ORIGINAL CONTRIBUTION

Immunolocalization of a Guinea Pig Sperm Surface Antigen Recognized by a Monoclonal Antibody E74

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E74 is a mouse monoclonal antibody raised against the acrosome-reacted guinea pig spermatozoa. This study describes immunolocalization of the E74 antigen in guinea pig spermatozoa. Immunoelectron microscopy of guinea pig spermatozoa shows that the E74 antigen is localized on the equatorial segment plasma membrane following the acrosome reaction but not associated with the surface of the acrosome-intact spermatozoa. Immunoblot analysis of Triton X-100 extract of cauda epididymal guinea pig spermatozoa following one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis shows that E74 antibody recognizes a protein with an apparent molecular weight of 45,000 dalton. Immunoblot of sperm extracts separated by two dimensional gel electrophoresis indicates a broad spot of 45,000 dalton in the 5 to 7.5 isoelectric focusing range.

INTRODUCTION

The cell biology of events immediately surrounding fertilization (attachment to the zona pellucida, adhesion to the oocyte plasma membrane, and sperm-egg membrane fusion) appears to be entirely dependent on the molecular properties of the surface of the sperm head. The mammalian spermatozoon has a highly region- alized plasma membrane as demonstrated on many occasions by differential binding patterns of the surface molecules [1]. The surface domains of the sperm plasma membrane are thought to correlate with the specific functions of the sperm surface [2]. For instance, functions associated with the interaction of sperm and eggs are localized to regions of the sperm head and major subsets of those functions are restricted to its peri-acrosomal plasma membrane. The peri-acrosomal plasma membrane is involved in attachment of sperm to the zona pellucida and the acro- some reaction [3, 4].

A variety of approaches has been taken to study the molecular composition

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a To whom all correspondence should be addressed: Isurani Ilayperuma, Department of Anatomy, Faculty of Medicine, University of Ruhumna, P.O. Box 70, Galle, Sri Lanka. Tel.: 94-9-2344; Fax: 94-9-22314; E-mail: isurani @anato.ru.ac.lk.
b Abbreviations: 2D, two-dimensional; mAb, monoclonal antibody; pl, isoelectric focusing point. Received: October 3, 2001; Returned for revision: January 28, 2002; Accepted: March 4, 2002.
of the sperm surface. For example, lectin probes have been used to identify saccharide components [5], and freeze fracture techniques have been used to identify discrete patterns of intramembranous particle distribution [6]. Monoclonal antibodies provide valuable probes for identification and characterization of sperm surface molecules. Several putative fusion proteins on mammalian spermatozoa have been identified using monoclonal antibodies [7-13] of which only PH-30/fertilin [8] has been well characterized. PH-30/fertilin is a sperm protein present on the post-acrosomal plasma membrane of guinea pig spermatozoa [8, 14]. It is an integral membrane glycoprotein composed of two subunits; the α subunit and β subunit. The α subunit contains a putative fusion peptide sequence, while the β subunit is an integrin ligand (disintegrin) [15, 16]. Peptides derived from the putative fusion sequence have fusigenic activity [17]. The PH-30 monoclonal antibody partially blocks sperm-egg fusion in the guinea pig [8]. Peptides derived from the disintegrin sequence impair fertilization [18]. However PH-30/fertilin is not present on the equatorial segment plasma membrane of guinea pig spermatozoa that is widely considered as the site of sperm-egg membrane fusion and does not undergo processing in parallel with the acrosome reaction [15]. This is in contrast with the observation that acrosome-intact spermatozoa cannot fuse with the zona pellucida-free oocytes [19] and the currently widely accepted notion that the equatorial segment is the site of sperm-egg membrane fusion [20-23]. Furthermore, the fertilin α gene is non-functional in the human [24] and mice lacking fertilin β produce spermatozoa that are capable of fusion with the oocyte [25]. This does not rule out a role for PH-30/fertilin in sperm-egg fusion, but it does suggest that there may be redundant adhesion pathways.

This paper describes immunolocalization of a guinea pig sperm surface antigen that appears on the equatorial segment plasma membrane, a region strongly implicated in sperm-egg membrane fusion, following the acrosome reaction.

**MATERIALS AND METHODS**

**Animals**

This study was approved by the animal ethics committee, University of Otago, New Zealand. Sexually mature male guinea pigs were killed by CO₂ asphyxiation and sperm were flushed from single excised vasa deferentia and caudae epididymis as described previously [11].

