Identification of a 71 kDa protein as a putative non-genomic membrane progesterone receptor in boar spermatozoa

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Abstract

A putative non-genomic progesterone receptor was identified by Western blot analysis from the membrane fraction but not the cytosolic fraction of boar spermatozoa using monoclonal antibody (mAb) C-262. When the membrane and the cytosolic fractions of boar liver, kidney, uterus and spermatozoa were analyzed with mAb C-262, protein bands with molecular masses of 86 and 120 kDa were detected from the cytosolic fraction of the uterus, whereas a 71 kDa protein was detected from the membrane fraction of spermatozoa. Apparently, while the 86 and 120 kDa proteins from the uterus correspond to the genomic progesterone receptor isoforms A and B in boar, the 71 kDa protein of the sperm membrane fraction seems to be a novel membrane-associated progesterone receptor. Ligand blot assay of the membrane and the cytosolic fractions of boar spermatozoa performed with peroxidase-conjugated progesterone revealed that only the 71 kDa membrane protein binds specifically to progesterone, reinforcing the results obtained from the Western blot analysis. Also ligand blot assays performed in the presence of mAb C-262 demonstrated that mAb C-262 inhibited progesterone binding to the 71 kDa protein in a dose-dependent manner. Ligand blot assays performed in the presence of free progesterone, RU486 or estrogen revealed that binding of peroxidase-conjugated progesterone to the 71 kDa protein was inhibited by free progesterone and RU486 in a dose-dependent manner but not by estrogen, which further confirms that progesterone binds to the 71 kDa protein specifically. Furthermore, the progesterone-induced acrosome reaction was inhibited by mAb C-262 in a dose-dependent manner. These results strongly imply that spermatozoa possess a progesterone receptor in a membrane-bound form and can be influenced by progesterone via non-genomic progesterone receptor.

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Introduction

In mammalian fertilization, sequential intracellular events should occur in spermatozoa for successful fertilization. Following ejaculation, spermatozoa need to undergo a process of biochemical and morphological modifications during transit through the female reproductive tract and initial contact with the zona pellucida (ZP) before they are capable of fertilizing the ovum (Yamagata *et al.* 1999, Baldi *et al.* 2002). These changes, known as capacitation and the acrosome reaction (AR), are prerequisites for successful fertilization (Saling & Storey 1979). The AR is a specialized form of exocytosis involving the fusion of the outer acrosomal membrane with the sperm plasma membrane overlying the acrosome, followed by vesiculation of fused membranes and release of the acrosomal contents (Russell *et al.* 1979). Thus, only acrosome-reacted spermatozoa are able to pass through the ZP and bind the oocyte plasma membrane (Yanagimachi *et al.* 1994). The AR can be induced by follicular fluid, cumulus cells (Tesarik *et al.* 1993) or the ZP (Cross *et al.* 1988). Identified as the active component of follicular fluid, progesterone, a steroid hormone known to induce transcriptional activations in the nucleus mediated by an intracellular receptor, has been reported to induce the AR in many mammalian spermatozoa (Osman *et al.* 1989, Meizel *et al.* 1990, Melendrez *et al.* 1994, Baldi *et al.* 1995, Meyers *et al.* 1995, Sirivaidyapong *et al.* 1999, Patrat *et al.* 2000, Somanath *et al.* 2000, Jang & Yi 2002), which strongly implies that progesterone exerts its effect on mammalian spermatozoa non-genomically via a still unidentified membrane-bound progesterone receptor (mPR). Although the mechanism is poorly understood, the AR induced by progesterone is regulated by signal transduction processes upon progesterone binding onto a still unidentified mPR. This represents an integration between changes in ionic conductance and second messenger generation (Kopf 2002). Since the mPR has not been unequivocally identified yet from boar spermatozoa, here we report a 71 kDa sperm membrane protein as a novel putative non-genomic mPR in boar spermatozoa employing Western blot and ligand blot analyses.

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Materials and Methods

Materials

Fresh boar testes were collected from Daejean Meat Co. (Incheon, Korea). Other tissues were kind gifts from the National Livestock Research Institute (Suwon, Korea). Ham's buffer was from GIBCO Laboratories. Mouse monoclonal antibody, mAb C-262 (Catalog No. SRA-1110), which was raised against the progesterone-binding C-terminal domain of human genomic progesterone receptor, was purchased from StressGen Biotechnologies Corp. (Victoria, BC, Canada), and peroxidase-conjugated progesterone (P-POD) was purchased from Fitzgerald Industries International, Inc. (Concord, MA, USA). Western blot detection reagent WEST-ZOL was from iNtRON (Seoul, Korea). Progesterone, 17β-estradiol and RU486 were purchased from Sigma Chemical Co. All other chemicals were obtained in molecular biology or extra-pure grade from Sigma and Amersham Biosciences.

