Functional and immunological analysis of the human sperm proteome

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This review has been accepted as a thesis together with ten previously published research papers by the Faculty of Health Sciences, Aarhus University, the 28th of November 2011 and defended on the 20th of January 2012.

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1. Preface and acknowledgements

The present dissertation is based on experimental research in the field of Reproductive & Developmental Biology performed during my employment at the Department of Cell Biology, University of Virginia Medical School (1993-1998), which has in part been finalized during my present employment at the Department of Clinical Immunology, Aalborg Sygehus, Aarhus University Hospital. The dissertation is composed as a review of 10 selected research articles published in peer-reviewed international journals between 1997 and 2010.

I am indebted to all my former colleagues at the Department of Cell Biology, UVa, for providing an open, interactive and humorous scientific environment. In particular the inspiring discussions and professional guidance of Professors John C. Herr and Charles J. Flickinger are greatly appreciated.

While "brain-storming sessions" at our weekly lab meetings facilitated problem solving, regular presentations and discussions of progress reports ensured a focussed and dynamic research environment, which was instrumental for the advance of the studies presented in this dissertation. I would like to express my genuine appreciation and thanks to my former colleagues in the laboratories of Professors Herr and Flickinger of whom several have made important contributions to the reviewed studies.

Our fruitful collaboration has continued over the years and has now focussed on the function of cancer-testis antigens during malignant transformation and validation of their efficiency as biomarkers.

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Finally, I wish to express my appreciation for the financial support the presented research projects have received from the Andrew W. Mellon Foundation, the National Institutes of Health, USA, and the Karen Elise Jensen Foundation.

2. The dissertation is based on the following 10 original research papers published in peer-reviewed journals:

I. Naaby-Hansen,S., Flickinger,C.J., and Herr,J.C. (1997) Two-dimensional gel electrophoretic analysis of vectorially labelled surface proteins of human spermatozoa, Biology of Reproduction, 56, 771-787.

II. Naaby-Hansen,S., Roof,R.W., Flickinger,C.J., Grafer,C.M., Parson,S.J., and Herr,J.C. (1997) A novel negative imaging technique for accurate localization of stainable proteins on complex 2-D autoradiograms, Electrophoresis, 18, 2065-70.

III. Mandal, A., Naaby-Hansen, S., Wolkowicz, M.J., Klotz, K., Shetty, J., Retief, J.D., Coonrod, S.A., Kinter, M., Sherman, N., Cesar, F., Flickinger, C.J., and Herr, J.C. (1999) FSP95, a testis specific 95 kDa fibrous sheath antigen that undergoes tyrosine phosphorylation in capacitated human spermatozoa, Biology of Reproduction, 61,1184-1197

IV. Coonrod, S., Naaby-Hansen, S., Shetty, J., and Herr. J.C. (1999) PI-PLC releases a 20-40 kDa protein cluster from the hamster oolemma and affects the sperm penetration assay, Molecular Human Reproduction, vol.5 no.11 pp. 1027-1033.

V. Coonrod, S.A., Naaby-Hansen, S., Shetty, J., Shibahara, H., Chen, M., White, J.M., and Herr, J.C. (1999) Treatment of mouse oocytes with PI-PLC releases 70 kDa (pI 5) and 35- to 45-kDa (pI 5.5) protein clusters from the egg surface and inhibits sperm-oolemma binding and fusion, Developmental Biology, 207, 334-349.

VI. Soren Naaby-Hansen+, Michael Wolkowicz, Ken Klotz, Leigh Ann Bush, V. Anne Westbrook, Hiroaki Shibahara, Jagathpala Shetty, Scott A. Coonrod, Prabhakara P. Reddi, John Shannon*, Michael Kinter*, Nicholas E.Sherman*, Jay Fox*, Charles J. Flickinger and John C. Herr. (2001) Co-localization of the inositol 1,4,5-triphosphate receptor and calreticulin in the equatorial segment and in membrane

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VIII. Michael J. Wolkowicz, Soren Naaby-Hansen, Marco Kallio, Angela Rinker, P. Prabhakara Reddi, Charles J. Flickinger and John C. Herr (2002) Human tektin B1 demonstrates flagellar localization in human sperm. Biology of Reproduction, 66, 241-250.

