Developmental expression of GTP-binding proteins in rat testes

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The expression of guanine nucleotide-binding proteins (G proteins) during the development of rat testes was investigated. Immunohistochemical studies on frozen sections and isolated testicular cells demonstrated that the expression of the GTP-binding proteins was developmentally regulated and specific for different cell types. The α subunit of the cholera toxin-sensitive stimulatory G protein (G_{α}) was first detected in testes from 7-day-old rats; its value reached a maximum at 23 days and then decreased to very low or undetectable amounts in testes of 45-day-old and adult rats (60–90 days of age). The G_{α} subunit appears to be expressed by Sertoli, peritubular myoid and interstitial cells. The common β subunit (G_{β}) was present at all ages during development and was more prominent around the periphery of the tubules in younger animals but then became more evident in the cytoplasm of germ cells with increasing age. The pertussis toxin-sensitive inhibitory G proteins, $G_{i1/2}$ and G_{i3} , showed a similar pattern of expression. Sertoli cells and peritubular cells expressed $G_{i1/2}$ and G_{i3} at very low levels at all ages, whereas pachytene spermatocytes and round spermatids expressed the inhibitory binding proteins only at later ages of development (45-day-old and adult testis). Northern blot analysis showed that with increasing age the G_{α} mRNA in the testis decreased and this was confirmed by *in situ* hybridization. These latter studies showed localization of the transcripts to somatic cells but not to germ cells. Thus, the cellular expression of G proteins is temporally linked to testicular development and this suggests that there is an age-dependent regulation of the effects of hormones and intratesticular factors acting via G protein-linked receptor and effector systems.

Introduction

Guanine nucleotide-binding proteins (G proteins) are involved in signal transduction, providing a link between membrane receptors and intracellular effectors. Cell surface receptors activated by their specific ligands catalyse GTP-binding by G proteins which then alters enzyme activity resulting in the initiation of membrane effector mechanisms. These effectors include a variety of systems such as adenylyl cyclase, phosphodiesterases and ionic channels (Birnbaumer *et al.*, 1989, 1990).

The peptide hormones FSH and LH play an important role in the development of the testes and in the regulation of spermatogenesis (Means *et al.*, 1980; Platts *et al.*, 1988). FSH acts on Sertoli cells by binding to its cell-surface receptor which is coupled to the stimulatory G protein (G_{α}) and thus activates adenylyl cyclase to catalyse the production of cAMP, the second messenger (Casey and Gilman, 1988). Other enzymes, namely phospholipase C and A_2 , which may also be regulated by G proteins, have been shown to be involved in Sertoli cell function (Cockcroft and Gomperts, 1985; Jelsema and Axelrod, 1987). Sertoli cells provide the cytoarchitectural support for the developing germ cells and are responsible for their metabolic needs (Parvinen *et al.*, 1986). This cell–cell interaction between Sertoli cells and germ cells is an essential feature of spermatogenesis. Germ cells appear to lack hormone-sensitive adenylyl

cyclase (Hildebrandt *et al.*, 1985), although a soluble Mn^{2+} -dependent hormone-insensitive adenylyl cyclase activity has been observed in round spermatids (Gordeladze *et al.*, 1981). However, a 0.9 kb mRNA species hybridizing to a G_{α} probe has been reported in rat haploid germ cells (Haugen *et al.*, 1990).

Using immunohistochemistry, we have previously shown that the α and β subunits of the stimulatory G protein are present in 10-day-old rat testis (Dym *et al.*, 1991). The presence of both pertussis and cholera toxin-sensitive GTP-binding proteins in isolated testicular cells has also been reported (Paulssen *et al.*, 1991). In this study, we sought to investigate further the expression of G proteins at the cellular level and to determine their distribution during development using immunocytochemical and *in situ* hybridization procedures.

Materials and Methods

Tissues

Sprague–Dawley rats at different ages (1, 7, 12, 23, 45, 60, 80 and 90 days) were killed using CO_2 asphyxiation. The testes were then removed, frozen in liquid nitrogen, and used for cryostat sectioning, RNA preparation, or for membrane preparation.