Preparation of spermatozoa and other cells

Boar epididymides were dissected from freshly excised tissue and spermatozoa were flushed with Ham's buffer (pH 7·4) containing 50 mM benzamidine, which was added as an activation inhibitor of trypsin-like enzyme (Yi 1999). The flushed spermatozoa were washed via centrifugation at 10 000 ***g*** through 11% Ficoll containing 50 mM benzamidine for 30 min (Yu & Yi 2001). The spermatozoa pellets were stored at −80 °C and used for further study. The slices of other boar tissues, i.e. liver, kidney and uterus, were washed with washing buffer (50 mM Tris–HCl, 150 mM NaCl, 150 mM sucrose, pH 7·7) at 4 °C for 1 h. Tissues were then homogenized twice by Ultra-Turrax T25 (IKA-Labortechnik, Staufen, Germany) at 13 500 ***g*** for 30 s each in a homogenizing buffer (25 mM Tris–HCl, 50 mM EDTA, 1 mM dithiothreitol and 0·1 mM phenylmethysulfonylfluoride (PMSF), pH 7·7). The homogenates were centrifuged at 10 000 ***g*** for 30 min at 4 °C, and the cell pellets were stored at −80 °C until used.

Preparation of membrane and cytosolic fractions

Spermatozoa and the other cell pellets were washed twice with PBS (pH 7·4) containing 2 mM benzamidine at 10 000 ***g*** for 10 min, and then resuspended in a small volume of the same buffer. The samples were sonicated by ultrasonication (30 s, six times) with a Sonosmasher (Ulsso Hitech Co. Ltd, Chongwon-Gun, Korea). The sonicated suspensions were centrifuged at 100 000 ***g*** for 2 h at 4 °C. The supernatant was saved as the cytosolic fraction of the respective tissue. Pelleted membrane was then solubilized with buffer containing 0·02 M phosphate buffer, 1% Triton X-100, 5 mM glycerophosphate and 0·005% PMSF, pH 7·0 and centrifuged at 100 000 ***g*** for 1 h to remove the unsolubilized materials. The membrane fraction of the respective tissue was obtained by saving the solubilized fraction of the membrane.

SDS-PAGE

SDS-PAGE was performed according to Laemmli (1970). Samples were treated with 5 × sample buffer (60 mM Tris–HCl, 25% glycerol, 2% SDS, 14·4 mM 2-mercaptoethanol, 0·1% bromophenol blue) at room temperature for 1 min, vortexed briefly and loaded onto the gel (final protein concentration 1 μg/μl). The electrophoresis was performed at 4 °C on a 0·75 mm slab gel with a 5% stacking gel and a 12·5% separating gel at 20 mA constant current until the dye front reached the bottom of the gel. After the gel electrophoresis was completed, the gel was stained with Coomassie Brilliant Blue R and destained with 50% methanol. Molecular masses of protein bands were estimated using the Image Analysis Program Labwork 4·5 (UVP, Inc., Upland, CA, USA).

Western blot analysis

Western blot analysis was performed according to Towbin *et al.* (1979) with minor modifications. Briefly, the proteins separated by SDS-PAGE were electrophoretically transferred to Immobilon-P membrane (Millipore, Bedford, MA, USA) using transfer buffer (15·6 mM Tris base, 120 mM glycine, pH 8·2) at a constant voltage of 30 V overnight. After electro-transfer, the membrane was incubated for 1 h in a blocking solution (Tris-buffered saline–Tween 20 (TBST), 5% skim milk, pH 7·4), and then overnight at 4 °C with monoclonal antibody (mAb) C-262, which was diluted to 2 μg/ml (1:500) in the blocking solution. Following overnight incubation, the membrane was washed twice in 50 ml TBST for 15 min each, and incubated for 2 h with peroxidase-conjugated goat mAb anti-mouse IgG (used at 1:2000 dilution in the blocking solution). After washing in TBST twice, the membrane was reacted with the Western blot detection reagent WEST-ZOL for 2 min. The membrane was exposed to X-ray film for 5 min in a light-tight film cassette. The exposed X-ray film was then developed and fixed.

Ligand blot analysis

Ligand blot analysis was performed according to Luconi *et al.* (1998) with minor modifications. Briefly, the proteins separated by SDS-PAGE were electrophoretically transferred to Immobilon-P membrane as described for Western blot analysis. After electro-transfer, the membrane was incubated in PBS containing 3% Nonidet-P40 for 30 min, in PBS containing 0·3% BSA and 0·1% Tween 20 for 2 h, and then in PBS containing 0·1%

Tween 20 for 10 min sequentially. After the membrane was incubated overnight in PBS containing 1 μM P-POD in the presence of varying concentrations of free progesterone, 17β-estradiol, progesterone antagonist RU486 or mAb C-262, the membrane was washed twice in PBS containing 0·1% Tween 20. The membrane was then reacted as described for Western blot analysis.

