Mouse GGN1 and GGN3, Two Germ Cell-specific Proteins from the Single Gene Ggn, Interact with Mouse POG and Play a Role in Spermatogenesis

Baisong Lu‡§ and Colin E. Bishop‡¶

From the ‡Department of Obstetrics and Gynecology and ¶Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas 77030

The germ cell-deficient (gcd) mutation is a recessive transgenic insertional mutation leading to a deficiency of primordial germ cells (PGCs). We have recently shown that the gene underlying this mutation is Pog, which is necessary for normal proliferation of PGCs. Here we show that Pog is also involved in spermatogenesis in that meiosis is impaired in Pog-deficient mice. Yeast two-hybrid screening revealed that POG interacted with GGN1 and GGN3, two proteins formed by alternate splicing of the same gene (Ggn). Ggn had more than 10 different splice variants giving rise to three proteins, GGN1, GGN2, and GGN3. The three proteins had different subcellular localizations, with GGN1, GGN2, and GGN3 localized along the nuclear membrane, in the cytoplasm, and in the nucleus/nucleoli respectively. The expression of Ggn was confined to late pachytene spermatocytes and round spermatids, a time window concomitant with the occurrence of meiosis. Mouse Ggn and Pog were both expressed in primary spermatocytes. Co-expression of POG with GGN1 or GGN3 in HeLa cells changed the localization of POG to the perinuclear localization or the nucleoli, respectively. Our data showed that in addition to functioning in proliferation of primordial germ cells, POG also functioned in spermatogenesis. Two spatial and temporal regulated proteins, GGN1 and GGN3, interacted with POG, regulated the localization of POG, and played a role in spermatogenesis.

Mouse primordial germ cells (PGCs) are generated ~6 days after fertilization by the epiblast cells along the border of the epiblast and extraembryonic ectoderm (1). Bmp4 and Bmp8 secreted by the neighboring extraembryonic ectoderm cells are two important signals for this induction (2, 3). Thereafter, the PGCs move out of the epiblast, and at 7.5–8 days postcoitum (dpc) they are at the base of the allantois as a pool of about 100 tissue-nonspecific alkaline phosphatase-positive cells (4). A process of proliferation and migration of the PGCs is followed until, at 13.5 dpc, they number about 20,000 in each gonad (5). Several genes are known to function in this process. Mutations in Kit and its ligand Kitl affect the proliferation and migration of PGCs (6–8), deletion of Tial1 (mTLAR) affects their survival (9), and Itgb1 (integrin β1) and Cdhl (E-cadherin) function in migration (10, 11).

After 13.5 dpc, germ cells in the male and female take different developmental pathways (12). In the male, PGCs are arrested in mitosis at 13.5 dpc. They resume mitosis after birth, the prospermatogonia, establish a stem cell pool, and start spermatogenesis (13). In the female, the germ cells enter meiosis and are arrested at the diplotene stage of meiosis I, 5 days after birth. Following a growth period, the oocytes resume meiosis at puberty and arrest again at metaphase II. Only after fertilization do the oocytes complete the meiosis. Unlike in the male line, female germ cells do not form a self-renewing stem cell population. They exist as a finite population, the number of which is fixed at birth.

The prophase of meiosis I in both sexes is notably long, which includes stages of leptotene, zygotene, pachytene, and diplotene (13). In the prophase, a series of meiosis-unique events take place. For example, chromosomal axes begin to condense in leptotene, chromosome pairing and synapsis initiate in zygonema, synopsis completion and genetic recombination occur in patchnema, and desynapase happens in diplonema (14). A number of meiosis-specific proteins are known to express during this period, these including the synaptonemal complex proteins (SYCP1 to -3) (15), germ cell-specific transcription factor SPRM1, and germ cell-specific histone protein H1t (H1Ft) (16, 17). Identifying all of the players in the process is necessary for the better understanding of meiosis.