**Monoclonal antibody**

The monoclonal antibody (mAb) E74 used in this study belongs to a panel of mouse monoclonal antibodies that was established against the acrosome-reacted guinea pig spermatozoa [11]. The mAb E74 was established as IgG₁ using an Amersham isotyping kit (Amersham, Little Chalfont, Buckinghamshire, United Kingdom).

**SDS polyacrylamide gel electrophoresis and western blotting**

SDS polyacrylamide gel electrophoresis was performed on 10 percent acrylamide gels according to the method of Laemmli [26]. Approximately 10⁶ spermatozoa were extracted with 2 percent Triton X-100 for 10 minutes on ice. Samples were heated to 100°C for five minutes in non-reducing SDS sample buffer (2 percent SDS, 62.5 mM Tris-HCl, pH 6.8, 10 percent glycerol, 0.001 percent bromophenol blue).

Two-dimensional (2D) SDS-PAGE was performed according to the method of O'Farrell [27]. The sperm sample prepared as described above was dissolved in solubilisation buffer (8 M urea, 10 mM 2-mer-
captoethanol, 4 percent Triton X-100, 1 percent pharmalyte, pH 3 to 10). First-dimensional analysis to determine the isoelectric focusing point (pl) was performed on Immobiline DryStrips (Pharmacia, Uppsala, Sweden) containing an immobilized linear pH 3-10 gradient. Second-dimensional analysis was performed on conventional vertical SDS-PAGE.

Proteins were either stained with silver [28] or electrophoretically transferred onto nitrocellulose membranes (Bio-Rad, Richmond, California) by western blotting [29]. Western blots were blocked with 5 percent skim milk powder (Anchor) in TBST (Tris-buffered saline, pH 7.6, (20 mM Tris base, 0.137 mM NaCl, 3.8 ml of 1 M HCl) containing 0.1 percent Tween-20 (BDH Laboratory Supplies, Poole, United Kingdom) and probed with mAb diluted (1:100) in PBS containing 1 percent BSA (Sigma, St Louis, Missouri) for 60 minutes. After washing with TBST (three changes over 45 minutes), the blots were incubated with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (secondary antibody) (DAKO, Glostrup, Denmark) for 60 minutes, washed with TBST as before and bound secondary antibody detected with enhanced chemiluminescence (ECL, Amersham, Little Chalfont, Buckinghamshire, United Kingdom).

**Immuno-Electron microscopy**

The acrosome reaction was induced with 38 μm Ca ionophore A23187 at a sperm count of ~10^7 spermatozoa ml^-1 [30]. Electron microscopy was performed according to the method of Green [30]. Briefly, the spermatozoa were fixed in 2.5 percent gluteraldehyde in 0.1 M cacodylate buffer, pH 7.4, followed by fixation in 1 percent osmium tetroxide in cacodylate buffer for two hours. The pellet was stained with 2 percent uranyl acetate in 0.1 M sodium acetate buffer, pH 5.1, dehydrated in a graded series of ethanol and infiltrated with propylene oxide. It was embedded in Spurr (ProSciTech,

![Figure 1. Transmission electron micrograph of acrosome-reacted guinea pig spermatozoa immunolabeled with (Panel A) mAb E74 and (Panel B) hybridoma supernatant without the mAb (Control). * indicates anterior and posterior boundaries of the equatorial segment. Abbreviations: n, nucleus; iam, inner acrosomal membrane; pmp, plasma membrane over the post equatorial segment; pme, plasma membrane over the equatorial segment. Note the intense localization of gold particles to the equatorial segment plasma as membrane in (A). (Bar = 0.5 μm).](image-url)
Thuringowa Central, Queensland, Australia), a low-viscosity epoxy resin [31] and ultrathin sections were cut with a diamond knife. Sections were incubated with mAb E74 for five minutes, washed three times with PBS and incubated with 10 nm gold-labeled goat anti-mouse IgG (Zymed Laboratories, South San Francisco, CA). Parallel control experiments were carried out with the mAb E74 replaced by the hybridoma supernatant. Sections were contrast-enhanced by double staining with uranyl acetate and lead citrate and viewed on a Philips electron microscope at 80 kV.