AR assay

The capacitated boar spermatozoa were treated with varying concentrations of mAb C-262 (1/500, 1/100 and 1/10) diluted with 0·1% dimethylsulfoxide with or without 20 μM progesterone, and incubated at 37 °C for 20 min in a humid atmosphere of 5% CO_2:95% air. AR was assessed by a chlortetracycline (CTC) fluorescence assay according to Fraser & Herod (1990). Briefly, CTC solution was freshly prepared containing 250 μM CTC in a buffer containing 130 μM NaCl, 5 mM cysteine and 20 mM Tris (pH 7·4). Cells in 50 μl sperm suspension were stained with 50 μl CTC solution. Following fixation with 10 μl 12·5% (w/v) paraformaldehyde in 0·5 M Tris buffer (pH 7·4) for 3 h, the stained suspension was placed on a clean slide glass and AR was assessed immediately.

Statistical analysis

Percentages of AR obtained in different experiments were expressed as means ± S.E.M. Each experiment was repeated five times and the significance of the results was examined at $P<0·05$. In instances where one treatment and control were compared, a one-way ANOVA was applied, and a Tukey test was used for the post-hoc test.

Results

SDS-PAGE and Western blot analyses of the whole extracts, the membrane and the cytosolic fractions of boar spermatozoa with mAb C-262

For identification of a potential progesterone receptor in boar spermatozoa, SDS-PAGE and Western blot analyses were performed on the whole extracts, the membrane and the cytosolic fractions of boar spermatozoa. The Western blot analysis of these proteins with mAb C-262 revealed a protein band with an apparent molecular mass of 71 kDa in the whole extracts and the membrane fraction but not in the cytosolic fraction of boar spermatozoa (Fig. 1a), while pre-immune serum did not detect any protein band from the whole extracts of boar spermatozoa (Fig. 1b).

Western blot analysis of the membrane and the cytosolic fractions of boar liver, kidney, uterus and spermatozoa with mAb C-262

Since the identity of the detected 71 kDa protein has not been reported previously, comparative Western blot analysis was performed using boar liver, kidney, uterus and spermatozoa. The membrane and the cytosolic fractions of these tissues were separated and analyzed by Western blot using mAb C-262. While 71 kDa protein was detected from the membrane fraction of spermatozoa, 86 and 120 kDa proteins, which correspond to the genomic progesterone receptor isoforms A and B in boar, were detected from the cytosolic fraction of the uterus (Fig. 2). Neither the membrane nor the cytosolic fractions of the liver and kidney extracts, on the other hand, showed any detected protein bands by mAb C-262.

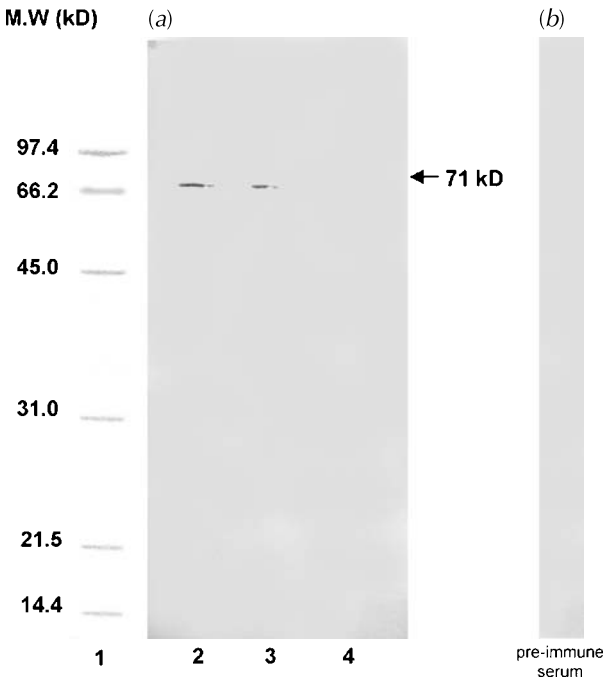

Figure 1 Western blot analysis of whole extracts and membrane and the cytosolic fractions of boar spermatozoa. (a) Whole extracts and membrane and cytosolic fractions of boar spermatozoa were prepared as described in Methods, and 30 μl of each sample were loaded and electrophoresed in a 12·5% separating gel at 20 mA constant current. Following electrophoresis, the proteins were transferred to Immobilon-P membrane, and the membrane was screened with mAb C-262 (1:500). The protein bands detected by mAb C-262 were visualized using the Western blot detection reagent WEST-ZOL. Lane 1: molecular mass standard, lane 2: whole extracts of spermatozoa, lane 3: membrane fraction of spermatozoa, lane 4: cytosolic fraction of spermatozoa. Numbers at the left denote the respective molecular masses of the molecular mass standards. The arrow denotes the 71 kDa sperm protein. (b) The whole extracts of boar spermatozoa were screened with mouse pre-immune serum. Preparation of the whole extracts and the screening procedures were as described in (a), except the same concentration of pre-immune serum replaced mAb C-262.