The germ cell-deficient (gcd) mutant is a transgenic insertional mutation showing a reduced number of PGCs in genital ridges of homozygotes from 9.5 dpc (18–20). Recently, we have shown that the gene responsible for this phenotype is Pog (proliferation of germ cells), which encodes a novel protein containing a plant homeodomain (PHD) motif at its C terminus (19). During the embryonic stage, Pog is involved in proliferation but not migration of PGCs. It is also involved in other aspects of the embryonic development, since in certain genetic backgrounds deletion of Pog leads to lower embryonic viability. Further investigation of the gametogenesis of Pog-deficient mice revealed that Pog-deficient females were sterile throughout their lives, whereas Pog-deficient males eventually became fertile at the age of 3–4 months due to the population of the tubules with spermatogonial stem cells and the resumption of
the spermatogenesis (21). Here we show that in Pog–/− males, although spermatogenesis is qualitatively normal after age 9–12 weeks, it is abnormal quantitatively. Pog–/− testes have a lower percentage of primary spermatocytes and round spermatids than normal controls, although they have the same percentage of proliferating spermatogonia. The impaired transition from the primary spermatocytes to spermatids in Pog–/− testis indicates that Pog is also involved in spermatogenesis in adult testis.

In an attempt to identify interacting protein partners of POG in the testis, we carried out yeast two-hybrid screening using an adult testis cDNA library. A novel germ cell-specific gene, gametogenin (Ggn), was found to encode proteins interacting with POG. Consistent with the functioning of Pog in spermatogenesis, Ggn was highly expressed in the adult gonad, specifically in germ cells from the late pachytene spermatocyte to the round spermatid stage. Single spermatocyte RT-PCR showed that Ggn and Pog were both expressed in the primary spermatocytes. Multiple splicing of Ggn pre-mRNA gave rise to at least three different proteins, GGN1, GGN2, and GGN3, showing a perinuclear, cytoplasmic, and nuclear localization, respectively. When POG was co-expressed with GGN3 in HeLa cells, the localization of POG switched from an ubiquitous intracellular localization to an essential nuclear specific localization, whereas when POG was co-expressed with GGN1, it changed to a perinuclear localization. Our data suggested that Pog also functioned in spermatogenesis. GGN1 and GGN3, two proteins produced from Ggn by alternative splicing, interacted with POG and regulated its subcellular localization.

**EXPERIMENTAL PROCEDURES**

**Testis Histology—**Generation of Pog-deficient mice has been previously described (19). Tissues were fixed overnight at 4 °C in either 4% paraformaldehyde or Bouin’s solution and were dehydrated, embedded in Paraplast X-tra (Fisher). 8-μm sections were stained with hematoxylin/eosin or periodic acid-Schiff reagent. Reuss’s system was adopted for staging of the seminiferous tubules (13).

**Flow Cytometry Analysis of Testicular Cells—**A monocellular suspension of testicular cells was prepared as described (22). Briefly, the tunica albuginea was removed, and the seminiferous tubules were minced in PBS (calcium- and magnesium-free) to release the testicular cells. The minced tissue was gently aspirated for 2 min, and the cells were washed in PBS and spun down at 800 × g for 10 min. The cells were resuspended in PBS, filtered through 80-μm nylon mesh, fixed in cold 70% ethanol, and kept at 4 °C until further analysis. For propidium iodide staining, 2 × 10^6 cells were washed twice with PBS and incubated in 300 μl of 0.5% pepsin in 0.9% saline, pH 2.0, at 37 °C for 10 min. After spinning down, the cells were incubated with propidium iodide staining solution (25 μg/ml propidium iodide, 40 μg/ml RNase, 0.3% Nonidet P-40), 2 ml of RNase inhibitor (Invitrogen), 1.33 ml of 10 mmol/liter Tris, pH 8.0) and 0.67 μl of 50 μm random decamers (Ambion, Austin, TX). The lysate was incubated at 37 °C for 3 min and cooled at room temperature for 3 min. 4 μl of lysis from each cell was incubated with 0.5 μl of reverse transcriptase at 37 °C for 30 min, and the other 4 μl of lysis from the same cell was similarly treated without reverse transcriptase to serve as a control. 1.5 μl of the RT product (with or without reverse transcriptase) was used as the template for both Ggn and Pog PCR. Primers for Pog PCR were GCCD2F (5′-TCAACACAGAAATGAGGACTC-3′) and SplicingR (5′-CAGATTCCACAGCTCCTAGT-3′). Primers for Ggn PCR were 215F (5′-GAGCTAGATCTGATTTGTCG-3′) and 481R (5′-ATTGATGTTGCTGCGGATG-3′). PCR was performed using the following cycle profile: one cycle for 4 min at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. For Ggn, the first round PCR product was used for electrophoresis. For Pog, a second round of PCR (30 cycles) was performed using the product of the first PCR as the template.