IX. Naaby-Hansen, S. and Herr, J.C. (2010) Heat shock proteins on the surface of human sperm. Journal of Reproductive Immunology, 84, 32-40.

X. Naaby-Hansen, S., Alan Diekman, Jagathpala Shetty, Charles J. Flickinger, V. Anne Westbrook, and John C. Herr (2010) Identification of calcium-binding proteins associated with the human sperm plasma membrane. Reproductive Biology and Endocrinology, 8, 6-17.

List of related articles

1) *Naaby-Hansen, S*., and Bjerrum, O.J. (1985) Auto- and Isoantigens of the human spermatozoa detected by immunoblotting with human sera after SDS-PAGE. J.Reprod.Immunol., 7, 41-57.

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8) Diekman, A.B., Westbrook-Case, V.A., *Naaby-Hansen, S*., Klotz, K.L., Flickinger, C.J., and Herr, J.C. (1997) Biochemical characterization of Sperm Agglutination Antigen-1, a human sperm antigen implicated in gamete interactions, Biol. Reprod., 56, 771-787.

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3. Introduction

3A. Spermatogenesis and epididymal maturation

The mammalian spermatozoon is a highly differentiated cell produced in the testis with the sole purpose of delivering the haploid male genome to the oocyte. The spermatozoon is the product of a complex series of changes that are organized spatially and temporally into a set of cell associations known as the cycle of the seminiferous epithelium (1). During spermatogenesis, round undifferentiated spermatogonia are transformed via spermatocytes and spermatids into highly asymmetric and motile spermatozoa (sperm); in humans, 12 steps can be recognized in the differentiation of spermatids alone (2). As a result of these numerous differentiation events, sperm are unusual cells in many respects. The nuclear chromatin becomes highly condensed and inactive in synthesis of RNA, and unique cytoplasmic components appear, such as the acrosome of the sperm head and the outer dense fibers and fibrous ribs of the tail. Although not immediately apparent from its microscopic appearance, even the sperm plasma membrane undergoes extensive differentiation during spermatogenesis. Different membrane domains are formed on the sperm surface (3), as shown in freeze-fracture preparations (4, 5), in immunocytochemical studies of differences in the distributions of sperm membrane components (6), and in the relocalization of surface antigens prior to fertilization (7). However, even though the sperm plasma membrane has been the subject of

numerous studies over the years, our comprehension of its composition, molecular interactions, and the architectural alterations that occur during genital tract transit and capacitation, is still rudimentary at present.

Upon completion of spermatogenesis the highly differentiated but still immotile sperm are transported from the seminiferous epithelium of the testis to the epididymidis. During epididymal transit the sperm plasma membrane undergoes further alterations and redistribution of its components, and both nuclear protamines and cytoskeletal structures in the tail are strengthened by extensive cross-linking (reviewed by Yanagimachi, 8). As sperm mature within the epididymis they gradually acquire the signalling pathways necessary for their ability to undergo capacitation at a later stage (9). Epididymal maturation additionally activates unknown regulatory mechanisms, which keep the mean cytosolic calcium concentration within narrow boundaries and regulate the amplitude and frequency of intracellular calcium oscillations and wave formations. This strict regulation of $[Ca^{2+}]_i$ is critically important for sperm to develop the signalling pathways that regulate the motion of the flagellum (10, 11, 12) and to establish the signalling network that controls the capacitation process (13). As a consequence of these alterations of its molecular structures and activities the sperm acquire the ability to move progressively, as well as the potential for capacitation and ultimately for fertilization.