Isolation and culture of testicular cells

Sertoli and myoid cells were isolated from testes of 15-day-old rats as described by Hadley *et al.* (1985). Sertoli cells were

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Fig. 1. The restriction sites of the two cDNA fragments (268 and 189 bp) generated from the original full-length *Gsa* cDNA, subcloned and used as templates for transcription of riboprobes, are shown.

Fig. 2. Immunocytochemical localization of different G proteins (*Gsa*, *Gβ*, *Gi1/2*, *Gi3*) at the indicated developmental ages of rat testis. Frozen sections (10 μ m) were cut, thaw mounted on glass slides, and fixed in cold methanol. Incubation with antibodies directed against the indicated G proteins was performed using methods of Dym *et al.* (1991). NRS: normal rabbit serum.

placed onto coverslips in serum-free defined medium (SFDM: Dulbecco's minimum essential medium (DMEM) containing 100 U penicillin ml^{-1} , 100 μg streptomycin ml^{-1} , 2 μg insulin ml^{-1} , 10 ng epidermal growth factor ml^{-1} , 5 μg human transferrin ml^{-1} , 50 ng vitamin A ml^{-1} , 200 ng vitamin E ml^{-1} , 10 nmol hydrocortisone l^{-1} , 100 pmol testosterone l^{-1} , 10 pmol oestradiol l^{-1} , 2 mmol glutamine l^{-1} , 5 ng sodium selenate ml^{-1} , 1 mmol sodium pyruvate l^{-1} , and 22 mmol sodium lactate l^{-1}). Myoid cells were plated on coverslips in 10% fetal bovine

serum. DMEM was purchased from Gibco BRL (Gaithersburg, MD); all other chemicals were obtained from Sigma Chemical Co. (St Louis, MO). The cells were cultured for 3 days before use for the immunocytochemical studies. The myoid cell population was generally a homogeneous population and was not contaminated by other cell types; the Sertoli cell cultures were contaminated with approximately 2% myoid cells.

Pachytene spermatocytes and round spermatids were isolated from testes of 90-day-old Sprague-Dawley rats by the

Fig. 2. (contd).

Fig. 3. Immunoblot analysis of (B) $G\alpha$, (C) $G\beta$, (D) $G\iota 1/2$ and (E) $G\iota 3$ proteins in membranes of testes of 12-day-old rats. Immunoblot analysis was performed using specific rabbit anti-G-protein antisera or normal rabbit serum (A). Membrane preparations were resolved on 10% SDS-PAGE gels and then the proteins were transferred to nitrocellulose membranes and incubated with the antibodies for 2 h.

method described by Onoda and Djakiew (1991) and used immediately for immunohistostaining. We generally obtained >95% purity in the spermatid fraction and 85% purity in the pachytene spermatocyte fraction.

Antibodies

Rabbit anti-G-protein antisera ($G\alpha$, $G\beta$, $G\iota 1/2$, and $G\iota 3$) were purchased from Dupont/NEN (Boston, MA). Fluorescein-conjugated and peroxidase-conjugated goat anti-rabbit antibodies were obtained from ICN Immunobiologicals (Lisle, IL).

RNA probes

Rat olfactory $G\alpha$ full length cDNA was a gift from R. R. Reed (Johns Hopkins University, School of Medicine, Baltimore, MD) (Jones and Reed, 1987). Two fragments of the original cDNA were subcloned into pGEM vectors (Promega Biotec., Madison, WI) and used as templates for the transcription of riboprobes complementary (antisense) and noncomplementary (sense) to mRNA (Fig. 1). Riboprobes were synthesized in the presence of ^{35}S CTP to a specific activity of 10^6 c.p.m. μg^{-1} .

Membrane preparation

Testicular membranes were obtained from testes of 12- and 90-day-old rats by the method previously described by Dym *et al.* (1991).

Immunohistochemistry

Immunohistochemistry was performed essentially as described by Dym *et al.* (1991). Alterations to the protocol included longer washing times after antibody incubations and a 1:3000 dilution of the fluorescein-conjugated goat anti-rabbit antibody.