**Co-immunoprecipitation—**Full-length Pog cDNA was cloned in the EcoRI site of pC-INF (Clontech, CA) and pcDNA3.1His/C (Invitrogen, CA), to express the GAL4 DNA binding domain-tagged POG and Xpress-tagged POG in mammalian cells. Ggn1 cDNA was cloned in the EcoRI site of pcDNAsmyc/HisA to express Myc-tagged GGN1. 8 μg of plasmid DNA was transfected into 70% confluent COS-1 or HeLa cells (grown in 10-cm tissue culture dishes) using Fugene-6 (Roche Molecular Biochemicals). 60 h after transfection, the cells were washed with PBS and lysed with 0.8 ml of Nonidet P-40 lysis buffer (150 mmol/liter sodium chloride, 1.0% Nonidet P-40, 50 mmol/liter Tris, pH 8.0) including a protease inhibitor mixture (Roche Molecular Biochemicals) and 50 μg/ml phenylmethylsulfonyl fluoride. Anti-Myc antibody (Invitrogen) or anti-GAL4 DNA binding domain antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used in the precipitation. 300 μl of each clear lysate was incubated with the suitable antibody and 50 μl protein A/G beads in a volume of 600 μl at 4 °C for 2 h. The beads were spun down at 4 °C and washed three times with ice-cold PBS. The beads were then resuspended in 40 μl of SDS loading buffer and used for Western blotting analysis.

**Subcellular Localization—**pEGFP-C2 and pEGFP-N2 vectors (Clontech, Palo Alto, CA) were used to make green fluorescent protein (GFP) fusion proteins with the target proteins. In some cases both C-terminal and N-terminal fusion constructs were made to ensure the GFP did not interfere with the subcellular localization. The constructs were transfected into HeLa cells, COS-1 cells, or GC-1 cells (grown on glass cover slides). 36 h after transfection the cells were washed twice with PBS and fixed in 4% phosphofomonic acid at 4 °C for 30 min. The cells were mounted with Vectashield (Vector Laboratories, Inc.), and localization of the GFP signal was checked by confocal microscopy (Zeiss LSM 510). For checking the nuclear localization, the same field was examined under UV for the GFP signal and under bright field for the nucleus.

**In Vitro Hybridization—**The 3′ 300 bp of the Ggn1 cDNA coding region was amplified and cloned into the EcoRI site of pBluescript KS/I. Digoxigenin-labeled antisense and sense RNA probes were prepared using a digoxigenin RNA-labeling kit (Roche Molecular Biochemicals). Hybridization was carried out at 65 °C for 18 h. After subsequent washing, bound probe was detected by alkaline phosphatase-conjugated anti-digoxigenin antibody and BM purple (Roche Molecular Biochemicals). Serial sections were stained with periodic acid-Schiff and staged using Russell’s system to define the stage and cell type that express Ggn1.

**Single Primary Spermatocyte RT-PCR Analysis—**Testicular germ cell stages were dissociated as described above. Single primary spermatocytes were picked under a Leica microscope by virtue of their large cell size. Single cell RT-PCR was performed as described (23) with modifications. Briefly, each cell was lysed in 8 μl of first strand cDNA synthesis buffer, which was made by mixing 96 μl of DNA/lysis buffer (1× alternate first strand buffer from the Ambion Reverse transcript kit, with 0.52% Nonidet P-40), 1.35 μl of 0.5 μg/ml phenylmethylsulfonyl fluoride, and 0.67 μl of 50 μm random decamers (Ambion, Austin, TX). The lysate was incubated at 65 °C for 3 min and cooled at room temperature for 3 min. 4 μl of lystate from each cell was incubated with 0.5 μl of reverse transcriptase at 37 °C for 30 min, and the other 4 μl of lystate from the same cell was similarly treated without reverse transcriptase to serve as a control. 1.5 μl of the RT product (with or without reverse transcriptase) was used as the template for both Ggn and Pog PCR. Primers for Pog PCR were GCCD2F (5′-TCAACACAGAAATGAGGACTC-3′) and SplicingR (5′-CAGATTCCACAGCTCCTAGT-3′). Primers for Ggn PCR were 215F (5′-GAGCTAGATCTGATTTGTCG-3′) and 481R (5′-ATTGATGTTGCTGCGGATG-3′). PCR was performed using the following cycle profile: one cycle for 4 min at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. For Ggn, the first round PCR product was used for electrophoresis. For Pog, a second round of PCR (30 cycles) was performed using the product of the first PCR as the template.

