Characterization of testicular mouse glucosamine 6-phosphate deaminase (GNPDA)

Markus Montag*, Katrin van der Ven, Christine Dörbecker, Hans van der Ven

Department of Endocrinology and Reproductive Medicine, University of Bonn, Sigmund-Freud-Str. 25, D-53105 Bonn, Germany

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Abstract Mammalian glucosamine 6-phosphate deaminase (GNPDA) was first detected in hamster spermatozoa. To further elucidate its role, we have cloned mouse GNPDA and produced a polyclonal rabbit anti-GNPDA antibody. This antibody recognized a 33 kDa protein in soluble extracts from mouse brain, liver, kidney, muscle, ovary, testis and sperm. Immunofluorescent analysis of the localization of GNPDA in male reproductive tissue revealed its presence in spermatids and in spermatozoa. In spermatids, GNPDA localized close to the developing acrosome vesicle and in spermatozoa close to the acrosomal region. Following the induction of the acrosome reaction, GNPDA fluorescence in spermatozoa was either reduced or GNPDA was absent. These data suggest that GNPDA might play a role in the acrosome reaction.

Key words: Spermatogenesis; Mouse; Glucosamine 6-phosphate deaminase; Acrosome

1. Introduction

An initial element of mammalian fertilization is the activation of the oocyte and this process is dependent on the sperm cell and its ability to induce intracellular Ca^{2+} oscillations [1]. It is now accepted that an oocyte activating capacity is present in sperm cytosol from a variety of species [2–5]. Recently, a cytosolic sperm factor from hamster spermatozoa was identified, which seemed to correlate with the ability of extracts to cause calcium oscillations and activation when injected into mouse oocytes [6]. This factor, named oscillin, showed a high sequence homology to bacterial glucosamine 6-phosphate deaminase (GNPDA). Initially only found to be present in hamster testis and spermatozoa [6], Wolosker et al. [7] reported a ubiquitous distribution of mammalian (rat) GNPDA by Northern and Western blot analysis. Further, these authors showed that recombinant as well as purified GNPDA failed to induce calcium oscillations when injected into mouse eggs and similar data were recently reported by others [8,9]. Thus the role of GNPDA in mammalian reproductive tissue is still under investigation.

This work aimed towards further understanding of the role of GNPDA in mouse spermatozoa. We cloned mouse GNPDA and produced anti-GNPDA antiserum which allowed the localization of GNPDA by immunofluorescence analysis in mouse testis and in spermatozoa before and after induction of the acrosome reaction.

*Corresponding author. Fax: (49) (228) 287 4651.
E-mail: m.montag@uni-bonn.de

2. Materials and methods

2.1. Cloning and sequencing of mouse GNPDA

A 550 bp GNPDA fragment was cloned from a mouse testis phage library (Stratagene, La Jolla, CA, USA) by polymerase chain reaction (PCR) using PCR primers designed according to published sequences [6] as follows: upstream primer, 5′-ACC TTC AAC ATG GAC GAG-3′; downstream primer, 5′-AGT CCA CAT ATG GTT CAC-3′. Screening by plaque-lift filter hybridization was performed with the PCR fragment labelled with digoxigenin by the random primed method (High prime kit, Boehringer Mannheim, Germany). Positive clones were harvested and rescreened twice. From the final screen we isolated five individual plaques which were treated with helper phage for excision of the clone in pBluescript. Restriction analysis showed that all cloned inserts had the same size and similar restriction patterns. One clone was chosen for nucleotide sequence analysis. An automated sequencer (A.L.F. Express DNA Sequencer, Amersham Pharmacia Biotech, Freiburg, Germany) and a cycle sequencing protocol (Cycle Sequencing Kit, Amersham Pharmacia Biotech) were used according to the manufacturer's recommendations and using primer-specific modifications. All sequences were reconfirmed by forward and reverse sequencing.