The sperm plasma membrane continues to be modified following ejaculation through its interactions with seminal plasma components, of which small membrane-bound vesicles produced by epithelial cells lining the prostatic acini and by the epididymal epithelium have attracted particular attention in recent years. The composition and function of these exosomes termed prostasomes and epididymosomes respectively, have recently been reviewed by Burden et al. (14) and by Sullivan et al. (15).

The maturation of sperm continues during their journey through the female reproductive tract, and requires constant adjustments to the regulation of intracellular calcium before it culminates with the induction of capacitation. While exposure to chemoattractants such as progesterone in female genital tract fluids appears to modulate sperm motility through elevation of $[Ca^{2+}]_i$ (16), adhesion to the tubal epithelium is thought to extend sperm life through depression of $[Ca^{2+}]_i$ (12).

3B. The fertilization process

Fertilization capacity, the ability of mammalian sperm to bind to and fuse with the metaphase II arrested oocyte, is acquired after residence of sperm in the distinct microenvironment of the uterus or oviduct (depending on the species) for a finite period of time through a series of molecular changes called capacitation, as first described independently by Chang (17, 18) and Austin (19). Capacitation involves molecular changes in both the sperm head and tail which allow defined physiological endpoints to occur, such as regulated acrosomal exocytosis (8) and a whiplash-like sperm tail motion, termed motility hyperactivation, which is observed when sperm reach the protective barriers surrounding the oocyte and increase their flagellar bend amplitude and beat asymmetry (20, 21). Hyperactivated motility is thought to enhance the ability of sperm to penetrate the egg investments by increasing the forward progression and amount of lateral flagellar thrust (22).

Variations in the amplitude and frequency of intracellular calcium oscillations and a gradual increase in the mean cytosolic free calcium concentration ($[Ca^{2+}]_i$) during capacitation are essential for spermatozoa to attain motility hyperactivation and to undergo the acrosome reaction (AR) (8, 22, 23). These two pre-fertilization events occur independently (8, 24), suggesting either that they are driven by different Ca^{2+}-regulated pathways or that the pathways are activated at different times in distinct subcellular locations (22). However, the molecular mechanisms that control motility hyperactivation, the involved signal transducers and calcium binding proteins, and the resulting changes in the organization and/or activity of flagellar proteins, have remained elusive.

In mammals, fertilization begins with the direct interaction between sperm and the egg investments. First, sperm use hyperactivated motility and surface situated hydrolytic enzymes, including the GPI-anchored hyaluronidase PH-20, to penetrate the cumulus cell barrier that surrounds the ovulated egg (25). Subsequent binding of sperm to the mesh of glycoproteins that constitute the egg's extracellular coat, the zona pellucida (ZP), initiates the acrosome reaction, which results in the release or exposure of lytic enzymes from the acrosomal vesicle that is contained in the head of the sperm. This highly localized increase in hydrolase activity around the front of the sperm head facilitates its penetration through the ZP. However, the sperm plasma membrane receptor(s) that mediates ZP-binding has not been unequivocally identified (26) and the receptor-induced signalling cascades that culminate in sustained calcium influx and acrosomal exocytosis still remain to be fully delineated, and their effector molecules identified.