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RESULTS

Impaired Spermatogenesis in Pog-deficient Mice—Pog-deficient males eventually become fertile at 3–4 months of age due to the population of the tubules with spermatogonial stem cells and the resumption of spermatogenesis (21). Since Pog is highly expressed in the testis, we were interested in determining whether the spermatogenesis in Pog-deficient males was affected. A careful examination of periodic acid-Schiff stained testis sections revealed that although spermatogenesis appeared qualitatively normal in Pog−/− mice, in that four waves of spermatogenesis could be seen in all populated tubules, it was quantitatively abnormal. In Pog−/− testis, there were consistently fewer round spermatids in the seminiferous epithelium than stage-matched tubules from normal littersmates (Fig. 1, A and B).

Flow cytometry was used to quantitatively examine the germ cell populations in normal and Pog-deficient mice. Five populations could be distinguished according to the amount of propidium iodide the cells bound. They were elongating and elongated spermatid (HC, H indicating hypostainability of compacted DNA during spermiogenesis), round spermatid (1C), spermatogonia and somatic cells (2C), spermatogonia and preleptotene spermatocytes synthesizing DNA (S), and primary spermatocyte (4C). Since in the Pog-deficient mouse, the lumens of the seminiferous tubules were not completely normal, possibly affecting spermatiation, the elongating and elongated spermatids were not included in the comparison. The data clearly showed that Pog-deficient mice had a lower percentage of 1C (round spermatids) and 4C (majority primary spermatocytes) cells (Fig. 1C), whereas they had the same percentage of testicular cells in S phase and a higher percentage of 2C cells. The higher percentage of 2C cells in Pog-deficient mice could be the result of hyperproliferation of somatic cells in the testis. Since in the adult testis, germ cells are the only cell type to synthesize DNA, the 4C/S and 1C/S ratios were used to compare the spermatogenesis of Pog-deficient and normal mice. Both ratios were significantly lower in Pog-deficient mice (Fig. 1D), indicating that some primary spermatocytes are lost and that the transition from primary spermatocytes to round spermatids (meiosis) is impaired. These data indicate that Pog is involved in spermatogenesis in addition to its function in proliferation of primordial germ cells.

POG Interacts with GGN1 and GGN3 in Yeast Two-hybrid Screening—Since POG is a novel protein, little is known about its function in the cell. The yeast two-hybrid system was used to search for proteins interacting with POG in the adult testis in order to shed some light on its potential role. Since the presence of the POG C-terminal PHD domain in the bait caused autoactivation of the reporter gene upon transformation into AH109, the PHD domain-coding region was deleted from the bait construct and used to screen a pretransformed mouse adult testis cDNA library (Clontech). Four million clones were screened using SD-Trp-Leu-His selection, and six independent clones were identified and sequenced. Three matched the cDNA sequence of mouse RanbpM (24), and the other three matched the mouse Unigene Mm.63529. Further testing the interaction by co-transformation of the bait and pray plasmid into yeast AH109 revealed that interaction with RanbpM plasmids was nonspecific, whereas activation of the reporter gene by the other three plasmids was specific and dependent on the presence of POG. Since no homologous full-length cDNA sequences were found in the public data base, we used the primer extension strategy to get the complete coding region of the cDNA based on the genomic DNA sequence and the expressed sequence tag records in the data base. We named this gene gametogenetin (Ggn) because of its germ cell-specific expression and its involvement in gametogenesis (see below).

