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EVIDENCE FOR A NOVEL SPLICE VARIANT OF THE α SUBUNIT OF G_o IN RAT MALE HAPLOID GERM CELLS

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Northern analysis shows that $G_o\alpha$ mRNA is highly expressed in immature germ cells from rat. Whereas α_{o2} mRNA is the major form in pachytene spermatocytes, a message of shorter chain length is present in large amount in haploid germ cells. This mRNA was detected with an oligonucleotide specific for the 3'-coding region of α_{o2}, but did not hybridize to oligonucleotides specific for the 5'-untranslated and 5'-coding regions. The results indicate the presence of a novel splice variant of α_o mRNA, which may code for a $G_o\alpha$ protein important for germ cell development.

GTP-binding regulatory proteins (G proteins) are involved in transduction of signals from activated receptors to intracellular effectors (for review, see 1-3). They consist of three distinct subunits (α, ß and γ), and the diversity in these subunits contributes to a large number of possible G-protein heterotrimers and interactions. Molecular cloning has revealed the existence of at least 16 G_α genes. Moreover, some of these genes ($G_s\alpha$ and $G_o\alpha$) have more than one product due to alternative RNA splicing.

Among the G protein family, a G_o protein was originally isolated from bovine brain (4,5), and its expression in mammals is shown to be restricted to nervous, heart and endocrine systems (6-11). Functionally, G_o may be a stimulator of phospholipase C (12,13), an inhibitor of Ca^{2+} channels (14), and a stimulator of K^+ channels (15).

The human $G_o\alpha$ gene has been cloned and shown to contain at least 11 exons, 9 of them in the coding region (16). Two classes of mammalian $G_o\alpha$ cDNAs have been isolated, α_{o1} and α_{o2}, which are derived from alternatively spliced mRNAs (16-19). They have exons 1-6 in common, but differ in exons 7 and 8. The products are proteins of 354 amino acids which differ in the carboxyl-terminal one-third of the molecule, a

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region important for interaction with both receptor and effector (20). Moreover, two α_{o1} mRNA subclasses which differ in the 3'-untranslated region have been reported (19,21). Toxin-labeling and biochemical purification have revealed the existence of at least two distinct forms of the G_o protein in both heart and brain (22-24).

We have recently shown that pachytene spermatocytes from rat testis contain high levels of both immunoreactive α_o protein as well as mRNA (25). No α_o was found in the somatic cells of the testis. In this study we have used oligonucleotides specific for each of the two classes of α_o in Northern analyses, to examine their mRNA distribution in germ cells from rat testis. The results indicate the existence of an additional splice variant of α_o which is identical to α_{o2} in the C-terminal coding region and differs in the 5'-untranslated and N-terminal coding regions. This splice variant may be specific to haploid germ cells.

MATERIALS AND METHODS

Cell preparation

The isolation of Sertoli cells from 19-day-old rats was carried out as described by Dorrington et al. (26). 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%.

Pachytene spermatocytes and round spermatids were obtained from 32-day-old rats. 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. (27). The cells were further purified by Percoll gradient centrifugation as described by Meistrich et al. (28) and Jutte et al. (29).

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, as well as by probing the filters with cDNAs which detect cell-specific mRNAs (30). The pachytene spermatocytes and round spermatids were 90% and 99% pure, respectively.

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 (31).

Probes

Plasmid containing a cDNA clone encoding rat α_{o1} was kindly supplied by Dr. Lutz Birnbaumer (Houston, Texas, USA). Gel-purified insert (1312 nucleotides) was labeled by using [^{32}P]dCTP (Amersham, UK) and a standard nick-translation kit (Amersham).

Oligonucleotides complementary to different regions in rat α_{o1} and α_{o2} cDNAs were purchased from Biotechnology Centre of Oslo, University of Oslo (Norway) and end-

labeled as described by Maxam and Gilbert (32), using [^{32}P]ATP (Amersham) and T4 polynucleotide kinase (Amersham). The following oligonucleotides were used: Probe 1, nucleotides (nts) 916 to 951 of α_{o1} (exon 7); probe 2, nts 916 to 951 of α_{o2} (exon 7); probe 3, nts −391 to −350 (5'−untranslated); probe 4, nts 13 to 49 (exon 1) (16,17,19).

Northern analysis

Total RNA (20 μg) was denaturated in 50% formamide and 6% formaldehyde and size−fractionated on a 1.5% agarose gel containing 6.7% formaldehyde. 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 (33). The hybridization was performed according to the ICN procedure with the nick−translated cDNA probe.

The Northern nylon filters were probed with the oligonucleotides 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 at 42°C. The filters were washed at 50°C with 0.5X SSC, 0.1% SDS for probes 1−3 and 0.2X SSC, 0.1% SDS for probe 4.

Autoradiography was carried out with Amersham HMP film.

RESULTS AND DISCUSSION

Multiple molecular weight forms of $G_o\alpha$ mRNA can be identified by Northern analysis. Whereas the α_{o1} and α_{o2} classes of mRNA differ in the open reading frame (16−19), two types of α_{o1} diverge in the 3'−untranslated region (19,21). The ratio of expression of these mRNA species shows widely tissue−specific variations (18,19).