Immunoblot analysis

Membrane preparations from testes of 12-day-old and adult (60–90-day-old) rats were resolved on a 10% SDS-PAGE gel (Laemmli, 1970). Separated proteins were then electrophoretically transferred to nitrocellulose membranes (0.45 μm ; Hoefer Scientific, San Francisco, CA) and then incubated at 4°C overnight in 5% non-fat milk in PBS to prevent non-specific binding. Primary and peroxidase-conjugated secondary antibodies were diluted at 1:2000 and 1:3000, respectively, in 1% non-fat milk made up in PBS, 0.1% (v/v) Triton X-100 and 0.05% (v/v) Tween 20. Both primary and secondary antibodies were incubated with the membranes for 2 h at room temperature and the nitrocellulose strips were washed three times for 10 min each to remove unbound antibodies. Immunoreactivity was visualized using 3-amino-8-ethyl carbazole as the enzyme substrate.

RNA (northern) analysis

RNA was isolated from testes of rats of various ages using the method of Chomczynski and Sacchi (1987). The RNA was subjected to electrophoresis and then transferred to a Nytran filter (Schleicher and Schuell, Keene, NH) for hybridization with the antisense and sense strands of the $G\alpha$ riboprobe. The filters were prehybridized for 4 h at 60°C in hybridization solution containing 50% formamide, 5 $\times$ SSPE (20 $\times$ SSPE = 174 g of NaCl, 27.6 g of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and 7.4 g EDTA, pH 7.4 in 1 l), 10% Denhardt's, 0.1 mg tRNA ml^{-1} and 0.2% SDS. For hybridization, 25 $\times 10^6$ c.p.m. of probe was added to the filters in 5 ml and allowed to incubate overnight at 60°C. The filters were then washed twice for 60 min each time with 0.1 $\times$ SSPE, 0.1% SDS and then dried and exposed to autoradiography with X-ray film (X-Omat AR, Eastman Kodak, Rochester, NY).

In situ hybridization

Cryostat sections (7 μm) were processed for *in situ* hybridization essentially following the procedures described by Richardson *et al.* (1991) and Sandberg and Vuorio (1987). Briefly, sections were fixed in 4% paraformaldehyde-PBS, passed through a graded series of alcohols and then treated with 1 μg proteinase K ml^{-1} for 5 min. The reaction was stopped by washing with 2 mg glycine ml^{-1} in PBS and the tissue was then acetylated with 0.1 mol acetic anhydride l^{-1} in 0.1 mol triethanolamine buffer l^{-1} , pH 8.0, to minimize non-specific binding of the probe. The sections were washed with PBS, dehydrated and air dried.

Treated sections were incubated for 1 h at 55°C in pre-hybridization mix (1.2 mol NaCl l^{-1} , 20 mmol Tris-HCl l^{-1} , pH 7.5, 4 mmol EDTA l^{-1} , 2 $\times$ Denhardt's solution, 1 mg yeast tRNA ml^{-1}) containing 50% formamide. The probe (3 $\times 10^6$ c.p.m.) in prehybridization mix containing 10% dextran sulfate was added to each slide and incubated at 55°C overnight. After hybridization, the slides were washed for 1 h with a high salt solution of 2 $\times$ SSC (20 $\times$ SSC = 175.3 g NaCl and 88.2 g sodium citrate in 1 l, pH adjusted to 7.0) containing 50% formamide and 1% 2 β -mercaptoethanol, treated for 30 min at 37°C with 20 μg RNase A ml^{-1} , and rewashed twice for 15 min each time with the high salt solution. This was

Fig. 4. Immunocytochemical localization of $G\alpha$, $G\beta$, $Gi1/2$, and $Gi3$ in cultured (S) Sertoli cells and (M) peritubular myoid cells, and in (P) purified preparations of pachytene spermatocytes, and (T) round spermatids using specific rabbit anti-G-protein antisera or normal rabbit serum (NRS).

followed by two further washes in more stringent conditions ($0.1 \times$ SSC, 0.5% 2 β -mercaptoethanol) and dehydration with alcohol. Autoradiography was performed by dipping the slides in Kodak NTB-2 (Eastman Kodak, Rochester, NY) and exposing them for 4–7 days at 4°C. The slides were developed and then counterstained with haematoxylin.