In RT-PCR experiments using several primer sets aligned to the 5′- and 3′-end of the mouse Ggn cDNA, we consistently obtained more than 10 DNA bands of different sizes from the testis and the ovary. Six major bands were sliced out of the gel and sequenced directly or after subcloning. Eight sequences were obtained (three sequences were from the smallest band after subcloning), showing all to be specific products of variant splicing of the Ggn pre-mRNA (Fig. 2A). Three related proteins could be deduced from the cDNAs sequenced and were named GGN1, GGN2, and GGN3. GGN1 has two potential transmembrane domains in the N terminus (amino acids 45–64) and the middle of the protein (amino acids 268–287) and two argi-
nine/lysine-rich domains at the C terminus (Fig. 2B). GGN3 has the C-terminal 137 amino acids of GGN1 together with the two arginine/lysine-rich domains. The Ensemble mouse genome assembly indicated that mouse Ggn is located on Chr 7 and is relatively small, spanning about 5 kb in the mouse genome.

Interaction between POG and GGN Proteins in Yeast and Mammalian Cells—The three clones obtained from the yeast two-hybrid screening all contained the cDNA coding the C-terminal part of GGN1, with the shortest coding the C-terminal 122 amino acids. Thus, both GGN1 and GGN3 contained the sequences mediating the interaction with POG in yeast. To further confirm the interaction between POG and GGN3 in yeast, the Pog cDNA and cDNA coding for GGN3 were switched between the DNA binding domain vector pGBKT7 and the activation domain vector pACT2. The resulting two constructs were co-transformed into yeast strain AH109, and the interaction was tested again. Only cells transformed with both pGBKT7/Ggn3 and pACT2/Pog survived the selection, whereas cells transformed with pGBKT7 and pACT2/Pog did not (Fig. 3A). These data indicate that the interaction between the POG and GGN3 proteins is specific in yeast.

Co-immunoprecipitation was used to further confirm the interaction in mammalian cells. In one direction, C-terminal Myc-tagged GGN1 co-immunoprecipitated with GAL4-tagged POG when the two proteins were co-expressed in COS-1 cells (Fig. 3B). In the other direction, Xpress-tagged POG co-immu-
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noprecipitated with Myc-tagged GGN1 when they were co-expressed in HeLa cells (Fig. 3C). These experiments provide further evidence that the two proteins interact specifically in mammalian cells.

To determine which part of POG mediated the interaction with GGN3, cDNA coding for amino acids 182–274 of POG was tested in the yeast two-hybrid system and was found to be sufficient to mediate the interaction (Fig. 3A). Using a similar strategy, it could be shown that the C-terminal 65 amino acids of GGN3 was not able to mediate the interaction. Sequences further N-terminal to this region were therefore needed for GGN3 to interact with POG.

In addition to binding to POG, GGN3 was able to form a homodimer with itself and a heterodimer with GGN1 in yeast two-hybrid experiments (Fig. 3D). The region mediating the homodimerization was mapped to the C-terminal 65 amino acids of GGN3, a region that could not mediate the interaction with POG. When pACT2/Ggn3 was co-transformed into AH109 cells with either pGBK7/Ggn1 or pGBK7/Ggn3, the transformed clones could grow on selection medium. In the case of pACT2/GgnC-65, only when it was co-transformed with pGBK7/Ggn3 but not pGBK7/Ggn1 could the cells survive the selection. It appears that although the C-terminal 65 amino acids of GGN3 are enough to mediate the interaction, the presence of the N-terminal sequences added further strength to the interaction. With the construct pGBK7/Ggn1, the larger protein size and the potential trans-membrane domain of GGN1 might limit the amount of protein getting into the nucleus; thus, a stronger protein-protein interaction was needed to activate the reporter gene.

GGn and Pog Were Both Expressed in Primary Spermatocytes—The expression of Ggn in the adult tissues was first analyzed by RT-PCR. A specific product could only be amplified from testis and ovary RNA but not from adult liver, kidney, lung, heart, spleen, or brain (data not shown). In situ hybridization was used to check the cell type and stage that expressed Ggn in the adult testis using a probe containing 300 bp of the 3′ coding region, present in all splice variants. Ggn was expressed only in the germ cells and not in the somatic, Sertoli, or Leydig cells (Fig. 4A). In the germ cells, Ggn expression is tightly related to the developmental stage. The expression started in stage VIII pachytene spermatocytes (Fig. 4, B and C), increased in stage IX and X pachytene spermatocytes, and culminated in stage XI diplotene spermatocytes and the meiotic cells in stage XII (Fig. 4, D and E). Expression decreased slightly in step 1–3 spermatids, further decreased in step 4–11 spermatids, and was no longer detectable in step 12 spermatids and beyond (Fig. 4E). The expression of Ggn in the testis is summarized in Fig. 4G.

