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A UNIQUE mRNA SPECIES FOR THE α SUBUNIT OF G_s IS PRESENT IN RAT HAPLOID GERM CELLS

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SUMMARY: The 1.9 kb mRNA encoding the α subunit of G_s observed in the somatic cells of the rat testis was not seen in the germ cells (pachytene spermatocytes and round spermatids) as revealed by Northern blot analysis. Interestingly, the cDNA probe for $G_s\alpha$ detected a mRNA of shorter chain length (0.9 kb) exclusively in the haploid germ cells (round and elongated spermatids). However, no ADP ribosylated protein was detected in membranes from germ cells after cholera toxin treatment. The 0.9 kb transcript may represent a haploid germ cell-specific variant of $G_s\alpha$, which is functionally different from the stimulatory component of the adenylate cyclase system.

The activity of hormone-sensitive adenylate cyclase is modulated by several guanine nucleotide-binding regulatory proteins or G proteins (for review, see (1-3)). These proteins have a trimeric structure, consisting of α, β, and γ subunits. The α subunits of the stimulatory G proteins are ADP ribosylated by cholera toxin and exist as species with apparent molecular weights of 45,000 or 52,000 on SDS-polyacrylamide gels (4). Although most tissues contain both forms of the polypeptide, their relative amounts vary (5).

In rat testis, the germ cells have been found to lack the hormone-sensitive adenylate cyclase (6,7). In round spermatids there is, however, a soluble $Mn2^+$-dependent adenylate cyclase activity which is hormone-insensitive (7). Furthermore, no ADP ribosylated substrates for cholera toxin action have been found in dog sperm (8). These findings indicate that testicular germ cells lack the stimulatory component (G_s) of the adenylate cyclase system.

In our attempts to localize the mRNA coding for $G_s\alpha$ in rat testicular cells, we discovered a unique mRNA species in the late germ cells, which was not seen in the other cell types.

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MATERIALS AND METHODS

Preparation of whole testes.

Testes were obtained from Sprague-Dawley rats (Møllegaards Breeding Centre Ltd., Denmark) of various ages (5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 80, 100 days of age), frozen i liquid N_2 and stored at - 70 °C for later RNA extraction.

Cell preparation.

The isolation of Sertoli cells from 19-day-old rats was carried out as described by Dorrington et al. (9). The cells were incubated at 34 °C in a humidified atmosphere of 5% CO_2 in air. On the third day after plating in Eagle's Minimal Essential Medium (MEM, Gibco, Grand Island, N.Y.) containing 10% foetal calf serum (Gibco), the cells were incubated further in serum free MEM. RNA was extracted from the cells on day 5 after plating. The contamination of germ cells in the Sertoli cell preparation was less than 2%.

Peritubular cells were isolated from 19-day-old rats by the method of Hutson and Stocco (10). The cells became confluent after 10-12 days and were harvested for RNA extraction on day 13. Sertoli cells containing lipid droplets were occasionally seen (<1%) in these preparations.

Germ cell preparations were obtained from 32-day-old (pachytene spermatocytes and round spermatids) and 44-day-old rats (elongating spermatids), respectively. The fractionation of the germ cells was performed in BSA gradients at unit gravity in a velocity sedimentation cell separator (STA-PUT) according to Grootegoed et al. (1977) (11). RNA extraction from the cells was carried out immediately after the fractionation was completed. The germ cells were examined both by phase contrast microscopy and by regular light microscopy after fixation and staining. The purities were evaluated by counting cells: pachytene spermatocytes (PS) 90-95%; round spermatids (RST) 85-90%, elongated spermatids (ES) 40% (contamination mostly RST).

1 ml of Leydig cell tumor (H-540), distintegrated in 0.9% NaCl, was transplanted s.c. on the back of 30- to 35-day-old rats (12). After 2-3 wk, the rats were killed and the tumors were excised immediately and stored in liquid N_2 for later RNA extraction.

Preparation of total RNA

RNA extraction from whole testes and from cell preparations were performed by homogenization in guanidinium isothiocyanate. Total RNA was isolated by centrifugation through a cesium chloride cushion and purified by phenol/chloroform extractions (13).