In this study we carried out Northern analysis of α_o with RNA from Percoll gradient−purified germ cells and isolated cultured Sertoli cells. The filters were probed with oligonucleotides complementary to sequences both common to and specific for α_{o1} and α_{o2} cDNAs, as well as with α_o cDNA. With the cDNA probe, several transcripts in the germ cell fractions were detected (Fig. 1A). Highest level of the 6.9 kb transcript, encoding α_{o2}, was in the pachytene spermatocyte fraction. This transcript was also seen in the round spermatids, however, these cells expressed a high level of a 4.5 kb mRNA species. The transcript coding for α_{o1} has approximately this size (17−19). The Sertoli cells did not contain any of these two types of transcripts. The 2.4 kb band detected with the cDNA probe in both the Sertoli cells and germ cells did not appear when the filters were probed with the oligonucleotides. Moreover, washing the filters at 60°C resulted in a significant decrease in the amount of hybridization to this band compared with the others (data not shown). These observations indicate that the message in Sertoli cells is due to cross−hybridization to a non−$G_o\alpha$.

Thus, while α_{o2} seems to be the major form expressed in pachytene spermatocytes, the type α_{o1} could be the dominating form in the haploid germ cells. To examine these possibilities we used probes with different exon specificity.

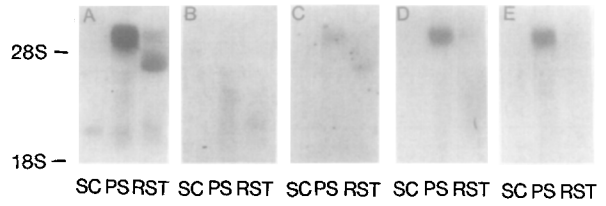

Figure 1. Northern blot analysis of total RNA from various rat testicular cell types (SC, Sertoli cells; PS, pachytene spermatocytes; RST, round spermatids). 20 μg of RNA from PS and RST, respectively, was loaded in each lane. The resulting filter was probed with ^{32}P-labeled cDNA for α_{o1} (A) and ^{32}P-labeled oligonucleotides described in Materials and Methods: Probe 1 (specific for exon 7 in α_{o1}) (B); probe 2 (specific for exon 7 in α_{o2}) (C); probe 3, (specific for 5'-untranslated region) (D), probe 4 (specific for exon 1 within the open reading frame) (E).

With probe 1, which is specific for exon 7 in α_{o1}, only very weak signals of smaller transcripts were seen in pachytene spermatocyte fraction (Fig. 1B). The 6.9 kb band as well as the 4.5 kb band in the round spermatid fraction were completely absent. This indicates that the 4.5 kb band do not correspond to α_{o1} mRNA. The weak signals in the pachytene spermatocyte fraction may reflect expression of type α_{o1} mRNA, although very low, since the Northern analysis with the cDNA probe revealed a band slightly smaller than 4.5 kb in these cells.

Surprisingly, when we hybridized with probe 2 which is specific for exon 7 in α_{o2}, both the 6.9 kb band in the pachytene spermatocytes and the 4.5 band in the haploid germ cells were detected (Fig. 1C). This suggests that the 4.5 kb may be an alternative mRNA splice variant of α_{o2}.

We also used two probes which are specific for common regions in rat α_{o1} and α_{o2} (Fig 1D and E). Probe 3 is specific for the 5'-untranslated region, whereas probe 4 is specific for exon 1 within the open reading frame. These probes should therefore hybridize to both the 6.9 kb mRNA and the 4.5 kb mRNA. Surprisingly, only the 6.9 kb band was detected with these probes (Fig. 1D and E). This confirms that the 4.5 kb band is not an α_{o1}-type transcript.

Thus, there seems to exist an additional alternative splice variant of α_o which is identical to α_{o2} in the region corresponding to the C-terminal domain, but differs in the sequence encoding the N-terminal part and the 5'-untranslated region. So far, only splice variants in 3'-coding region and in the 3'-untranslated region have been cloned from mammals (16-19,21). However, two $G_o\alpha$-like cDNAs which have been cloned from Drosophila melanogaster, are splice variants that use alternate first exons, which code for the 5'-untranslated sequence of the mRNA and the first 39 amino acids

(34,35). The deduced amino acid sequences of both proteins are more than 80% identical to that of the mammalian $G_o\alpha$s, and it is more α_{o2}-like than α_{o1}-like (17,34,35). Furthermore, Bertrand et al. (19) have compared the nucleotide sequence of rat, hamster and bovine α_o cDNAs and pointed out that there was an abrupt divergence in sequence composition of the bovine 5'-untranslated region compared with those of rat and hamster which were almost identical, suggesting a position of splice junction.

The germ cells seem to be in an exceptional position regarding unique mRNAs. We have previously reported the existence of smaller-sized $G_s\alpha$ mRNA in haploid germ cells compared with somatic cells from rat (36). Moreover, the expression of unique mRNAs coding for several of the regulatory subunits of cAMP-dependent protein kinases, have been demonstrated in rat haploid germ cells (37). The open question is whether the $G_o\alpha$ message detected in the round spermatids is translated into a biologically active protein, and if so, whether this protein has another function than the other $G_o\alpha$ proteins. In this connection, it is worth noting that this mRNA differs from the known α_o mRNAs in a sequence encoding a region which is important for interaction with the $\beta\gamma$ dimer and membrane attachment (38-40).

We have recently reported that phosphpolipase C activity in germ cell membranes is stimulated by 5'-guanylyl imidophosphate, making phospholipase C to an effector candidate for G_o in these cells (25). Furthermore, the first indication of G protein-coupled receptors in germ cells has recently been reported by Meyerhof et al. (41) who have isolated a cDNA encoding a putative G protein-coupled receptor from a rat testis cDNA library. The corresponding mRNA was exclusively expressed in spermatocytes and spermatids. Although no ligand has been identified, there may exist a unique signal transduction system i haploid germ cells which is essential for spermatid differentiation.

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