Consistent with the expression pattern in the adult testis, the expression of Ggn in the developing postnatal gonad is also developmentally regulated. It is not expressed in 6-day-old testes in which the germ cells are almost exclusively spermatogonia or 14-day testis in which the most advanced germ cells are early pachytene spermatocytes. However, it is expressed in 21-day testis tubules containing late pachytene spermatocytes or spermatids (Fig. 4F). Thus, in the postnatal male testis, Ggn expression is strictly confined to late pachytene spermatocytes through spermatids, a time during which meiosis takes place.
Pog has been shown to expressed in the adult testis and the ovary (19), although it is not known which individual cell types express Pog. For unknown reasons, we have been unable to obtain a Pog antibody suitable for immunohistochemistry; nor have we been able to obtain clear in situ hybridization data using several different Pog probes and multiple different techniques. Thus, to test whether Pog was also expressed in the same cell type that expressed Ggn, we picked single primary spermatocytes by virtue of their morphology (large cell size) and performed single cell RT-PCR. As shown in Fig. 4H, three cells (cell numbers 2, 3, and 5) were positive for both Ggn and Pog, which indicated that Ggn and Pog are expressed in primary spermatocytes. From the sequenced splicing variations shown in Fig. 2, three DNA products of 922, 600, and 452 bp would be expected from the testis cDNA using primer pair 215F and 481R for PCR, which was also confirmed in our experiments (data not shown). In primary spermatocytes, only the 600- and 452-bp products could be amplified from the primary spermatocytes. This suggested that either the splicing of Ggn in 4N, 2N, and 1N germ cells was regulated or the 922-bp product was inefficiently amplified due to the longer template in 4N, 2N, and 1N germ cells was regulated or the 922-bp product could be amplified from the primary spermatocytes. From the sequenced splicing variations, which indicated that Ggn and Pog are both expressed in primary spermatocytes, we performed in situ hybridization with Ggn probe on Pog−/− testis sections to see whether there was any change in Ggn expression compared with the expression in normal testis sections. No difference was noticed between normal and Pog−/− testis sections in terms of Ggn expression (data not shown), which indicated that deletion of Pog did not affect the expression of Ggn.

GGN1, GGN2, and GGN3 Had Different Subcellular Localization—In the absence of suitable antibodies, we used EGFP (green fluorescent protein) fusion proteins to determine the subcellular localization of GGN1−3 and POG in vitro transfected HeLa cells. GGN1 was localized to the perinuclear region when it was fused to either the C terminus or the N terminus of EGFP (Fig. 5B). The same localization was found when the protein was transiently expressed in HeLa, COS-1, or GC-1 cells (a germ cell line resembling type B spermatogonia). When the C terminus of GGN1 containing the two nucleolar targeting signals (NTS) was deleted, the protein was confined to the cytoplasm (Fig. 5C).

GGN2 shares the N-terminal 217 amino acids with GGN1. When GGN2 was fused to the N terminus of GFP, the fusion protein was found to localize exclusively to the cytoplasm (Fig. 5D), consistent with the cytoplasmic localization of GGN1 after the two arginine/lysine-rich domains were deleted.

A GFP-GGN3 fusion protein was found exclusively in the nucleus, with the majority of the protein accumulating in the nucleoli (Fig. 5, E–G). GGN3 also had two arginine/lysine-rich domains. Similar domains have been found in other nucleoli-located proteins such as TERF, FGF3, and TAT. All of these sequences contain NTSs (25), which have the ability to target the protein to the nucleus and nucleolus.