Probes

Plasmid containing a cDNA clone encoding $G_s\alpha$ isolated from rat olfactory cDNA library was kindly supplied by Drs. David T. Jones and Randall R. Reed (14). Gel-purified cDNA insert was excised with Eco RI endonuclease resulting in a fragment of 1123-base-pair with poly(A)-tail. This probe was labeled by using [^{32}P]dCTP (Amersham, UK) and a standard nick-translation kit (Amersham).

A $G_s\alpha$-specific oligonucleotide (28-mer) complementary to the 3'-nontranslated region of rat $G_s\alpha$, nucleotides 1589-1616 (14), was purchased from Genetic Designs Inc. (Houston, Texas, USA). This probe was end-labeled as described by Maxam and Gilbert (1977) (15), using [^{32}P]ATP (Amersham) and T4 polynucleotide kinase (Amersham).

Northern analysis

Total RNA (20 µg) was denaturated in 50% formamid and 6% formaldehyd and size-fractionated on a 1.5% agarose gel containing 6.7% formaldehyd. The RNA was visualized by staining with ethidium bromide and then transferred to BioTrans nylon filter (ICN)

Schwarz/Mann Biotech, Cleveland, OH) by capillary blotting technique (16). The hybridization was performed according to the ICN procedure with the nick-translated cDNA probe.

The Northern nylon filters were probed with the oligonucleotide using the following prehybridization/hybridization conditions: 40% formamide, 5XSSC, 5X Denhardt's solution, 50 mM sodium phosphate (pH 6.5), 0.1% SDS, 50 µg/ml tRNA, 42°C. The filters were washed with 0.5X SSC, 0.1% SDS at 50°C.

Autoradiography was carried out with Amersham HMP film.

ADP ribosylation

Crude membrane suspensions containing 5 mg membrane protein per ml, were prepared essentially as described elsewhere (17). These suspensions were diluted 1:8 with 1 mg/ml DNase I and assayed as described by Ribeiro-Neto et al. (18). Incubations were started by mixing 20 µl diluted membrane suspension, 30 µl toxin mixture, 10 µl pre-activated cholera toxin and 10 µl [^{32}P]NAD^+ ($18x10^6$ cpm). Incubations were conducted for 40 min at 35°C. Final constituent concentrations were: 10 µM NAD^+, 300mM potassium phosphate, 50 mM ADP ribose, 10 mM thymidine, 1 mM ATP, 0.1 mM GTP, 1 mM EDTA, 10 mM $MgCl_2$, 25 mM Tris-HCl pH 7.5, 0.02 mg/ml DNase I, 7.5 µg/assay BSA, 1.66 mM DTT and 100 µg/ml cholera toxin.

The reactions were terminated with 1 ml ice cold 20% TCA. The pellets were washed with diethyl ether and allowed to dry at room temperature. Each sample was added 100 µl SDS-PAGE sample buffer (Laemmli) and subjected to ultrasound sonication in a water bath for 5 min followed by heating to 80°C for 5 min.

35 µl aliquots of solubilized ADP ribosylated protein corresponding to 30 µg of total protein were analyzed on SDS-PAGE (Laemmli). Gels were blotted onto Immobilon filters and subjected to standard autoradiography.

RESULTS

Northern blot analysis of total RNA from testicular cells with the $G_s\alpha$ probe demonstrated a 1.9 kb band in the somatic cells, in agreement with similar analysis of other tissues (14,19). Only a very small amount of this message was detected in the germ cell samples and may well be ascribed to contamination (4-8%) of these cell preparations with somatic cells. However, the round spermatids express a message with a shorter chain length (approx. 0.9 kb) neither present in the somatic cells of the testis (fig. 1) nor in other tissues or cells reported so far.

To analyze further the cellular localization of the smaller mRNA, we carried out Northern blot analysis of germ cell RNA including a fraction containing elongating spermatids (40%) (fig. 2). Although the amount of RNA in this latter sample was 65% compared with the others, the expression of the 0.9 kb transcript was most pronounced in this fraction.