3C. Immunity to sperm and subfertility

Metalnikov first demonstrated the auto- and iso- immunogenic nature of sperm more than a century ago (27). However, it was not until 1954, when Rumke (28) and Wilson (29) independently reported the presence of antisperm antibodies in infertile men, that autoimmunity was conceived as a potential cause of infertility. A decade later Edwards (30) and McLaren (31) showed that immunity to sperm induced by intraperitoneal inoculation of female mice with sperm impaired their reproductive performance. At the same time Franklin and Dukes (32) described the presence of sperm agglutinins in the sera of women with unexplained infertility, and Isojima & Ashitaka (33) subsequently demonstrated the presence of sperm-immobilizing antibodies in serum samples from the same women. Shortly after, Rumke and colleagues (34) revealed an inverse correlation between the titre of sperm agglutinating antibodies found in sera from men and their fertility. The association between humoral immunity to sperm and subfertility implicated by these early reports has since been the focus of numerous studies. High titers of anti-sperm antibodies (ASA) are found in infertile patients (35-37) and in men after vasectomy (38-41), and it is now generally believed that the presence of ASA in the reproductive tract can reduce fertility in both men and women (42-44). The incidence of ASA in infertile couples is 9%-55% depending on the antibody assay used and the reporting center (37, 45-48). However, serum ASA have also been detected in up to 19% of fertile men (48, 49) and in as many as 43% of fertile women by the immunobead binding test (48). These findings indicate that not all ASA cause infertility, and emphasize the necessity for the development of new more specific diagnostic assays, before the significance of ASA in infertility can be definitively established.

Fertility reducing ASA are thought to block essential sperm functions, reduce sperm viability, or directly interfere with gamete interaction. Agglutinating ASA may reduce the forward progressive motility of sperm and/or obstruct their ability to penetrate the cervical mucus (50, 51). Cytotoxic ASA may act as opsonins facilitating the recognition and destruction of sperm by

phagocytes (37) or they may evoke the complement cascade resulting in sperm lysis (52-54). ASA directed against functionally important sperm surface antigens may impair the induction of capacitation and/or the acrosome reaction, or they may diminish sperm' ability to interact with and penetrate the egg investments (43, 55-57). Finally, maternal ASA have finally been shown to affect early embryo development in both rats and humans (58,59).

Improper sequestration of testis-specific antigens or defective regulation of the immune system may cause men to develop autoimmunity to sperm (reviewed in 37, 48 & 60). The acquisition of immunocompetence and tolerance occurs in the prenatal and neonatal stages of ontogeny, whereas spermatogenesis occurs much later, at puberty. Because sperm-specific antigens first appear long after immunological tolerance to other self-antigens is established, they would be thought to be recognized as foreign by the adult male immune system. However, the development of autoimmunity to sperm is rare. A combination of anatomic protection and immunosuppressive mechanisms appears to prevent testis-specific sperm antigens from inducing autoimmune reactions in the testis. During puberty tight junctions are formed between Sertoli's cells, creating an environment in the central portions of the seminiferous tubules that excludes large proteins and immune cells (61). While this so-called 'blood-testis barrier' protects postmeiotic spermatocytes and more mature germ cells in the adluminal compartment, spermatogonia and early spermatocytes are developed below (i.e., external to) this layer. In addition, studies in mice indicate that the tight junction barrier is not totally impermeable to testicular antigens (62, 63), suggesting that other immunoregulatory mechanisms also participate in preventing the development of autoimmunity to sperm. Tung et al. (64) have suggested, for example, that leakage of sperm-specific antigens across the blood-testis barrier leads to the development of late tolerance to these antigens. In addition, various immuno-inhibitory/modulating substances have been detected in seminal plasma (65), including the potent immunosuppressor 19-hydroxyprostaglandin E (66, 67) and inhibitors of complement-mediated lysis (68). These and other immunosuppressive factors in the seminal plasma and in secretions from the female reproductive organs are thought to have significant roles in protecting sperm from immunological damage during genital tract transit. A predominance of CD8 lymphocytes and soluble CD8 activators in semen may also participate in the protection of sperm from immune destruction (69). Moreover, Sertoli cells have been shown to modify immune reactivity in the testis through both direct and paracrine interactions with immune competent cells (70, 71).