Results

Immunocytochemistry and immunoblotting of $G\alpha$ in testis

The anti-G protein polyclonal antisera used in these studies were generated in rabbits against the carboxy terminus of the

proteins. These antibodies were used to detect the presence of G proteins in different developmental stages of rat testis (Fig. 2). $G\alpha$ was not detected in testes of 1-day-old rats, but could be seen in 7-day-old animals and appeared to increase in the 12-day- and 23-day-old animals. The staining was observed mainly at the periphery of the tubules presumably at the basal plasma membranes of Sertoli cells and peritubular cells. Staining was also present in the interstitial tissue of the testis. In 45-day-old and adult (60–90 days of age) rats, the intensity of fluorescence was low (if present at all) and was located at the periphery of the tubules in discrete thin lines. Some nonspecific staining was present in testes of 45-day-old and adult rats that could be attributed to nonspecific antibody binding to the acrosomes of the spermatids, as this was also seen in testes of the same

Fig. 5. Northern blot analysis of total RNA of rat testes using antisense and sense probes for the 268 bp fragment of *Gsa*. Samples were prepared from (A) 1-, (B) 7-, (C) 12-, (D) 23-, (E) 45-, and (F) 80-day-old rats. Panels G–L show a lack of hybridization in the sense controls at the same ages. Photographs of the RNA gels, stained with ethidium bromide, are shown in the upper panels.

developmental age stained with normal rabbit sera (see Fig. 2). The anti-G β antiserum gave a pattern of staining similar to that of G α for testes of 1-, 7- and 12-day-old rats, but in the older animals (23–90 days) staining was also present in the plasma membranes of cells within the seminiferous tubules. The Gi1/2 antiserum stained the testes of all rats investigated, but the pattern of the staining changed from being diffuse peripheral in 1-, 7- and 12-day-old animals to intratubular in testes of 23-day-old rats and intense germ cell staining in testes of 45-day-old and adult rats. Similarly, the Gi3 antiserum intensely stained germ cells in testes of 45-day-old and adult rats, but it was not consistently seen in the younger animals, suggesting a very low expression of the Gi3 protein.

Immunoblot analyses of testicular plasma membranes obtained from either 12-day-old (Fig. 3) or adult (60–90 days of age) rats (data not shown) indicate that the antisera used in this study recognize proteins of molecular weights identical to those described in other systems (Birnbaumer *et al.*, 1987). The anti-G α antiserum recognized two bands of 42 and 46 kDa (Fig. 3b); the anti-G β antiserum recognized a 35 kDa protein (Fig. 3c); antisera against Gi1/2 and Gi3 stained proteins of about 40 kDa (Fig. 3d and e, respectively).

To examine further the presence of G proteins in testicular cells, we isolated Sertoli cells and peritubular myoid cells from 15-day-old rats and pachytene spermatocytes and round spermatids from 90-day-old rats and stained them with the anti-G protein antisera (Fig. 4). Both Sertoli cells and peritubular myoid cells showed immunoreactivity for all the G proteins

investigated (Fig. 4, A2–A5 and B2–B5). The intensity of Sertoli cell staining was greater than that seen in the myoid cells. Pachytene spermatocytes and round spermatids were not stained by anti-G α antiserum (Fig. 4, C2 and D2) but showed staining with anti-G β , -Gi1/2 and -Gi3 antisera (Fig. 4, C3–C5 and D3–D5).

RNA (northern) analysis for *Gsa*

The results of the immunohistochemical studies indicated that differential expression of G proteins occurs in the testis. Northern blot analysis was performed on RNA from testis with a riboprobe for G α to investigate whether this takes place at the transcriptional or the translational level. Northern blot analysis of total RNA probed with both antisense and sense probes for a 268 bp segment of G α is shown (Fig. 5). G α mRNA is present from day 1 to adult (Fig. 5A–F), but a marked decrease is observed after 23 days (Fig. 5D–F). The corresponding sense probe showed no hybridization to the RNA (Fig. 5G–L). Another smaller fragment from the 3' end of the G α full-length clone was used to probe the RNA, and similar results were obtained (data not shown).