RESULTS

The pattern of mAb E74 binding to acrosome-reacted guinea pig spermatozoa, as determined by immunoelectron microscopy is illustrated in Figure 1A. The antigen was predominantly associated with the external surface of the equatorial segment plasma membrane. There was sporadic low level of binding of mAb E74 to the post-acrosomal plasma membrane of the head. The posterior boundary of the periacrosomal plasma membrane coincides with the point at which the plasma membrane becomes attached to the post-acrosomal sheath. This line was marked by a diminution in the amount of detectable E74 antigen lying posterior to the boundary. Analysis of a random sample of 100 sections of acrosome-reacted spermatozoa for gold particle localization revealed that there were 36 ± 12 particles on the equatorial segment and 12 ± 2.0 particles at post-acrosomal segment plasma membrane. No evidence of immunogold labelling was found either on the inner acrosomal membrane or on the plasma membrane of the tail. There was no evidence of immunogold labelling on the acrosome-intact spermatozoa or with the control (1B).
Western blot of guinea pig sperm extracts separated by 1D SDS-PAGE showed that mAb E74 recognize a broad band of 45 kDa (Figure 2). Immunoblot of sperm extract separated by 2D SDS-PAGE indicated that the antigen migrated as multiple spots at 45 kDa region with a pI value extending from 5 to 7.5 (Figure 2). The major spot was visible with silver staining of sperm extracts (Figure 2). No immunoreaction was found with the control (not shown).

**DISCUSSION**

Allen and Green [11] described the generation of a panel of mouse monoclonal antibodies against the acrosome-reacted guinea pig spermatozoa. The antibody used in this study (mAb E74) belongs to this panel. The acrosome reaction is an absolute precondition for zona penetration [32] and sperm-egg membrane fusion [19]. Sperm-egg membrane fusion occurs through the plasma membrane of the equatorial segment [20-23] and/or post-equatorial segment [19, 33]. The acrosome-intact spermatozoa cannot fuse with the oolemma [34] although both the equatorial segment plasma membrane and post equatorial segment plasma membrane are present and exposed. Therefore it is reasonable to assume that following the acrosome reaction these membranes are processed in a way that leads to the exposure of a novel epitope that renders spermatozoa fusogenic. The acquisition of fusion competence by spermatozoa following the acrosome reaction is likely to reflect molecular changes to the sperm surface and that such changes could be detectable with monoclonal antibodies. The production of monoclonal antibody E74 used in this study was based on this assumption [11].

The E74 antigen is expressed as a multiple protein with a molecular weight of 45 kDa and pI of 5 to 7.5. This may explain the broad immunoreactive band (45 kDa) observed in 1D SDS-PAGE and western blotting. These may be proteolytic degradation products of a precursor molecule. Alternatively, the antibody may recognize the same epitope on two different molecules.

For ease of experimental manipulations the acrosome reaction was chemically induced, in vitro, using Ca\(^{2+}\) ionophore A23187 [30]. The ionophore-induced acrosome reaction is morphologically similar to the physiological acrosome reaction induced by zona pellucida [35]. Furthermore it has been shown that ionophore treated spermatozoa can fertilize oocytes [36, 37].

Studies using monoclonal antibodies have clearly demonstrated the localization of surface antigens in distinct plasma membrane domains of the guinea pig sperm head [14]. However, there is no firm evidence that a domain corresponding to the equatorial segment of the acrosome exists in the plasma membrane prior to the acrosome reaction. Ultrastructural localization of E74 antigen using immunogold labelling indicates that the antigen is localized to plasma membrane over the equatorial segment following the acrosome reaction but is absent from the acrosome-intact sperm.

The primary location of E74 antigen and exactly how it is presented on the plasma membrane overlying the equatorial segment is not clear. Two mechanisms have been proposed to explain the appearance of novel antigens on the equatorial segment plasma membrane following the acrosome reaction [11, 13]: (1) through modification of existing antigens by released acrosomal enzymes. There is evidence for surface modifications in the equatorial segment plasma membrane following acrosome reaction [7, 11]. (2) through diffusion or direct translocation of antigens from an intra-acrosomal site. Evidence for such a mechanism has yet to be found.
The pattern of immunolocalization shown by mAb E74 is similar to those reported for several other putative fusion proteins present on mammalian spermatozoa. Monoclonal antibody M29 recognizes a 40 kDa protein on the equatorial segment plasma membrane of acrosome-reacted mouse spermatozoa [7]. In guinea pig sperm, mAb G11 [11] recognizes an equatorial segment antigen following the acrosome reaction. Monoclonal antibody M1 recognizes a protein on the equatorial segment plasma membrane of acrosome-reacted hamster spermatozoa [13]. Further investigations are necessary to study the expression of E74 antigen in relation to the acrosome reaction and its effect on sperm-egg membrane fusion.

Given the importance of the equatorial segment plasma membrane in fertilization, an analysis of its molecular composition will be valuable in identifying proteins that are central to an understanding of fertilization. Identification of the equatorial segment plasma membrane antigens is the first step in characterizing these proteins.

ACKNOWLEDGMENT: Financial assistance from New Zealand Ministry of Foreign Affairs and Trade is acknowledged.

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