Autoimmunity to sperm might thus be the result of defects in the sequestration of testis-specific antigens from the immune system or caused by a decrease in the normally occurring suppression of immune responses to testis-specific sperm antigens in men (reviewed in 37 & 60). Obstruction of sperm egress has been associated with development of autoimmunity to sperm (48). Serum antibodies against sperm have been reported in approximately half of men who have undergone vasectomy (38, 39). Vasectomy induced sperm antibodies have been shown to block IVF (72) and the presence of ASA within reproductive tract secretions at the time of vasovasostomy (surgical reversal of vasectomy) have been correlated with a reduced chance of subsequent fertility (73). Indeed 30-60% of vasectomized men remain infertile after surgically successful vasovasostomy (74), which has been attributed to the presence of circulating ASA (40). It is generally believed that the strong immune responses to sperm antigens observed following vasectomy are due to the testis-antigen sequestrating barrier being severely weakened at the same time as local immuno-suppressive mechanisms are destroyed by the surgical procedure (48).

Although seminal plasma hypersensitivity in women appears to be a relatively common phenomena (75), and cases of women who develop immediate allergic reactions to seminal plasma proteins following/during coitus have been reported (76, 77), this response is not usually associated with the development of immunity to sperm, even in women who are regularly inoculated intra-vaginally with sperm (40). Indeed, several studies of the immune responses of women to intrauterine sperm insemination, direct intraperitoneal insemination with large numbers of sperm, or fallopian tube sperm perfusion, found no or only marginal increases in the incidence of ASA after the treatment (78-84). The immuno-modulating agents of seminal plasma are thought to be critical in preventing sperm from inducing severe iso- or autoimmune responses in the female partner (i.e., directed against testis- or nuclear-specific antigens) (65). While immuno-inhibitory substances in seminal plasma may prevent sensitization of women to sperm antigens after coitus (48), adherence of semen-derived immuno-modulators and opsonins to the surface of sperm may also ensure that dead or dying sperm can be removed from the female genital tract without triggering undesired inflammatory and/or immune responses (X). Taken together, these studies imply that despite the general immuno-suppressive action of seminal plasma, some of its constituents may trigger immune responses in the female reproductive tract under certain conditions (85). However, sperm antigens appear to have negligible roles in these responses (85).

Although the etiology of antisperm immunity in women still remains to be fully elucidated, data from several studies imply that many, if not most, cases of antisperm immunity in women might be triggered by a pelvic/genital tract infection with microorganisms that express immunogenically cross-reactive antigens (86-88, IX). However, the repertoire of sperm surface antigens that are involved in immuno-subfertility and their roles in the fertilization process, still remains to be defined.

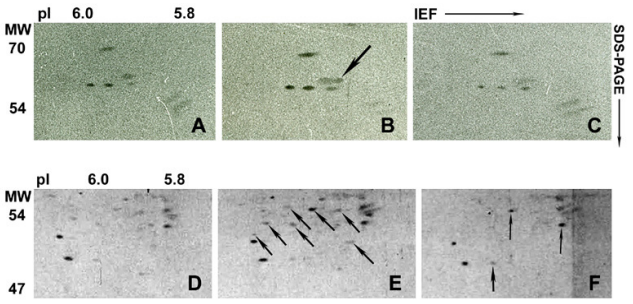

Figure 1
Vasectomy-induced humoral immune responses against sperm antigens demonstrated by 2DE-IB with serum samples taken prior to (left images), 18 days (centre images), and 223 days (right images) following surgery.
A-C: Enlarged gel areas illustrating temporal changes in IgM antibody reactivity.
D-F: Distinct gel areas showing qualitative and quantitative alterations in serum IgG antibody binding to human sperm antigens (oblique arrows indicate antigens with increased antibody binding). The upward vertical arrows in F indicate sperm antigens that show a sustained or even increased IgG response in the months following sterilization.

3D. Rationale for the research program

The research program we initiated in 1994 was aimed at investigating the human sperm proteome, defining its antigenicity, and studying its alterations during the capacitation process. Most of the studies included in this dissertation were accordingly instigated based on the following working hypothesis:

A combination of holistic and function-targeted analysis of the human sperm proteome is likely to:

Provide new insights into the unique regulatory mechanisms that control capacitation, motility hyperactivation, and/or the acrosome reaction.