Ligand blot assays of the membrane and the cytosolic fractions of boar spermatozoa

To determine whether the detected 71 kDa protein can actually bind to progesterone, a ligand blot assay was

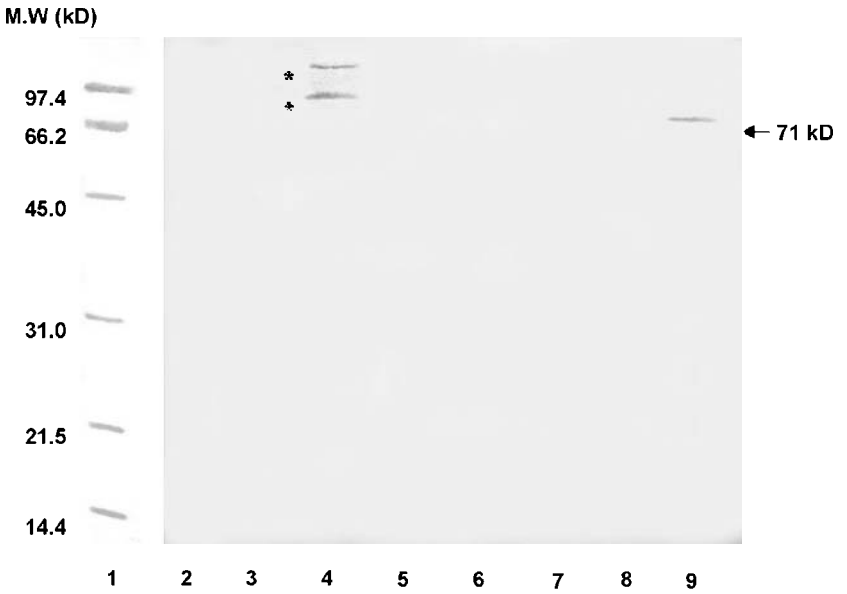

Figure 2 Western blot analysis of the membrane and cytosolic fractions of boar liver, kidney, uterus and spermatozoa. The membrane and cytosolic fractions of boar liver, kidney, uterus and spermatozoa were prepared as described in Methods, and SDS-PAGE and Western blot analyses were performed as described in Fig. 1. Lane 1: molecular mass standard, lane 2: cytosolic fraction of liver, lane 3: cytosolic fraction of kidney, lane 4: cytosolic fraction of uterus, lane 5: cytosolic fraction of spermatozoa, lane 6: membrane fraction of liver, lane 7: membrane fraction of kidney, lane 8: membrane fraction of uterus, lane 9: membrane fraction of spermatozoa. Numbers at the left denote the respective molecular mass standards. The arrow denotes the 71 kDa protein band detected by mAb C-262 from the membrane fraction of spermatozoa. Asterisks denote the 86 and 120 kDa protein bands detected by mAb C-262 from the cytosolic fraction of uterus.

performed using P-POD in the absence or presence of varying concentrations of free progesterone. In the absence of free progesterone, P-POD bound a single protein band of 71 kDa on the membrane fraction of boar spermatozoa (Fig. 3a), whereas the intensity of the same protein band gradually decreased in the presence of free progesterone up to 5 μM and completely disappeared above 10 μM (Fig. 3b). This signifies that free progesterone competes for binding onto the 71 kDa protein with P-POD and the effectiveness of inhibition by free progesterone on P-POD binding increased as the concentration of free progesterone in the incubation media increased (Fig. 3b). The specificity of the 71 kDa protein for progesterone was further substantiated by two additional ligand blot assays. First, when ligand blot assays were performed in the presence of varying concentrations of progesterone antagonist RU486, the intensity of the 71 kDa protein band decreased as the concentration of RU486 increased, implying that P-POD binding on the 71 kDa protein is inhibited by RU486 (Fig. 4). Secondly, when ligand blot assays were performed in the presence of varying concentrations of the other steroid, 17β-estradiol (estrogen), the results clearly demonstrated that estrogen did not block P-POD binding on the 71 kDa protein even at 50 μM (Fig. 5). These results strongly suggest that progesterone binds the 71 kDa protein specifically.