2.2. Production of polyclonal antibody

The 550 bp fragment used for screening the phage library was subcloned in frame into pThioA expression vector (His-Patch Thio-Fusion Expression System, Invitrogen, De Schelp, The Netherlands). Expression of a fusion protein was performed according to the instructions of the supplier. The fusion protein was eluted from preparative SDS gels using a mini whole gel eluter (Bio-Rad, Hercules, CA, USA). Eluted proteins were directly used for immunization of rabbits using Ribi adjuvant (Sigma, St. Louis, MO, USA). For each injection we used up to 1 mg of protein. A first booster injection followed 28 days after the start of the immunization and another 21 days later. Polyclonal serum was obtained by bleeding the rabbits 2 weeks after the final injection. The serum was affinity purified and the specificity was assessed by Western blot analysis.

2.3. Isolation of GNPDA from mouse tissues and injection into mouse oocytes

Mouse tissues were isolated, washed once in ice-cold phosphate buffered saline (PBS), pH 7.4 and cut into small pieces. Single pieces were immediately frozen in liquid nitrogen. Mouse spermatozoa were isolated from the epididymis, washed once in PBS, suspended at a concentration of 10^9/ml in PBS and frozen in 5–10 μl aliquots. For extraction of soluble proteins, tissue or sperm samples were subjected to three subsequent freeze-thaw cycles followed by centrifugation in a microfuge (Biofuge, Heraeus, Osterode, Germany) and the supernatants containing soluble proteins were used for injection experiments. The protein concentration was determined using a protein assay kit (Sigma). SDS-PAGE and Western blot analysis were carried out as described previously [10].

Stimulation, isolation of oocytes and denudation of oocyte cumulus complexes followed essentially the protocol described earlier [11], except that mice were not mated and isolation occurred at 14 h after application of human chorionic gonadotropin. For microinjection of mouse oocytes we applied the protocol given by Rybouchkin et al. [12] with the modification that we used HEPES-buffered medium (Gamete-100, Scandinavian IVF Sciences, Göteborg, Sweden) throughout the manipulation procedure. Micromanipulation was performed at an inverted microscope (DMIRB, Leica, Bensheim, Germany) equipped with micromanipulators (Narishige, Tokyo, Japan)

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and using commercial injection needles (Cook, Brisbane, Australia). The protein concentration of all injection solutions was adjusted to approximately 50 μg/μl. Activation of oocytes was judged from the presence of a second polar body and the formation of a female pronucleus within 5–7 h after injection [13].

2.4. Immunocytochemistry

Immunocytochemistry was performed on isolated mouse spermatocytes and spermatids and on capacitated and acrosome reacted mouse sperm. Mouse spermatocytes and spermatids were isolated from the testis essentially as described [14]. Cell suspensions were washed with PBS, smeared on clean slides and air dried prior to immunocytochemistry [10]. Slides were treated with primary antibody (polyclonal rabbit anti-GNPDA antibody diluted 1:300) or with pre-immune serum for 1 h. Primary antibodies were washed off with PBS and were detected using a fluorescein-conjugated goat anti-rabbit secondary antibody (1:100; Sigma). The preparations were washed again and the DNA was counter-stained with Hoechst 33342 (Sigma). Immunofluorescence microscopy was performed using an inverted microscope (DMIRB, Leica) equipped with a 100× fluotar objective and appropriate filter sets for UV and combined blue/green excitation.

2.5. Capacitation and acrosome reaction

Mouse spermatozoa were isolated from the epididymis [15]. Capacitation and acrosome reaction were performed as described [16]. In short, spermatozoa were capacitated after isolation by incubation in culture medium for 2–3 h at 37°C, 5% CO_2. The acrosome reaction was induced by the addition of calcium ionophore A23817 to 2 μM and incubation for another 30 min. For immunofluorescent analysis, capacitated and acrosome reacted spermatozoa were fixed as described above. The acrosome reaction was assessed with *Pisum sativum* lectin coupled to FITC (100 μg/ml in PBS; Sigma). Detection of GNPDA followed the protocol given above. For each evaluation at least 200 spermatozoa were examined.

For Western blot analysis, 20×10^6 spermatozoa were pelleted after capacitation and after the acrosome reaction. The culture medium supernatant was concentrated on 3K size exclusion filter to yield a minimum amount of medium (20 μl) which was used for SDS-PAGE. From the sperm pellet, soluble GNPDA was isolated as described above. SDS-PAGE and Western blot analysis were performed with equivalents of 20×10^6 spermatozoa and corresponding supernatant.

