genet. 5:* 201–209, 1971

Roy, A. K. and Chatterjee, B.: Sexual dimorphism in the liver. *Annu. Rev. Physiol. 45:* 37–50, 1983

Shreffler, D. C., Atkinson, J. P., Brown, L. J., Parker, K. L., and Roos, M. H.: Genetics, structure and function of murine S region gene products. *In* M. B. Zaleski, C. J. Abeyounis, and K. Kano (eds.): *Immunobiology of the Major Histocompatibility Complex*, pp. 78–88, Karger, Basel, 1981

Yamanaka, H., Kirdani, R. Y., Saroff, J., Murphy, G. P., and Sandberg, A. A.: Effects of testosterone and prolactin on rat prostatic weight, 5 α-reductase, and arginase. *Am. J. Physiol. 229:* 1102–1109, 1975

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