Inhibitory effects of mAb C-262 on progesterone-induced AR in capacitated boar spermatozoa

Since mAb C-262 detected a putative mPR from the membrane fraction of boar spermatozoa, we investigated whether mAb C-262 can exert its effects on progesterone-induced AR. When capacitated boar spermatozoa were incubated with varying concentrations of mAb C-262 in the presence of 20 μM progesterone, the proportion of acrosome-reacted spermatozoa clearly decreased with the increasing concentrations of mAb C-262 in a dose-dependent manner (Fig. 6). The proportion of acrosome-reacted spermatozoa decreased from 26·0% of the spermatozoa treated only with 20 μM progesterone to 25·0, 20·2 and 14·2% in the spermatozoa treated with 20 μM progesterone added with 1/500, 1/100 and 1/10 diluted amounts of mAb C-262 (original concentration 1 mg/ml) respectively. However, when the spermatozoa were incubated with mAb C-262 in the absence of progesterone, the proportion of acrosome-reacted spermatozoa did not decrease compared with the control group,

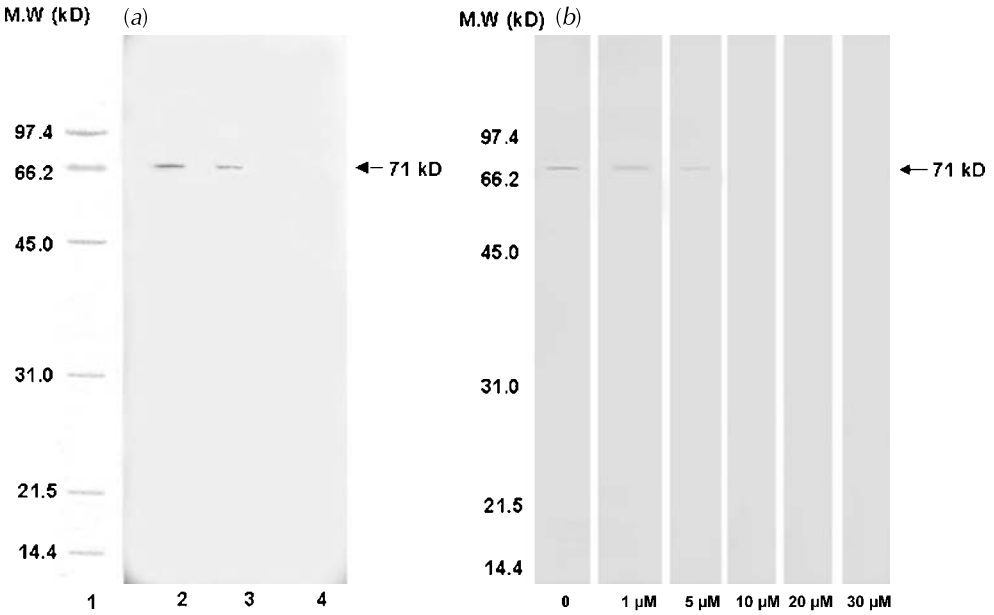

Figure 3 Ligand blot assays of whole extracts and membrane and cytosolic fractions of boar spermatozoa in the absence or presence of varying concentrations of free progesterone. The preparation of whole extracts and membrane and cytosolic fractions of boar spermatozoa and SDS-PAGE were performed as described in Fig. 1. P-POD was used as a ligand, and the progesterone-bound protein bands were visualized using WEST-ZOL. (a) Ligand blot assay was performed in the absence of free progesterone. Lane 1: molecular mass standard, lane 2: whole extracts of spermatozoa, lane 3: membrane fraction of spermatozoa, lane 4: cytosolic fraction of spermatozoa. (b) Ligand blot assay was performed with the membrane fraction of spermatozoa in the presence of varying concentrations of free progesterone. Numbers at the left denote the respective molecular mass of the molecular mass standards. Numbers shown at the bottom (b) denote the concentration of free progesterone added to the incubation media. The arrow denotes the 71 kDa protein band bound by P-POD.

which was treated with buffer only, suggesting that mAb C-262 alone does not have any inhibitory effects on the AR when treated alone to the capacitated spermatozoa (Fig. 6). In contrast, treatment of equivalent doses of pre-immune serum did not show any effects on progesterone-induced AR (Fig. 6). To determine whether these inhibitory effects of mAb C-262 on AR can be correlated with actual inhibition of P-POD binding, the ligand blot assay was performed with varying concentrations of mAb C-262. As the concentration of mAb C-262 in the incubation medium increased, the actual binding of P-POD on the 71 kDa protein diminished and was completely undetected at 20 μg (1/50 dilution) of mAb C-262, demonstrating that mAb C-262 inhibited P-POD binding on the 71 kDa protein in a dose-dependent manner (Fig. 7).