The above findings were confirmed by the results of an age study (fig. 3). The 1.9 kb message was present in testis from

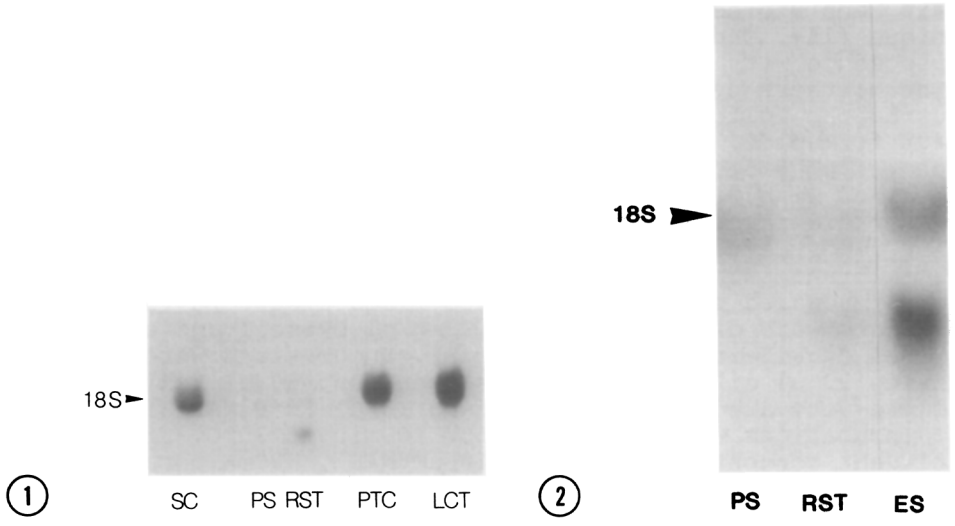

Figure 1. Northern blot analysis of total RNA from various rat testicular cell types (SC, Sertoli cells; PS, pachythene spermatocytes; RST, round spermatids; PTC, peritubular cells; LCT, Leydig cell tumor). 20 µg of RNA was loaded in each lane. The resulting filter was probed with ^{32}P-labeled cDNA for $G_s\alpha$.

Figure 2. Northern blot analysis of total RNA from different germ cell fractions from rat (PS, pachytene spermatocytes; RST, round spermatids; ES, elongating spermatids). 20 µg of RNA from PS and RST, respectively, was loaded in lanes 1 and 2, whereas 13 µg of RNA from elongating spermatids was loaded in lane 3. The resulting filter was probed with ^{32}P-labeled cDNA for $G_s\alpha$.

rats of all ages (5-100 days). The smaller band appears at day 30 when the production of haploid germ cells has just started. Furthermore, there was an age-dependent decrease in the expression of the 1.9 kb transcript, whereas the amount of hybridization to the 0.9 kb mRNA showed an increase with increasing age.

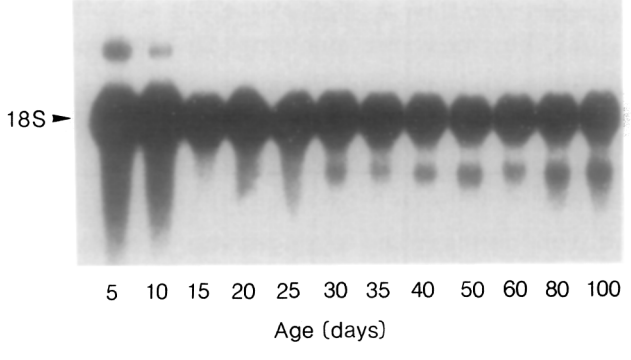

Figure 3. Northern blot analysis of total RNA from testes from rats of different ages. 20 µg of RNA was loaded in each lane. The resulting filter was probed with ^{32}P-labeled cDNA for $G_s\alpha$.

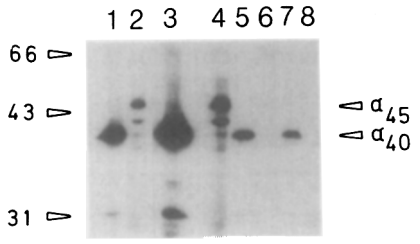

Figure 4. Toxin-catalyzed ADP ribosylation of G_α subunits in crude membrane preparations from peritubular cells (lanes 1 and 2), Sertoli cells (lanes 3 and 4), pachytene spermatocytes (lanes 5 and 6) and round spermatids (lanes 7 and 8). The membrane preparations were incubated with ^{32}P-labeled NAD^+ and pertussis toxin (lanes 1,3,5 and 7) or cholera toxin (lanes 2,4,6 and 8). Each lane was loaded with 30 µg protein. The migration of cholera toxin- and pertussis toxin-catalyzed ADP ribosylation products (α_{45} and α_{40}, respectively) are indicated, as are molecular weight standards of 31, 43 and 66 kDa.