Aid the identification of sperm auto- and iso-antigens involved in immuno-subfertility, and thereby beget characterization of new immunocontraceptive candidates.

Facilitate the discovery and characterization of unknown testis-specific proteins,improving our current comprehension of sperm-specific functions.

4. Investigation of the human sperm proteome

4A. Development and optimisation of high-resolution two-dimensional gel electrophoresis-based methods for separation and characterization of human sperm proteins

In the beginning of the nineteen nineties, two-dimensional gel electrophoresis (2DE) as developed by O'Farrell in the mid-seventies (89, 90), in combination with parallel protein purification, was the only well-established technique capable of both qualitative and semi-quantitative analysis of complex protein mixtures. By 1994, seven laboratories had applied 2DE-based techniques to the study of human sperm proteins (91-99). In one study, 2DE immuno blotting (2DE IB) with serum samples taken before and at various time points after vasectomy had revealed temporal changes in anti-sperm IgM and IgG antibody reactivities following the ligation of vas deferens (97). This demonstration of the 2DE IB technique's ability to record minor fluctuations in humoral immune responses (Figure 1) implied that high resolution electrophoresis and electroblotting might be similarly well attuned to study capacitation-induced changes in the post-translational modification and biochemical properties of sperm proteins. The versatility of the many analytical procedures associated with protein electrophoresis, along with the availibility of specialized computer softwares for 2D gel analysis and recent improvements in mass spectrometry-based protein analysis, further implicated 2DE-based techniques and mass spectrometry as the methods of choice for studying the molecular mechanisms that regulate sperm function.

In 1994, we therefore included a selection of 2DE-based analytical approaches and mass spectrometry as core methodologies in a new ambitious research proposal aimed at studying the human sperm proteome, defining its antigenicity, and investigating its modifications during in vitro capacitation. The proposed research program soon obtained financial support from the NIH and the Andrew W. Mellon Foundation, allowing the experimental work to commence.

A lack of standardized procedures, together with huge variations in electrophoretic resolution, reproducibility and pH-ranges in previously published 2DE-based studies of human sperm (91, 93-100), made a comparison of the reported protein patterns difficult, if not impossible. Our first objectives were therefore to develop and optimize a series of standardized procedures for reproducible sample handling, solubilization, electrophoretic separation, transfer, and immunological and biochemical analyses, which could serve as references in future studies of sperm proteins and provide the foundation for the building of a comprehensive database for the human sperm proteome. High-resolution 2D gel systems for separation of human sperm and seminal plasma proteins were developed using either isoelectric focusing (IEF) or nonequilibrium pH gradient electrophoresis (NEPHGE) for the first dimension electrophoresis, followed by polyacrylamide gel electrophoresis (PAGE) in the second dimension, enabling both acidic, neutral and basic sperm proteins to be analysed in a single study for the first time. Following the processing of several hundred 2D gels and comparison of a variety of different solubilization procedures, we finally attained the desired resolution and reproducibility in the electrophoretic separation. By that time we were also able to solubilize proteins from different sperm preparations in a highly reproducible fasion. After different gel and blot staining procedures had been optimized, we could therefore begin computer-aided comparison of the protein patterns in sperm samples obtained from different donors or from the same donor at different times, and initiate the construction of a 2D gel-based database of detergent/urea extracted sperm proteins.

4B. Establishment of a database for the human sperm proteome

Following 2D gel electrophoresis sperm proteins were either visualized by gel staining procedures or were electro-transferred to NC- or PVDF-membranes for further analysis. The resulting 2DE images were subjected to computer-aided analysis. Commassie and silver stained gels were scanned in a wet state with a high-resolution Kodak camera, and the information was digitized on a SUN computer. X-ray films from autoradiographic and chemiluminescence experiments were scanned with either the Kodak camera or a Howtek scanmaster 3+ laser scanner. The resulting 2D images were analysed using the Bio Image "2D Analyzer" version 6.1 (Figure 2). This software was supposed to automate the identification, quantification, and comparison of 2D-separated spots, but it was quickly noticed that the spot annotation program lacked sufficient accuracy. One major flaw was the program's inability to define correct spot boundaries regardless of how the image acquisition and spot annotation processes were tuned, as may be appreciated by close inspection of the computer annotated images in Figure 2. While the software provided reliable densitometry and comparison analysis, the necessity for careful re-evaluation and even manual redefinition of spot boundaries made this initial phase of image processing a rather laborious procedure.