Discussion

Progesterone has been known to function in the nucleus at the transcriptional level as a transcriptional activator mediated by a genomic progesterone receptor. Lately, non-genomic mPRs have been reported in conjunction with non-genomic functions of progesterone (Zhu *et al.* 2003). In mammalian reproduction, progesterone has been implicated to be involved in AR (reviewed by Calogero *et al.* 2000), in human (Osman *et al.* 1989, Baldi *et al.* 1995, Patrat *et al.* 2000), mouse (Melendrez *et al.* 1994), stallion (Meyers *et al.* 1995), golden hamster (Meizel *et al.* 1990), dog (Sirivaidyapong *et al.* 1999), goat (Somanath *et al.* 2000) and boar (Jang & Yi 2002). Progesterone, by binding to a non-genomic mPR, has been reported to induce AR via intracellular signal transduction cascades (Kopf 2002). Parinaud & Milhet (1996) demonstrated that progesterone-induced AR may involve a cAMP-mediated pathway by showing that concentrations of cAMP increase in a Ca^{2+}-dependent manner from human sperm. In addition, Blackmore *et al.* (1990) and Baldi *et al.* (1991) reported that progesterone induced a significant increase in intracellular Ca^{2+} concentration in sperm. However, despite the implications that AR might be induced by progesterone mediated by mPR, the identity of mPR in spermatozoa has not been conclusively reported yet. Previously, Sabeur *et al.* (1996) and Luconi *et al.* (1998, 2002) reported that two proteins with apparent molecular masses of 50–52 and 46–48 kDa and of 56 and 29 kDa were detected by mAb C-262 as potential mPRs in human

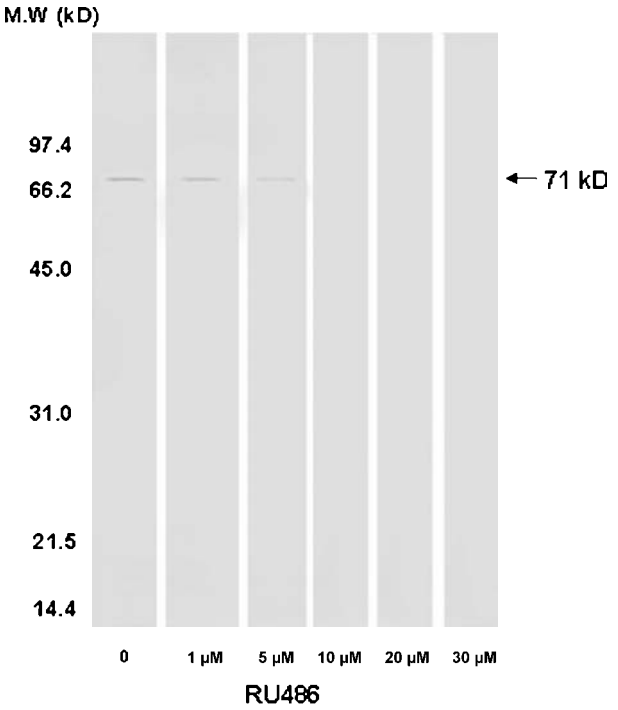

Figure 4 Dose-dependent effects of progesterone antagonist RU486 in P-POD binding onto the 71 kDa protein. The ligand blot assay was performed as described in Fig. 3 with the membrane fraction of spermatozoa, except that varying concentrations of RU486 were added with P-POD. Numbers at the left denote the respective molecular mass of the molecular mass standards. Numbers shown at the bottom denote the concentration of RU486 added to the incubation media. The arrow denotes the 71 kDa protein band bound by P-POD.