3. Results

3.1. Nucleotide and amino acid sequence of mouse GNPDA

The nucleotide sequence and the deduced amino acid sequence of the mouse GNPDA are shown in Fig. 1. The isolated cDNA clone was 2249 bp in length and contained an open reading frame starting from position 53 to 923. A comparison of mouse GNPDA based on published amino acid sequences revealed 14 amino acid changes to human and 10 to hamster GNPDA.

3.2. Presence and location of GNPDA

SDS-Page and Western blot analysis from soluble protein extracts prepared from mouse brain, kidney, liver, muscle, ovary, testis and sperm revealed the presence of soluble GNPDA protein in all examined mouse tissues. The size of the stained protein amounted to 33 kDa, except for brain tissue where an additional band occurred at 66 kDa probably due to protein dimerization (Fig. 2). The amount of GNPDA was much greater in tissues than in spermatozoa. We injected cytosol derived from mouse liver, testis or spermatozoa. Activation and pronuclear formation only occurred with cytosol from spermatozoa (11 oocytes activated out of 12 which survived the injection procedure; 92%) and from testis (8/12; 67%) but not with cytosol derived from liver (0/11; 0%).

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1    gggccgctagtgctgtagctgctgcagccccgggaagcctgcgagtctagcg ATG AAG CTC ATT ATC CTG GAA CAC TAT TCC CAG GCC AGT GAG
                                                          M   K   L   I   I   L   E   H   Y   S   Q   A   S   E
95   TGG GCG GCC AAG TAT ATT AGG AAC CGT ATC ATC CAG TTT AAC CCA GGG CCT GAC AAG TAC TTC ACC CTG GGG
     W   A   A   K   Y   I   R   N   R   I   I   Q   F   N   P   G   P   D   K   Y   F   T   L   G
167  CTC CCC ACT GGG AGC ACC CCG CTT GGC TGC TAC CAG AAG CTG ATT GAG TAC TAT AAG AAT GGG GAC CTG TCC
     L   P   T   G   S   T   P   L   G   C   Y   Q   K   L   I   E   Y   Y   K   N   G   D   L   S
239  TTT CAA TAT GTG AAA ACC TTC AAC ATG GAC GAG TAT GTG GGT CTT CCT CGA GAC CAC CCA GAG AGT TAC CAC
     F   Q   Y   V   K   T   F   N   M   D   E   Y   V   G   L   P   R   D   H   P   E   S   Y   H
311  TTC TTC ATG TGG GAT AAT TTC TTC AAG CAC ATT GAC ATC CAC CCT GAA AAC ACC CAC ATT TTG GAT GGA AAT
     F   F   M   W   D   N   F   F   K   H   I   D   I   H   P   E   N   T   H   I   L   D   G   N
383  GCG GCT GAC CTG CAG GCC GAG TGT GAT GCC TTT GAG GAG AAG ATC CAG GCT GCC GGA GGG ATC GAA CTC TTT
     A   A   D   L   Q   A   E   C   D   A   F   E   E   K   I   Q   A   A   G   G   I   E   L   F
455  GTC GGA GGC ATT GGC CCC GAT GGA CAC ATT GCC TTC AAT GAG CCA GGC TCC AGC CTG GTG TCC AGG ACC CGT
     V   G   G   I   G   P   D   G   H   I   A   F   N   E   P   G   S   S   L   V   S   R   T   R
527  GTG AAG ACT CTG GCT ATG GAC ACC ATC CTG GCC AAC GGT AGG TTC TTT GAT GGT GAT CTT GCC AAG GTG CCC
