Deficiency in the regulation of testicular galactolipid sulphotransferase in rats carrying the growth-and-reproduction-complex (*grc*) gene

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The regulation of the activity of testicular germ-cell galactolipid sulphotransferase was investigated in rats homozygous for the *grc* (growth and reproductive complex) gene. In the adult *grc* homozygotes, the activity was elevated relative to that in the wild-type animals, and a concomitant deficiency of a developmentally regulated sulphotransferase inhibitor was found. Spermatogenesis in the *grc* homozygotes is blocked at a stage that correlates temporally with the earliest detection of this inhibitor in the wild-type animal. In addition, there was a similar increase in the specific activity of the kidney galactolipid sulphotransferase in the *grc* homozygotes. This biochemical abnormality is the first to be associated with a genetically regulated, developmental defect linked to the major histocompatibility complex, and it is related to the pathogenesis of one of the major lesions controlled by the *grc*.

INTRODUCTION

In a variety of species, recessive genes influencing development are linked to the major histocompatibility complex (MHC): the *t*-complex in the mouse, the *grc* in the rat and similar genes in the human [reviewed by Gill *et al.* (1983)]. The transmission and phenotypic expression of these genes have been studied extensively, but the biochemical basis of their effects are still quite obscure. This paper presents the first study of a biochemical defect associated with the *grc* in the rat.

The *grc* influences fertility and body size: homozygous males are 30% smaller than the wild-type, their testes are 90% smaller and spermatogenesis is blocked at the primary pachytene spermatocyte stage (Kunz *et al.*, 1980). No hormonal or chromosomal defects have been associated with these developmental anomalies (Gill *et al.*, 1983; Kunz *et al.*, 1980; Greiner *et al.*, 1980), but there are morphological abnormalities in approximately half of the sperm in the heterozygotes (Gill *et al.*, 1983).

An investigation of the testicular galactolipid sulphotransferase activity of the *grc* homozygotes was included in a study of the regulation of the activity of this enzyme during spermatogenesis in the rat in order to examine the possibility of a relationship between the enzymic and testicular defects. SGG is the major testicular glycolipid (Ishizuka *et al.*, 1973; Kornblatt *et al.*, 1972), and its synthesis is restricted to the early primary spermatocyte stage (Kornblatt *et al.*, 1974). In the normal rat, SGG remains in the plasma membrane of the subsequent cell stages without turnover (Kornblatt, 1979), and the sulphotransferase activity measured *in vitro* for the later stages of spermatogenesis is decreased by a specific inhibitory activity. The testicular galactolipid sulphotransferase will sulphate both glycerol- and sphingosine-based galactolipid acceptors [the preceding paper (Lingwood, 1985)]. The enzyme inhibitor preferentially decreases the sulphation of GG and thereby causes a change in the enzyme's specificity. This change, in turn, causes a switch from the synthesis of SGG to that of SGC and SLC concomitant with an overall decrease in sulpholipid biosynthesis during spermatogenesis (Lingwood, 1985).

The present study shows that the adult *grc* homozygotes have an elevated testicular galactolipid sulphotransferase activity coincident with a deficiency of enzyme inhibitor. The testicular inhibitor also decreases the synthesis *in vitro* of SGG by the kidney sulphotransferase (Lingwood, 1985). In addition, the specific activity of the kidney galactolipid sulphotransferase is elevated in the *grc* homozygotes. These results suggest that the activity of the galactolipid sulphotransferase enzymes of both testis and kidney are under similar control.

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MATERIALS AND METHODS

Animals

The rats carrying the *grc* gene and the wild-type animals were obtained from the F2 hybrid progeny of the BIL/1 (*grc/grc*) × YO (*grc⁺/grc⁺*) cross. The homozygotes will simply be designated as '*grc*' and '*grc⁺*'.

Enzyme preparations

Tuniae were removed from freshly excised rat testes as described in the preceding paper (Lingwood, 1985). The seminiferous tubules were homogenized in 0.32 M-sucrose/1 mM-EDTA to give a protein concentration of

Abbreviations used: MHC, major histocompatibility complex; SGG, sulphatoxygalactosylacylalkylglycerol; GG, galactosylacylalkylglycerol; SGC, sulphatoxygalactosylceramide; GC, galactosylceramide; SLC, sulphatoxylactosylceramide; LC, lactosylceramide; PAPS, 3'-phosphoadenosine-5'-phosphosulphate.