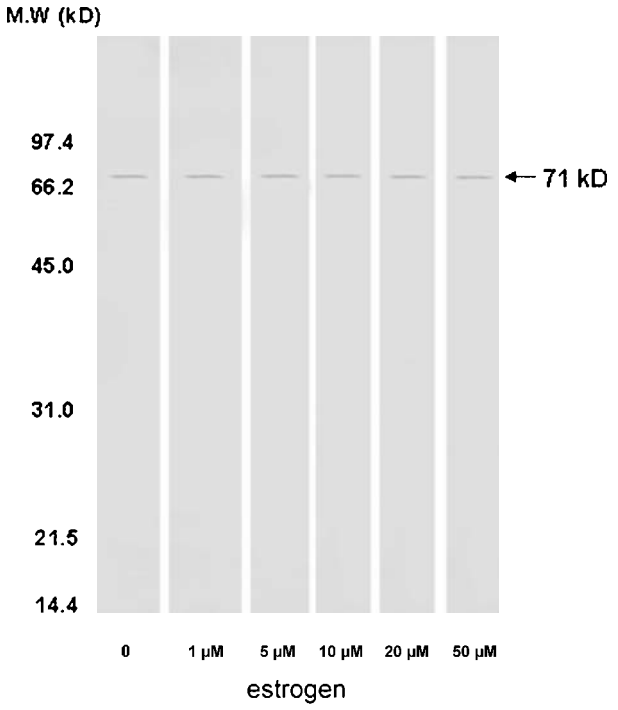

Figure 5 Dose-dependent effects of free estrogen in P-POD binding onto the 71 kDa protein. The ligand blot assay was performed as described in Fig. 3 with the membrane fraction of spermatozoa, except that varying concentrations of 17β-estradiol were added with P-POD. Numbers at the left denote the respective molecular mass of the molecular mass standards. Numbers shown at the bottom denote the concentration of 17β-estradiol added to the incubation media. The arrow denotes the 71 kDa protein band bound by P-POD.

spermatozoa respectively. Also there are several other proteins which are postulated to be potential mPRs detected by different antibodies. Most importantly, Meyer *et al.* (1998) and Falkenstein *et al.* (1999) reported that 28 and 56 kDa proteins, which do not share the classic hormone-binding domain with the genomic progesterone receptor, are detected by the ProgPep antibody raised against the N-terminus of porcine liver mPR. Interestingly, as reported for mAb C-262 by Sabeur *et al.* (1996), ProgRek, raised against the recombinant porcine liver mPR expressed in *E. coli* fused with an N-terminal His-tag, inhibited the progesterone-induced Ca^{2+} increase (Falkenstein *et al.* 1999). Our results of Western blot analysis with mAb C-262, however, detected a single protein band of 71 kDa in the whole extracts and the membrane fraction but not in the cytosolic fraction of spermatozoa (Fig. 2). Furthermore, when Western blot analysis was performed using the other boar tissues including uterus, which is known to contain genomic progesterone receptor isoforms A and B (Geisert *et al.* 1994, Slomezynska *et al.* 2000), the 71 kDa protein was detected only from the membrane fraction of spermatozoa in contrast to the 86 and 120 kDa proteins detected from the cytosolic fraction of uterus by mAb C-262, verifying that 71 kDa protein is a novel protein with a high probability of being a novel mPR. Even though mAb C-262 detected a single protein band of 71 kDa in the Western blot analysis, since the immunoreactivity cannot guarantee the presence of a functional progesterone receptor, we performed the ligand blot assay. When the specificity of interaction was evaluated by binding competitions with unlabeled progesterone, progesterone antagonist RU486 or estrogen, unlabeled progesterone and RU486 but not estrogen competed with P-POD for binding onto the 71 kDa protein from the membrane fraction of boar spermatozoa in a dose-dependent manner, confirming the results obtained from the Western blot analysis (Fig. 3) that progesterone binds the 71 kDa protein specifically. Furthermore, progesterone-induced AR was specifically decreased by the addition of mAb C-262 in a dose-dependent manner, reassuring that progesterone-induced AR could be mediated by this putative mPR and that mAb C-262 can inhibit the progesterone-induced AR by blocking the progesterone-binding domain of the putative mPR.