     V   K   T   L   A   M   D   T   I   L   A   N   G   R   F   F   D   G   D   L   A   K   V   P
599  ACC ATG GCC CTG ACA GTG GGG GTC GGC ACT GTC ATG GAT GCT AAA GAG GTG ATG ATC CTC ATC ACA GGC GCT
     T   M   A   L   T   V   G   V   G   T   V   M   D   A   K   E   V   M   I   L   I   T   G   A
671  CAC AAG GCC TTC GCT CTG TAC AAA GCC ATC GAG GAG GGC GTG AAC CAC ATG TGG ACG GTG TCC GCC TTT CAG
     H   K   A   F   A   L   Y   K   A   I   E   E   G   V   N   H   M   W   T   V   S   A   F   Q
743  CAG CAC CCC CGC ACT GTG TTT GTG TGT GAC GAG GAC GCC ACC TTG GAA CTA AAA GTG AAG ACA GTC AAA TAT
     Q   H   P   R   T   V   F   V   C   D   E   D   A   T   L   E   L   K   V   K   T   V   K   Y
815  TTC AAA GGT TTA ATG CTT GTT CAT AAC AAG CTG GTG GAC CCC CTG TAC AGT ATC AAG GAG AAG GAA ATT CAG
     F   K   G   L   M   L   V   H   N   K   L   V   D   P   L   Y   S   I   K   E   K   E   I   Q
887  AAA AGC CAA TCT GCT AAG AAG CCA TAC AGT GAC TAG cctgtgaccgacatggtattcagtacctcagagggacaggcaggtctttccggaaagtctctgtagga
     K   S   Q   S   A   K   K   P   Y   S   D
991  gagagagtagaattactttttagtccactctggctgctgcagatctgaggcttggtatatacatgttaaggagtttggctatggagaacattgttgattataattttctccttctttttcagtactgggggctgaacct
1131 ggggccttgcacatgccaaggaggtgccttgctaactgagctatgtccccaactttctgccccttttcataatggttttgtaaccatccgaagtctccatcactgactgtaattatattcttctgcccctatgcacatgg
1271 gcagcacacgccgtcgagaactccggtctgtatgtgtgctttttctagagttggcagggattaggcttgtgcactttggacataacctttctggagacttgagatccccctcttctgccagcctccccactgagaatttt
1401 cattttaaagaatacacttccagaacttgaattatgcacaaggaagcagcccttcctgccagaaccaagaacctgggggagaagcctgtcccttgttcccattgtgtggattgtctttcctgttgtcagtagctagcctt
1551 tctgtaaacaggccttctgggcatgtggggctaagatggctttcacaggtaaactcaaagcttacaaaggccagccttgtcttgttctctctcaagattccaacaagagaaaagtggaaaggcctcctgcctgagctctg
1691 ttttcctcaacagattcttacacactccctagtaaggagatcgcgtggtcacagacacgctatgatttcagtgtttttacccaaggtggggagctgacatctgcacatgcgctgtgctctgcggcctcctcagagctgag
1831 tgccaagattatacacgctactgctctctcctcgcttaactcaccccagcactcccaccttcgggtttatatcctctgtcctcagcatcagaactaaaccctttcaccccctctgtccttcaggctcccttcccaggctcct
1971 ctgcttggccttgctttatcttctctgcctctgtggagtagtcgctgcctccttctccgtccttgtgcctgtgtgaagctagagctgcctatttccaggagaggtctttcagaacattgcccaggccctccagaagtact
2111 gtgtttggtaccagcatttgtttgtagttttatatgtgattgtgctattgtgctgttaagctaatggatcaatggacttgtttacaatgtgatattttctattaaatttaatattttcaaaaaaaaaaaaaaaaaa 2249
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Fig. 1. The complete nucleotide sequence of mouse GNPDA cDNA and its deduced amino acid sequence are shown. The coding region starts at position 53 and ends at position 923. The sequence data have been deposited in the GenBank database under accession number AF088903.