GGN1 and GGN3 Determined the Localization of POG in Mammalian Cells—POG was an intracellular protein; it localized to both the cytoplasm and the nucleus whether it was fused to a short Xpress tag or the N or C terminus of EGFP (Fig. 6A and data not shown). Since GGN1 and GGN3 were localized to the perinuclear region and the nucleoli, respectively, we wanted to determine what would happen if POG was co-expressed with GGN1 or GGN3 in HeLa cells. When EGFP-POG and HA-GGN3 were co-expressed, EGFP-POG changed from a ubiquitous intracellular localization to a nucleolar specific localization in some cells (Fig. 6B). Depending on the ratio and the amount of the two plasmids used, it was possible that only a part of the transfected cells harbored both plasmids and that the cells showing nucleolar specific GFP signal expressed only EGFP-POG and HA-GGN3, whereas the cells showing a ubiquitous intracellular GFP signal expressed only EGFP-POG. To confirm this hypothesis and to check whether POG and GGN3 co-localized in the cell, we stained HA-GGN3 with anti-HA antibody. After treating the cells with 0.1% Triton X-100, the GFP signal from the cells showing ubiquitous distribution disappeared, indicating that EGFP-POG was a soluble protein and could not withstand the treatment. The GFP signal from cells showing nucleolar specific distribution persisted (Fig. 6C). These cells expressed HA-GGN3, and the HA-GGN3 signal colocalized with EGFP-POG (Fig. 6, D and E). These data strongly supported the view that the two proteins interacted with each other.

EGFP-GGN1 was similarly co-expressed with Xpress-tagged POG. In this experiment, POG showed a perinuclear localization similar to that of GGN1 and co-localized with GGN1 (Fig. 6, F–H). Thus, GGN1 and GGN3 determined the subcellular localization of POG. The ability of GGN1 and GGN3 to change the localization of POG in the cell suggested that the interaction between POG and GGN1/GGN3 might serve as a means to regulate the subcellular localization of POG and thus regulate the activity of POG in the cell.

Fig. 5. Subcellular localization of GGN proteins. A, control GFP was localized to the nucleus and the cytoplasm in HeLa cells. B, GFP-GGN1 fusion protein localized to the nuclear membrane. C, cytoplasmic localization of GFP-GGN1 after the C-terminal NTSs of GGN1 were deleted. D, cytoplasmic localization of GFP-GGN2 fusion protein. E–G, nucleus/nucleolar localization of GFP-GGN3. E, GFP-GGN3 was exclusively localized inside the nucleus stained with 4',6-diamidino-2-phenylindole. The fusion protein was enriched in the nucleoli (E) when the same cell was checked under bright field to visualize the nucleoli (G).
FIG. 6. GGN1 and GGN3 determined the localization of POG. A, EGFP-POG was localized to the cytoplasm and the nucleus in HeLa cells. B, EGFP-POG showed nuclear specific localization upon co-expression with HA-GGN3 (pointed arrows). C, EGFP-POG signal in the nucleoli resisted the 0.1% Triton X-100 treatment. D, rhodamine signal representing HA-GGN3 from the same field as C. E, merging of C and D indicated the co-localization of EGFP-POG and HA-GGN3. F, green fluorescence signal of EGFP-GGN1 co-expressed with Xpress-tagged POG. G, the same field as in F revealing the localization of POG stained by anti-Xpress antibody. H, merging of F and G, showing the co-localization of POG and GGN1.

DISCUSSION

Here we have described a novel function in gametogenesis for Pog, a gene previously shown to underlie the germ cell-deficient mutation, gcd, and to be involved in the proliferation of primordial germ cells (19). We have also described the identification and characterization of a novel germ cell-specific gene Ggn, which is involved in gametogenesis and encodes several proteins that interact with POG.

We have recently shown that Pog is necessary for primordial germ cell proliferation (19). Here we further show that Pog is also involved in gametogenesis in adulthood. We show that spermatogenesis in Pog−/− testis is qualitatively normal but quantitatively abnormal. The lower 4C/S and 1C/S ratios in Pog−/− testis suggested a deficiency in the differentiation of the primary spermatocytes and round spermatids. Thus, in the Pog−/− male, in addition to PGC deficiency, spermatogenesis is also impaired. Since Ggn and Pog are expressed in the ovary, it is possible that they may also function in oogenesis. However, Pog-deficient females have very few oogonia at birth, and since females do not form a stem cell population from which late onset population can occur, the role of Pog in the female could not be directly addressed. Female sl1271/sl1731 (Kitl mutant) mice have about 6% of the PGC of normal mice, and they are fertile (26). The fact that Pog−/− females have a similar degree of PGC deficiency (data not shown) but are infertile is consistent with Pog−/− females having an additional defect in oogenesis and that Pog may play a role in oogenesis in addition to functioning in PGC development.