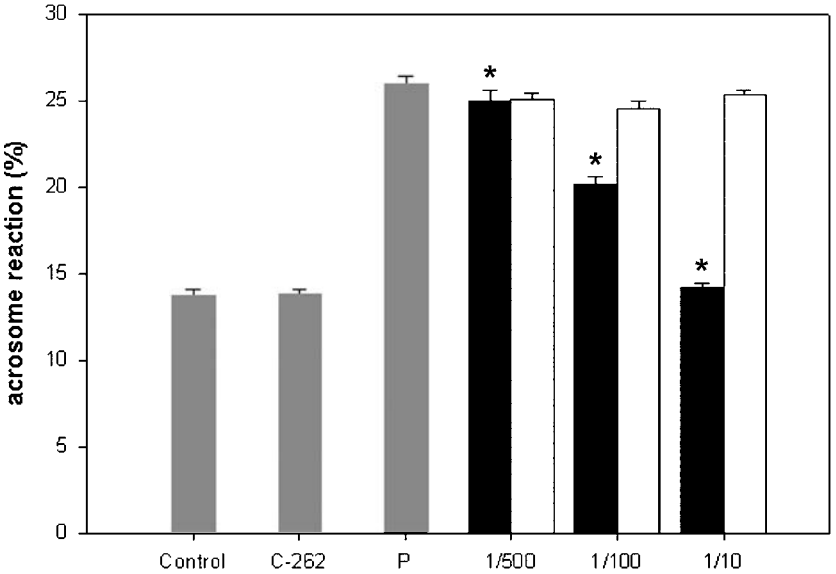

Figure 6 Inhibitory effects of mAb C-262 on progesterone-induced AR in capacitated boar spermatozoa. The boar spermatozoa were incubated with 20 μM progesterone containing varying concentrations of mAb C-262 or mouse pre-immune serum. Following 20 min incubation, the acrosome-reacted spermatozoa were assessed by staining with CTC. Control denotes the spermatozoa treated with 0·1% dimethylsulfoxide buffer only, C-262 denotes the spermatozoa treated with 1/10 diluted amounts of mAb C-262 only, and P denotes the spermatozoa treated with 20 μM progesterone only. The 1/500, 1/100 and 1/10 denote the group of spermatozoa treated with 20 μM progesterone added with 1/500, 1/100, and 1/10 diluted amounts of mAb C-262 (original concentration 1 mg/ml) or mouse pre-immune serum. Black bars denote the spermatozoa treated with mAb C-262, and the white bars denote the spermatozoa treated with mouse pre-immune serum. The results are presented as means ± S.E.M. ($n=5$ with 200 spermatozoa scored for each treatment). Statistical analysis of the inhibitory effects of mAb C-262 on progesterone-induced AR showed significance at $P<0·05$, and these are marked with asterisks in the figure.

In the present study, a heterologous mAb system was employed for the identification of a novel putative mPR in spermatozoa. mAb C-262, which was raised against the progesterone-binding C-terminal domain of the human genomic progesterone receptor, was chosen to identify the non-genomic forms of progesterone receptor for several reasons. First, mAb C-262 was reported to compete with progesterone binding (Sabeur *et al.* 1996), which suggests that mAb C-262 binds directly to a putative mPR. Secondly, the progesterone-binding C-terminal tail is 100% conserved among genomic progesterone receptors from different species (Weigel *et al.* 1992); thus, any protein detected from the membrane fraction by this antibody would have a high probability of being a putative progesterone-binding protein. Therefore, although mAb C-262 was raised against the genomic progesterone receptor, it was used for the screening of a non-genomic mPR in the present study. As for the ligand blot assay, a question could be raised about the progesterone-binding activity on the reduced protein sample. Structural analysis of the human nuclear progesterone receptor reported by William & Sigler (1998) could partially answer the question. The progesterone-binding domain of the reported receptor, which presumably shares structural similarity with the mPR reported in the present study, does not seem to have any intra-disulfide bonds, suggesting that reduction may not affect progesterone-binding activity. Additionally, two protein bands reported by Luconi *et al.* (1998), which were detected by mAb C-262, could also bind P-POD, even after the protein samples were reduced and treated at 95 °C for 5 min. Therefore, progesterone-binding activity of the putative mPRs detected by mAb C-262 may not be affected by the reducing conditions and the progesterone-binding domain may retain a biologically active structure.

In conclusion, our results demonstrate that spermatozoa possess a putative progesterone receptor, potentially as a membrane-bound form, and this 71 kDa membrane protein could be a novel mPR in boar spermatozoa. Furthermore, the inhibitory effects of mAb C-262 on progesterone-induced AR strongly imply that progesterone-induced AR can be mediated by this putative mPR in boar spermatozoa.

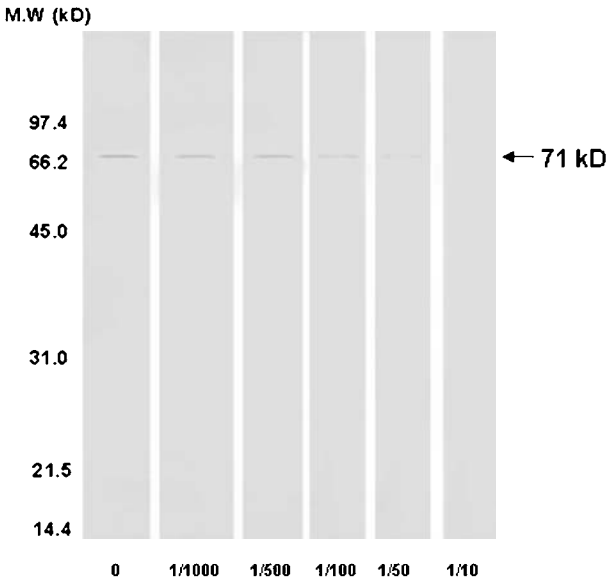

Figure 7 Dose-dependent inhibitory effects of mAb C-262 in P-POD binding onto the 71 kDa protein. The ligand blot assay was performed as described in Fig. 3 with the membrane fraction of spermatozoa, except that varying concentrations of mAb C-262 were added with P-POD. Numbers at the left denote the respective molecular mass of the molecular mass standards. Numbers shown at the bottom denote the dilutions of mAb C-262 added to the incubation media. The arrow denotes the 71 kDa protein band bound by P-POD.

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