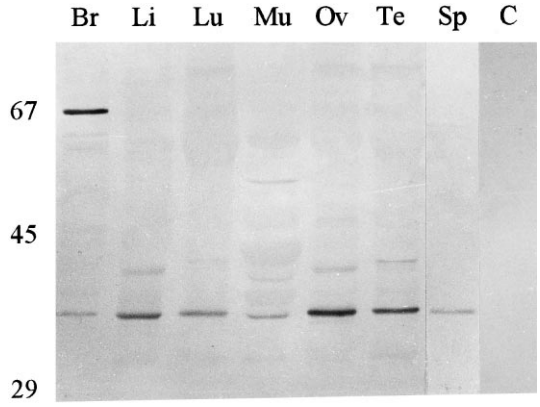

Fig. 2. Presence of GNPDA in different mouse tissues (Br = brain, Li = liver, Lu = lung, Mu = muscle, Ov = ovary, Te = testis, Sp = spermatozoa; C = Control: Sp reacted with secondary antibody alone). For each lane we used 20 μg of protein extract, except for spermatozoa, where the proteins derived from 10×10^6 spermatozoa were used, which is equivalent to 50 μg of total protein. The presence of 33 kDa GNPDA can be detected in every tissue. In brain an additional band at approximately 66 kDa is present which is reduced in intensity at higher concentrations of DTT or β-mercaptoethanol in the loading buffer.

We investigated the localization of GNPDA in isolated testicular cells by immunofluorescence. This study revealed a weak staining in the cytoplasm of isolated spermatocytes (Fig. 3A,B) and a stronger staining at a distinct site in isolated spermatids (Fig. 3C,D and E,F). Although we could not perform a double immunofluorescent staining with an anti-acrosomal antibody, the localization of GNPDA in isolated spermatids seemed to be close to the developing acrosomal vesicle (Fig. 3E,F). Pre-immune serum tested negative (data not shown).

3.3. Location of GNPDA in capacitated versus acrosome reacted spermatozoa

Next we examined the presence and localization of GNPDA in capacitated versus acrosome reacted mouse spermatozoa derived from the caput and cauda epididymidis. In capacitated spermatozoa, over 80% (165/200) spermatozoa presented with an intact acrosome as revealed by staining

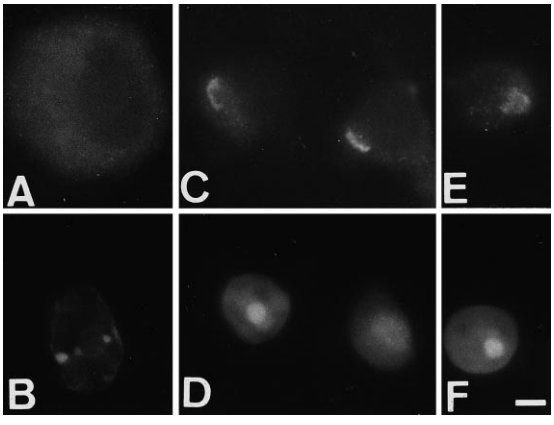

Fig. 3. Immunofluorescent localization of GNPDA in isolated testicular cells (A, C, E: GNPDA fluorescence; B, D, F: DNA staining). Spermatocytes (A, B) show a weak reaction in the cytoplasm. In spermatids GNPDA is predominantly located at a distinct cytoplasmic site (C–F) close to the nucleus. Bar in F = 4 μm.

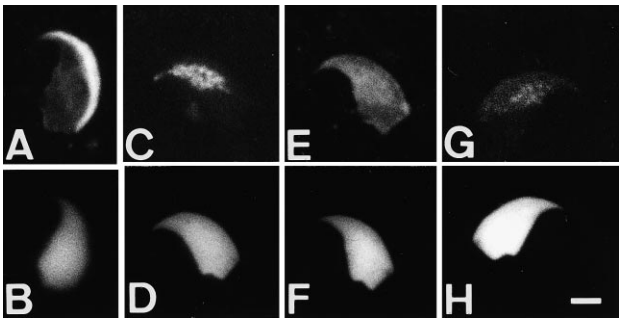

Fig. 4. Fluorescent localization of the acrosome (A, E), GNPDA (C, G) and corresponding DNA staining (B, D, F, H). In capacitated mouse spermatozoa with intact acrosome (A, B), GNPDA is localized underneath the acrosome (C, D). Following the induction of the acrosome reaction (E, F), GNPDA fluorescence is either reduced and distributed over the entire sperm head (G, H) or absent (not shown). Bar in H = 4 μm.