POG was shown here to interact with GGN1 and GGN3, two proteins from a single germ cell-specific gene, Ggn. The germ cell-specific expression of Ggn, especially its spatio-temporal regulated expression in the developing and adult testis, strongly suggested that Ggn could be involved in gametogenesis. Furthermore, the expression of the two genes in primary spermatocytes, the interaction between POG and GGN1/ GGN3, the change in the localization of POG upon interaction with GGN1 and GGN3, and the coincidence of the time window of Ggn expression with the occurrence of the lesion in gametogenesis of Pog−/− mice all suggest that the two genes are involved in this process.

How the interaction between POG and GGN1/GGN3 affects gametogenesis is not known at present. POG and GGN1/GGN3 are novel proteins, and the biochemical pathways they are involved in remain to be identified. POG is a PHD domain-containing protein and may have the ability to interact with chromatin to exert its activity (27). Without the co-expression of GGN1 or GGN3, POG is distributed in both the cytoplasm and the nucleus in transfected HeLa cells. While in the presence of GGN1 or GGN3, POG is localized near the nuclear membrane or in the nucleoli, respectively. Whereas this is the result from co-transfected HeLa cells, it is likely that the same process exists in germ cells in the testis, since the two genes are both expressed in some of the germ cells. Thus, the interaction between GGN1/GGN3 and POG may serve as a means to regulate the localization and thus the activity of POG.

Ggn spans only about 5 kb of genomic DNA, but it has more than 10 different splice variants and generates multiple proteins with different subcellular localizations. Variant splicing can be found in about 40% of the human genes, particularly in the testis (28, 29). As an extreme example, cAMP-response element-binding protein (CREB) and cAMP-response element mediator (CREM) are known to have more than 20 isoforms resulting from multiple promoters, alternative polyadenylation, and multiple alternative splicing (29). Ggn is another unusual example of a small testis-specific gene with many (>10) different splice variants. At least three different proteins with different subcellular localizations are produced from the same Ggn gene. GGN1 is a perinuclear protein; GGN2 is localized in the cytoplasm, and GGN3 is confined to the nucleus/nucleoli. Since Ggn is expressed in 4N primary spermatocytes, 2N secondary spermatocytes, and 1N spermatids, it is possible that the splicing is regulated and that the production of GGN1
and GGN3 is related to the stage of the germ cells. Thus, the localization of POG in the cell would be determined by the availability and the amount of GGN1 and GGN3.

GGN1 has two trans-membrane domains and two nucleolar-targeting signals. The additional findings that the N-terminal portion of GGN1 localized to the cytoplasm and the C-terminal part of GGN1 to the nucleus suggested that GGN1 could be a nuclear membrane protein. The topology of GGN1 is that the N terminus is cytoplasmic, the C terminus containing the two arginine/lysine-rich domains is in the nucleus, and the two trans-membrane domains span the two layers of the nuclear membrane. Since in the testis Ggn has its highest expression level in diplotele spermatocytes and meiotic germ cells, where the nuclear membrane breaks down and the nucleolus is disorganized (30), the fate and role of GGN1 and GGN3 during this process will be intriguing. We have recently found another novel gene, Ggnbp, showing a similar testis expression profile as Ggn and encoding a protein interacting specifically with the N terminus of GGN1.2 Thus, GGNBP, GGN1, and POG form a novel protein complex functioning in spermatogenesis.

In conclusion, we provide data to show that Pog is involved in gametogenesis in addition to functioning in PGC development. We have identified and characterized two germ cell-specific interacting proteins, GGN1 and GGN3, generated from Ggn by alternate splicing. In addition, we present data localizing these proteins in the cell and show that Pog and Ggn are both expressed in primary spermatocytes. Our work provides a basis for the eventual dissection of the biological functions of these proteins in regulating gametogenesis and human disease.

Acknowledgments—We thank Cavatina Truong for excellent technical assistance and Michael Mancini and David Stenoien for help with the confocal microscopy.

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B. Lu and C. E. Bishop, submitted for publication.