To examine the possibility that the two messages were different in the 3'-nontranslated end we used an oligonucleotide complementary to a sequence in this region (nucleotides 1589-1616 (14)) to probe the Northern filters. The oligonucleotide recognized both mRNA species (data not shown) indicating similarity in the 3'-nontranslated region.

Cholera toxin treatment of crude membrane fractions of Sertoli cells and peritubular cells resulted in the labeling of several substrates (fig. 4). The predominant form was a 45kDa polypeptide, reflecting ribosylation of a $G_s\alpha$ subunit. In spite of the long exposure time, no labeled band was detected in membranes from germ cells. Pertussis toxin-sensitive substrates were found in membranes from both the somatic cells and the germ cells of the testis (fig. 4).

DISCUSSION

By Northern analysis we found that the somatic cells of the testis contained the message encoding $G_s\alpha$ that is normally expressed in other tissues (14,19). This 1.9 kb band was not observed in the germ cell samples, a finding supported by the decrease in the amount of hybridization to this band during the rapid proliferation of germ cells (between day 20 and 60). Furthermore, no cholera toxin-sensitive substrates were present in the germ cell membranes, whereas ADP ribosylated bands were readily detected in membranes from the somatic cells. These results are in agreement with the fact that immature germ cells lack the adenylate cyclase activity which is coupled to $G_s\alpha$.

Surprisingly, the haploid germ cells were shown specifically to contain a message of chain lenght 0.9 kb as revealed by the Northern analysis. This message is probably expressed at higher levels in elongating spermatids than in round spermatids since a fraction enriched in elongating spermatids showed a higher amount of hybridization to this band than the round spermatid fraction (80-85% pure). The age study confirmed the cellular distribution of the 0.9 kb band by its appearance at day 30. The production of haploid germ cells in rat testis starts at about day 21-24, whereas the elongating spermatids appear between day 30 to 40.

It has recently been shown that germ cells may use alternative polyadenylation site signals giving rise to short length mRNAs encoding the subunits of protein kinases as well as the proto-oncogene c-abl (20,21). To examine the possibility that the 0.9 kb mRNA differs from the 1.9 kb mRNA by using an polyadenylation signal closer to the open reading frame, we carried out Northern analysis with a $G_s\alpha$-specific oligonucleotide complementary to a region in the 3'-nontranslated end of the cDNA for $G_s\alpha$. This sequence could be downstream of a putatively alternative polyadenylation signal accounting for the difference in chain length between the two messages. However, the 0.9 kb band as well as the 1.9 kb band were detected with the oligonucleotide probe.

This result, together with the fact that the germ cells did not contain cholera toxin-sensitive substrates, indicate that there are other explanations to the different expression of transcripts by the germ cells and the somatic cells of the testis.

Sequencing and expression of cDNA clones suggest that four closely related forms of $G_s\alpha$ may be produced by alternative splicing of a single precursor mRNA (22,23). These variants comprise two pairs of shorter and longer molecules, respectively, which differ by a single stretch of 45 nucleotides. Each pair in turn differs in the presence or absence of a 3 nucleotide sequence (CAG). In transfection/expression studies three of the splice variants have been synthesized and all were shown to stimulate both adenylate cyclase and calcium channels (24).

The 0.9 kb transcript observed in our study may represent another variant of $G_s\alpha$ produced by alternative splicing of the gene, which is haploid germ cell-specific and which is not ADP ribosylated by cholera toxin. The open question is whether this shorter message is translated into a biologically active protein.

So far neither receptors nor effectors known to be coupled by G proteins have been reported for the germ cells. The haploid germ cell specific adenylate cyclase is not an obvious candidate for such an effector in view of its lack of sensitivity to hormones and GTP (7), as well as its soluble nature (6). Approaches to clone the cDNA for the 0.9 kb message are now in progress in our laboratory.

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