with FITC-labeled *Pisum sativum* lectin (Fig. 4A,B). Immunofluorescent detection of GNPDA showed in over 70% (142/200) of spermatozoa a positive reaction at an intracytoplasmic site neighboring the acrosome (Fig. 4C,D). Following the induction of the acrosome reaction by Ca^{2+} ionophore, 75% (150/200) of spermatozoa reacted (Fig. 4E,F) and no longer showed a reaction with the FITC lectin. In over 60% (135/200) of spermatozoa we noted a weak immunofluorescence with the anti-GNPDA antiserum (Fig. 4G,H) and in the remaining spermatozoa GNPDA could no longer be detected (not shown). We therefore performed a corresponding Western blot analysis of capacitated and acrosome reacted spermatozoa and especially investigated the presence of soluble GNPDA in the culture supernatants. During capacitation, GNPDA was only detected in the spermatozoa and was absent from the culture medium (Fig. 5, lanes 1,2). Following induction of the acrosome reaction, GNPDA was found in spermatozoa as well as in the supernatant, indicative for a release of GNPDA from spermatozoa (Fig. 5, lanes 3,4).

4. Discussion

In this paper we report the sequence of mouse GNPDA cDNA clone. Our analysis shows that mouse GNPDA is the homologue to hamster oscillin and similarly exhibits a strikingly high sequence homology to all mammalian GNPDA sequences reported so far. Using a polyclonal antibody produced against a part of mouse GNPDA, we found soluble mouse GNPDA to be present in all examined tissues. This

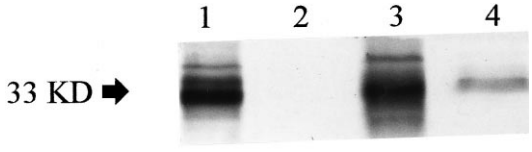

Fig. 5. Western blot analysis of the presence of GNPDA in capacitated (lanes 1, 2) and in acrosome reacted (lanes 3, 4) spermatozoa. In capacitated spermatozoa, GNPDA was only detected in the sperm pellet (lane 1) but not in the culture medium supernatant (lane 2). In acrosome reacted spermatozoa GNPDA was found in the sperm pellet and in the supernatant. In each lane the protein equivalent of 20×10^6 spermatozoa was used for SDS-PAGE and Western blot analysis.

finding is in contrast to the data presented in the initial paper on mammalian GNPDA [6], where GNPDA was only detected in the testis and was absent in liver. Mouse extracts from liver did not activate mouse oocytes after injection, whereas extracts prepared from spermatozoa and from testis did. These findings are in accordance with recently published work which showed that cloned hamster and human GNPDA did not exhibit oscillogen activity following expression in either prokaryotic or eukaryotic cells and injection into mouse oocytes [7–9]. Altogether, these data provide strong evidence that GNPDA, although it is a soluble sperm factor, might not be responsible for the oscillogen activity known to be present in sperm cytosol.

However, the role of sperm GNPDA is still unsolved. GNPDA catalyzes the formation of fructose 6-phosphate and ammonia from glucosamine 6-phosphate [17] and consequently Wollosker et al. [7] proposed that it may function as an energy source for sperm motility. This explanation seems to be unlikely, because GNPDA is localized within the cytosol of the sperm head, whereas the motor for sperm motility is located behind the sperm head. We propose that GNPDA plays a role within the cascade which leads to the initiation and completion of the acrosome reaction. During spermatid differentiation, GNPDA co-localizes to a region which resembles the developing acrosome vesicle. In differentiated spermatozoa, GNPDA is located close to the acrosome. Even more important, the localization of GNPDA can be changed by the induction of the acrosome reaction. In acrosome reacted spermatozoa, GNPDA immunofluorescence was either weak or completely absent. This observation and the presence of GNPDA in the medium supernatant of acrosome reacted spermatozoa support the idea that soluble GNPDA is released from spermatozoa during the acrosome reaction.