Table 1. Comparison of the inhibition of testicular or renal galactolipid sulphotransferase activity by inhibitors from wild-type (*grc*⁺) and homozygous *grc* testes

Results are from triplicate analyses on two to three animals in each group. The standard deviations of the measurements in each group were ± 3 –10%. *, Significantly different ($P < 0.05$). Abbreviation used: nd, not determined.

Enzyme source			Sulphotransferase activity (pmol of SGG/h per mg)		
			Testis		Kidney
Animal	Age of animal	Inhibitor source	2 μ M-PAPS	30 μ M-PAPS	30 μ M-PAPS
<i>grc</i> ⁺	20 Days	None	10.8	153.9	nd
	3 Months	None	nd	20.2	18.6
<i>grc</i>	3 Months	None	9.0	93.0	38.1
Inhibition (%)					
<i>grc</i> ⁺	20 Days	<i>grc</i> ⁺	43	27	nd
	20 Days	<i>grc</i>	4*	15*	nd
	3 Months	<i>grc</i> ⁺	nd	nd	28
	3 Months	<i>grc</i>	nd	nd	6
<i>grc</i>	3 Months	<i>grc</i> ⁺	38	24	64
	3 Months	<i>grc</i>	0*	4*	36

Fig. 1. Autoradiogram of kidney sulphotransferase reaction products

Kidney homogenate from wild-type (3 months old) (3 mo) or from *grc* animals (*grc*) were incubated with exogenously added GG (25 nmol) (assayed in triplicate) or GC (2 nmol) (assayed in duplicate) in the presence of 30 nmol [³⁵S]PAPS. The radiolabelled sulphoglycolipids were separated by t.l.c. (chloroform/methanol/water, 40:10:1, by vol.) and detected by autoradiography.

40–60 mg/ml and stored in liquid N₂ without loss of activity. Freshly excised kidneys were homogenized in a similar manner after removal of the capsule.

Galactolipid substrates

SGG was obtained from bovine testes (Lingwood, 1985). GG was prepared from SGG by anchimeric-assisted solvolysis (Lingwood *et al.*, 1983). LC was prepared from human erythrocytes (Laine *et al.*, 1974), and GC was purchased from Supelco, Bellefonte, PA, U.S.A.

Sulphotransferase assay

A 25 nmol portion of GG and 1 mg of Triton X-100 dissolved in chloroform/methanol (2:1, v/v) were dried together under N₂. Then 0.1 ml of 0.1 M-Tris/HCl, pH 8.6, containing 80 mM-K₂SO₄, 10 mM-ATP, 2.5 mM-

MgCl₂, 2 or 30 nmol of [³⁵S]PAPS (10⁵ d.p.m.) (New England Nuclear, Lachine, Que., Canada) and 25 μ l of enzyme preparation (approx. 0.1 mg of protein) was added. The mixture was incubated at 37 °C for 2 h and partitioned into 5 vol. of chloroform/methanol (2:1, v/v) and 5 vol. of 0.88% KCl. The mixture was centrifuged, the lower phase was dried under N₂, and glycolipids were separated by t.l.c. (chloroform/methanol/water, 40:10:1, by vol.). Radioactive products were detected by autoradiography, scraped from the plate and counted for radioactivity in a liquid-scintillation spectrometer.

Preparation of the enzyme inhibitor

A testicular homogenate prepared as described above was centrifuged at 8000 *g* for 2 min. The supernatant was passed through a Millipore filter (0.45 μ m pore size) and

portions were either used directly or freeze-dried and stored at -20°C . Samples were reconstituted to the original volume, and $25\ \mu\text{l}$ was included in the sulphotransferase assay described above to determine inhibitory activity.

Analysis of upper-phase glycolipids

Testes from individual animals were homogenized in a Waring blender in 20 vol. of chloroform/methanol (2:1, v/v). The homogenate was filtered, and the residue was extracted in 10 vol. of chloroform/methanol (1:1, v/v). The filtrates were combined, evaporated, resuspended in chloroform/methanol (2:1, v/v) and partitioned against water. The lower phase was repartitioned against 10 vol. of theoretical upper phase (chloroform/methanol/aq. 0.1% NaCl, 1:10:10, by vol.) (Laine *et al.*, 1974), and the upper phases were pooled and evaporated to a minimal volume. The extract was then dialysed overnight against water, freeze-dried and analysed by t.l.c. [chloroform/methanol/water (55:25:4, by vol.)]. The separated components were detected with orcinol spray, which distinguished carbohydrate containing species.