In situ hybridization with *Gsa* RNA probe

Localization of G α RNA in different developmental stages of testis by *in situ* hybridization is shown (Fig. 6) as seen under

Fig. 6. Localization of the $G\alpha$ RNA in testes from rats at different ages by *in situ* hybridization. (A) Phase microscopy and (B) dark field microscopy are shown. The arrowhead at day 12 indicates the tunica albuginea. Arrows at day 23 and adult indicate the intense labelling of interstitial cells and blood vessels.

dark field microscopy and under phase microscopy. In the younger animals, day 1–12, mRNA for $G\alpha$ can be seen throughout the whole testis. In 1-day-old testis, no specific pattern of hybridization is evident, but in 7- and 12-day-old testis, a pattern begins to emerge. A ring-like pattern of hybridization can be seen with transcripts located both in the centre of the tubules, possibly corresponding to the cytoplasm of the Sertoli cells, and in the interstitium. The tunica albuginea is also labelled (Fig. 6b, 12 days). At days 23 and 45 of age and in adults (60–90 days), mRNA for $G\alpha$ appeared to be preferen-

tially localized to the periphery of the tubules and very little or no hybridization was seen within the lumen. This change in localization of the mRNA parallels the morphological differentiation of cells occurring with age in the seminiferous tubule. The Sertoli and myoid cells gradually occupy a more narrow band around the periphery of the tubule, while proliferation of the spermatogonia results in an increased proportion of the tubule being occupied by the germ cells. The intensity of the hybridization signal decreased with age. Interstitial cells and blood vessels were labelled at all ages.

Discussion

The signal transduction mechanisms regulating germ-cell differentiation remain to be elucidated; however, it is likely that the guanine nucleotide-binding proteins (G proteins) are involved. Immunohistochemical studies indicated that the somatic testicular cells, as well as germ cells, express the inhibitory GTP-binding proteins, Gi1/2 and Gi3. These results confirm previous observations (Paulssen *et al.*, 1991), but differ in that these authors reported that pachytene spermatocytes and round spermatids were not immunoreactive for Gi1/2. However, our results are consistent with their findings that both types of cell contain significant mRNA transcripts for both subunits. In our study, germ cells were obtained from 90-day-old rats, whereas younger rats (30 days of age) may have been used by Paulssen and his colleagues (Paulssen *et al.*, 1991). From our results, germ cells begin to express Gi1/2 and Gi3 between days 23 and 45 after birth, and it is possible that the discrepancy in our findings could be attributed to the fact that at day 30, pachytene spermatocytes and round spermatids have yet to begin to express Gi1/2 in significant amounts. Karnik *et al.* (1992), in agreement with our results, also noted the presence of Gi proteins (Gi1, Gi2 and Gi3) in spermatogenic cells of the testes of 10–12-week-old mice. We did not study the distribution of G proteins in spermatogonia because of the difficulty in obtaining purified populations of these cells in rats. Examination of cross-sections of testes, however, revealed that there was no detectable staining of spermatogonia with either Gi1/2 or Gi3, although inhibitory G proteins have been shown to be involved in growth factor-induced mitotic division in Balb/c 3T3 cells (Grouch, 1991). The physiological role of G proteins in cell types that were thought to lack G protein-coupled receptors led to speculations about the role of paracrine factors in the regulation of effector systems. However, a cDNA clone encoding a putative G protein coupled receptor has recently been isolated from a rat testis cDNA library (Meyerhof *et al.*, 1991). *In situ* hybridization experiments using a cDNA probe localized the mRNA for this novel receptor to spermatocytes and spermatids but not to somatic cells or spermatogonia (Meyerhof *et al.*, 1991). In addition, expression of this mRNA is not seen at day 14 *post partum* but is first detected after day 28 and increases during the second and third months after birth. Although these observations suggest the presence of a G protein-coupled receptor, for a yet unknown ligand, in different types of germ cell, a role of G protein-mediated signal transduction induced by GTPase-activating proteins (Skene, 1989) in germ cells cannot be excluded.

Although Sertoli and myoid cells could readily be stained for Gi1/2 and Gi3 in the isolated cells, they showed only very low levels, or no expression, in sections taken from testes of rats of corresponding age. This could be explained by the fact that, although the cells do express the protein, the levels are not high enough to be detected in cross-sections of the testis, in which there may be low cell volume (Russell *et al.*, 1990). As expected, G β is seen in all types of cell examined. In younger animals, the immunoreactivity is located mainly in the somatic cells and upon further development, immunoreactivity becomes evident in the cytoplasm of the germ cells at about day 23 after birth. At this time, the germ cells have yet to express the inhibitory G proteins. G α was found in both Sertoli and myoid cells but not

in either type of germ cell examined; this finding is consistent with the view that germ cell membranes do not contain a cholera toxin-activated G protein (Hildebrandt *et al.*, 1985) and do not possess a hormone-sensitive adenylyl cyclase system (Haugen *et al.*, 1990).