Therefore, GNPDA might serve as an indicator for the integrity of the sperm acrosome. We showed recently that an equatorial localization of GNPDA in human spermatozoa might correlate with the ability to initiate fertilization after intracytoplasmic sperm injection (ICSI). Patients with spermatozoa showing mainly an equatorial distribution of GNPDA had higher fertilization rates compared to patients with GNPDA predominantly dispersed over the whole sperm head [10]. In human spermatozoa we only occasionally found spermatozoa with complete absence of GNPDA immunoreactivity. In view of the data presented in this paper, we may conclude that in the human, spermatozoa showing a localization nother than equatorial might have undergone the acrosome reaction. This situation would compare with the rearrangement and loss of GNPDA observed in acrosome reacted mouse spermatozoa. In the natural course of fertilization, the acrosome reaction is initiated prior to fusion of the spermatozoon with the oocyte [18]. In ICSI, which is a major treatment modality for severe male factor subfertility [19,20], initiation of the acrosome reaction does not occur because the spermatozoon is injected directly into the ooplasm. In view of our previous results [10] it is tempting to speculate that spermatozoa which underwent the acrosome reaction a long time prior to injection might have lost not only acrosomal proteins and GNPDA but also other soluble sperm proteins. If this included the oocyte activating soluble sperm oscillogen, these spermatozoa would be not able to initiate successful fertilization of an oocyte.

In conclusion, our data indicate that GNPDA in spermatozoa may play a role during the acrosome reaction. Further investigations are necessary to elucidate the involvement of GNPDA in the biochemical course and organization of the acrosome reaction cascade.

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References

[1] Swann, K. and Ozil, J.P. (1994) Int. Rev. Cytol. 152, 183–222.
[2] Stice, S.L. and Robl, J.M. (1990) Mol. Reprod. Dev. 25, 272–280.
[3] Swann, K. (1990) Development 110, 1295–1302.
[4] Meng, L. and Wolf, D.P. (1997) Hum. Reprod. 12, 1062–1068.
[5] Palermo, G.D., Avrech, O.M., Colombero, L.T., Wu, H., Wolny, Y.M., Fissore, R.A. and Rosenwaks, Z. (1997) Mol. Hum. Reprod. 3, 367–374.
[6] Parrington, J., Swann, K., Shevchenko, V.I., Sesay, A.K. and Lai, F.A. (1996) Nature 379, 364–368.
[7] Wolosker, H., Kline, D., Bian, Y., Blackshaw, S., Cameron, A.M., Fralich, T.J., Schnaar, R.L. and Snyder, S.H. (1998) FASEB J. 12, 91–99.
[8] Wu, H., He, C.L., Jehn, B., Black, S.J. and Fissore, R.A. (1998) Dev. Biol. 15, 369–381.
[9] Wolny, Y.M., Fissore, R.A., Wu, H., Reis, M.M., Colombero, L.T., Ergün, B., Rosenwaks, Z. and Palermo, G.D. (1999) Mol. Reprod. Dev. 52, 277–287.
[10] Montag, M., Parrington, J., Swann, K., Lai, F.A. and van der Ven, H. (1998a) FEBS Lett. 423, 357–361.
[11] Montag, M., van der Ven, K., Rink, K., Delacrétaz, G. and van der Ven, H. (1998b) Fertil. Steril. 69, 539–542.
[12] Rybouchkin, A., Benijts, J., De Sutter, P. and Dhont, M. (1996) Hum. Reprod. 12, 1693–1698.
[13] Kimura, Y. and Yanagimachi, R. (1995) Biol. Reprod. 52, 709–720.
[14] Olson, G.E., Nagdas, S.K. and Winfrey, V.P. (1997) Mol. Reprod. Dev. 48, 71–76.
[15] Hogan, B., Beddington, R., Costantini, F. and Lacy, E. (1994) in: Manipulating the Mouse Embryo. A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[16] Geussová, G., Peknicová, J., Capková, J., Kaláb, P., Moos, J., Philimonenko, V.V. and Hozák, P. (1997) Andrologia 29, 261–268.
[17] Comb, D.G. and Roseman, S. (1958) J. Biol. Chem. 232, 807–827.
[18] Yanagimachi, R. (1994) in: The Physiology of Reproduction (Knobil, E. and Neil, J.D., Eds.), pp. 189–281, Raven Press, New York.
[19] Palermo, G.D., Joris, H., Devroey, P. and Van Steirteghem, A. (1992) Lancet 340, 17–18.
[20] Van Steirteghem, A.C., Nagy, Z., Joris, H., Liu, J., Staessen, C., Smitz, J., Wisanto, A. and Devroey, P. (1993) Hum. Reprod. 8, 1061–1066.