Metabolic labelling

Testes were removed from rats immediately after they were killed. The seminiferous tubules were finely minced with a sharp razor blade and resuspended in phosphate-buffered saline (0.1 M-phosphate buffer containing 0.15 M-NaCl), pH 7.2. Tubular fragments were allowed to settle in a measuring cylinder for 10 min at room temperature. The supernatant was centrifuged at 800 g for 10 min, and the pellet was resuspended in phosphate-buffered saline containing 1% fructose to a concentration of approx. 10^6 cells/ml. A 1 ml portion was incubated for 5 h at room temperature in the presence of 0.1 mCi of H_3^3SO_4 . Glycolipids were extracted with chloroform/methanol (2:1, v/v) and partitioned against 0.88% KCl; the lower-phase glycolipids were separated by t.l.c. [chloroform/methanol/water (40:10:1, by vol.)]. Radiolabelled sulpholipids were detected by autoradiography.

RESULTS

The specific galactolipid sulphotransferase activity of the adult *grc* testicular homogenate is approx. 4-fold higher than that of the age-matched wild-type, whereas that of the kidney is about twice as high (Table 1). The high-speed (8000 g) supernatant of the adult testicular homogenate of normal animals contains an inhibitor of sulphotransferase activity. This inhibitory activity is most conveniently measured by using the testicular homogenate from 20-day-old animals, since the highest enzyme specific activity is observed at this age [the preceding paper (Lingwood, 1985)]. The inhibitor can also decrease the sulphation of GG *in vitro* by the kidney homogenate. Although SGG is not made in the kidney *in vivo* (Lingwood *et al.*, 1981), the kidney sulphotransferase will synthesize SGG from exogenous GG *in vitro* (Lingwood, 1985). The inhibitor preparation from the *grc* homozygotes contained little activity when compared with the adult wild-type animals, and this deficiency was apparent irrespective of the enzyme used in the assay (kidney or testis, *grc* or *grc*⁺) (Table 1).

Since SGG is not produced by the kidney *in vivo*, the sulphation of GG and of physiological substrate GC was examined *in vitro* by using kidney homogenate from *grc*

Fig. 2. (a) Comparison of the specificities of testicular galactolipid sulphotransferase from *grc* homozygotes and from wild-type (*grc*⁺) rats, and (b) metabolic labelling of isolated germ cells from the testes of *grc* homozygous and wild-type rats for the assay of sulphotransferase activity

(a) Testicular homogenates from 20-day-old wild-type rats (20 da), adult (3 months old) wild-type rats (3 mo), and adult *grc* homozygotes (*grc*) were incubated with 0.2 nmol of GC or with 1.0 nmol of LC in the presence of 30 nmol of [^{35}S]PAPS. Radiolabelled products were separated by t.l.c. as described in the Materials and methods section. Both the 20-day-old wild-type rats and the *grc* homozygotes synthesized SGG, whereas the wild-type adults did not. (b) Cell suspensions from 20-day-old wild-type rats (20 da), adult wild-type rats (3 mo) and adult *grc* homozygotes were incubated in the presence of H_3^3SO_4 , and radiolabelled sulpholipids were extracted, separated by t.l.c. and detected by autoradiography. The 20-day-old rats and the *grc* homozygotes synthesized predominantly SGG, whereas the adult wild-type animals synthesized SGC and SLC. The SGG/SGC ratios in the three groups were 4.8, 3.2 and 0.5 respectively, and SGG/SLC ratios were 6.3, 2.5 and 0.4 respectively.

Fig. 3. T.l.c. analysis of equal aliquots of the upper-phase lipid extract from the testes of individual adult *grc* homozygotes, *grc* heterozygotes and wild-type animals as described in the Materials and methods section

Two slow-migrating orcinol-positive bands are missing or greatly decreased in the *grc* homozygotes (arrows). *grc*, *grc* homozygotes; *grc*/+, *grc* heterozygotes; 3 mo, wild-type. All animals were analysed at 3 months of age.

and *grc*⁺ animals. The sulphation of both GG and GC was elevated when the enzyme from *grc* animals was used (Fig. 1).