G α is not expressed at the protein level in testes of 1-day-old rats, despite the large amounts of transcripts produced, as shown by northern blot analysis and *in situ* hybridization. As we have previously shown (Dym *et al.*, 1991), the immunostaining of G α appears to be located at the periphery of the seminiferous tubules, where the peritubular cells and the basal portion of Sertoli cells are in contact with the basement membrane (Hadley and Dym, 1987). Sertoli cell FSH receptors are found at the base of the seminiferous tubules (Orth and Christensen, 1977) and it is at this location that they are coupled to G α . The apparent peak in G α production in Sertoli cells, which occurs between day 12 and day 23, and its subsequent decrease in testes of 45-day-old and adult rats, could be attributed to the fact that Sertoli cells become insensitive to FSH stimulation via adenylyl cyclase in older animals. Several studies have shown that both *in vivo* and *in vitro*, before 20 days of age in rats, FSH increased DNA synthesis and mitotic activity (Griswold *et al.*, 1976; Means *et al.*, 1980). This response decreases with age and is not present in adults (Griswold *et al.*, 1976; Means *et al.*, 1980). Similar modulation of Gi1/2 and Gi3 in rat myometrium linked to hormonal status during gestation has been reported by Tanfin *et al.* (1991). Another explanation may be that there is differential expression of the two subunits of G α . The immunoblots showed that the antisera used in this study preferentially recognized the larger 46 000 subunit, although a minor faint 42 000 immunoreactive protein could also be seen. However, we have previously shown, using a different antiserum, that Sertoli cells strongly express both the 42 and 46 kDa Gs proteins (Dym *et al.*, 1991). To study this developmentally regulated expression of the G α protein further, we carried out northern blot analysis using total RNA from testes of rats of different ages. These studies were designed to determine whether the changes in protein concentrations were due to changes in the amount of mRNA encoding the protein. An RNA species migrating at 1.85 kb was noted, but the 0.9 kb species found in round and elongated spermatids (Gordeladze *et al.*, 1981; Eskild *et al.*, 1991) could not be detected. The absence of the latter may be due to the fact that total RNA from whole testis was used as opposed to RNA from individual types of cell. The results showed that there is a decrease in the amount of G α mRNA with age, but this could be due to the dilution factor of increased RNA contribution from types of germ cell that do not contain the mRNA for G α (Paulssen *et al.*, 1991).

In situ hybridization confirmed the results obtained by RNA (northern) analyses that indicated that the 1.85 kb transcripts for G α were present in all the different developmental ages examined. In 1-day-old rats, the mRNA was distributed throughout the whole testis and the intensity of hybridization supported the northern blot findings that large amounts of mRNA for G α are produced. Again, comparison with the immunohistochemistry studies, in which we were unable to detect the presence of G α protein, suggests that the expression of G α is regulated at the level of translation. In 7- and 12-day-old testis, when the seminiferous tubules are still mainly

composed of Sertoli cells, Gs hybridization was seen in the area of the tubule corresponding to the cytoplasmic portion of the Sertoli cells. As the germ cells in the seminiferous tubule undergo differentiation, the somatic cells become more confined to the periphery of the tubule and, correspondingly, hybridization is seen around the periphery of the tubule, and in the interstitium, but is absent in the germ cells. Although *in situ* hybridization is not a quantitative technique, there appears to be a decrease in G α mRNA in the somatic cells with age, suggesting that some modulation at the transcriptional level may also occur. The labelling of the tunica albuginea and blood vessels confirms the presence of G α in fibroblasts and endothelial cells as well as smooth muscle (Birnbaumer *et al.*, 1990).

In conclusion, in the present study, we have shown differential expression of G proteins in developing rat testes and it is possible that these G proteins are involved in the signal transduction mechanisms regulating spermatogenesis. Both transcriptional and translational regulation appear to be involved in this process, but the precise nature of these mechanisms remains to be investigated.

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