In the normal animal, the inhibitor effects a shift in the substrate specificity of galactolipid sulphotransferase during testicular development which can be demonstrated *in vitro* and *in vivo* (Lingwood, 1985). Since the *grc* homozygote is deficient in the inhibitor, the possibility that the switch in sulphotransferase specificity did not occur was examined. The relative specificities of the testicular homogenate for the sulphation *in vitro* of GG, GC and LC were determined (Fig. 2a). The homogenate from the 20-day-old wild-type animals and from the adult *grc* homozygotes still synthesized SGG, whereas the homogenate from the adult wild-type animals preferentially sulphated GC and LC. Similar results were obtained by metabolically labelling sulphoglycolipids in intact germ cells (Fig. 2b). The 20-day-old wild-type rats and the adult *grc* homozygotes showed high incorporation and synthesized predominantly SGG, but the major labelled sulphoglycolipids in the adult wild-type animals were SGC and SLC and [³⁵S]sulphate incorporation was greatly decreased.

Analysis of the upper-phase testicular lipids from *grc* homozygotes, *grc* heterozygotes and wild-type animals (Fig. 3) showed that two slow-migrating orcinol-positive bands were missing, or greatly decreased, in the *grc* homozygote, but were present in the heterozygote and in the wild-type. Fluorescamine spray demonstrated that these bands contained free primary amino groups, and this finding suggests that they are not glycolipids but, rather, glycopeptides. Since the partially purified inhibitory activity is associated with the presence of carbohydrate-containing primary amino components of similar mobility on t.l.c. (Lingwood, 1985), these species may contain the inhibitor.

DISCUSSION

Testicular SGG comprises a large portion of the male germ-cell plasma membrane, and its synthesis is restricted to the early primary-spermatocyte stage of development. Although its function is unknown, SGG undergoes considerable cell-surface redistribution during spermatogenesis (Lingwood & Schachter, 1981), and its biosynthesis is regulated by a soluble inhibitor (Lingwood, 1985). This molecule also alters the galactolipid acceptor specificity such that there is a switch from the sulphation of a galactosylglycerol lipid acceptor (GG) to the sulphation of sphingolipid (GC and LC) acceptors during spermatogenesis; nonetheless, sulphation of total galactolipids is greatly decreased during development. The results presented here show that the *grc* homozygote is deficient in inhibitor activity: the sulphotransferase activity remained at the high level observed in normal 20-day-old animals (Table 1), and the enzyme had a less effective switch to the sulphation of sphingolipid acceptors than did normal adult rats (Fig. 2a). Endogenous sulphoglycolipid biosynthesis as measured by metabolic labelling remained high, similar to that observed in the normal 20-day-old animal (Fig. 2b). The kidney sulphotransferase activity of the *grc* homozygotes was also elevated (Table 1, Fig. 1). Therefore the galactolipid sulphotransferase of the testis and the kidney may be related; indeed, a kidney inhibitor of galactolipid sulphotransferase activity has recently been detected (C. Lingwood, unpublished work). Despite the elevated sulphotransferase activity in the *grc* mutant, assays of kidney function were found to be within normal limits (results not shown).

Spermatogenesis in the *grc* homozygote is consistently blocked at the primary-spermatocyte stage (Kunz *et al.*, 1980), which corresponds to the first appearance of the sulphotransferase inhibitor in the normal rat. Since SGG is a major membrane component of all subsequent germinal cells, a defect in the regulation of its biosynthesis would have a drastic effect on cellular differentiation. The necessity to switch to the sulphation of sphingolipids during differentiation may result from the inability of glycerolipids to undergo lateral hydrogen-bonding (Pascher, 1976), which may be necessary to stabilize the cell membrane (Boggs, 1980) during the later stages of spermatogenesis.

The defect in the control of sulphotransferase activity in the *grc* homozygote is not confined to the testis, since the sulphotransferase activity was also abnormally high in the kidney. Whether these findings are a reflection of the common embryological origin of the testis and the kidney, or are part of a more widely distributed defect, is not yet known. Nonetheless, the primary genetically controlled defect in the *grc* homozygotes appears to be the result of the inappropriate quantitative control of galactolipid sulphotransferase inhibitor.

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