1397 human sperm proteins with a molecular mass between 5 and 160 kDa and isoelectric points from 4 to 11 were catalogued from silver-stained gels loaded with NP40/urea extracts of sperm harvested by Percoll density gradient centrifugation, and 1191 proteins were resolved following solubilization with a SDS/CHAPS/urea buffer (I). Although very similar patterns of silverstained proteins were observed following 2DE separation of different sperm preparations, computer comparison analysis revealed minor variations in the profiles of solubilized sperm proteins which originated from different donors (Figure 2). Detergent extracts of sperm obtained at different times from the same donor also showed minor variations in protein content. Most of these differences were observed within horizontal charge trains on 2D gels, suggesting that they reflected variations in posttranslational protein modifications rather than differences in protein expression among sperm isolated from different ejaculates.

4C. Analysis of human sperm proteins by affinity-based methods

Immuno- and lectin-blotting procedures, and affinity overlay assays with radioactive nucleotide triphosphates and ^{45}Ca were used to investigate the identity, biochemical nature and function of the newly catalogued sperm proteins.

Lectin blotting is an efficient method for fast identification of glycoproteins in complex mixtures of solubilized proteins (96). Differences in the substrate specifities among lectins can be used to achieve a partial characterization of carbohydrate sidechains (100), or to aid in planning purification/removal procedures for specific subsets of glycoproteins. Lectin specific binding to glycoproteins on the human sperm surface is demonstrated in Figure 5 (section 2E below).

Low molecular weight (LMW) GTP-binding proteins have important roles in the intracellular signalling cascades that control key cellular activities such as cytoskeleton regulation and differentiation (101). Unlike oligomeric G proteins, LMW monomeric GTP-binding proteins are capable of binding GTP following electrophoretic separation and blotting (102, 103). Small GTPases are present in mammalian sperm and are thought to be involved in the regulation of motility and capacitation (104-107). In order to identify GTP-binding human sperm proteins and examine their regulation during capacitation we accordingly used the [α-^{32}P]GTP-overlay procedure described by McGrath et al. (102), with the modifications suggested by Gromov and Celis (108). This overlay assay identified five small, slightly acidic GTP-binding human sperm proteins on blots from IEF/PAGE gels (Figure 3). Increased [α-^{32}P]GTP-binding to the two most acidic protein forms were observed in extracts from sperm that had been incubated in a capacitation inducing medium for 6 hrs. The amount of [α-^{32}P]GTP bound to the three basic proteins did not change significantly during in vitro capacitation, indicating that capacitation inducing signals specifically increase the GTP-binding capacity of the two most acidic proteins. The molecular weights and isoelectric points of the GTP-binding human sperm proteins suggest that they belong to the Rho-family of small GTPases, which are known constituents of the mammalian sperm.

Affinity overlay experiments with [γ-^{32}P]ATP revealed two basic ATP-binding proteins on blots from NEPHGE/PAGE gels (Figure 3). The protein spots were subsequently found to be accessible for surface
radioiodination and to bind lectins with affinity for α-D-mannosyl residues (see Figure 5 below), suggesting that the two basic ATP-binding human sperm proteins are glycosylated membraneproteins.

Calcium-binding sperm proteins were identified using a ^{45}Ca overlay assay (VI) modified from that described by Maruyama et al. (109) in combination with 2DE and mass spectrometry. More than a dozen acidic human sperm proteins consistently bound ^{45}Ca in the overlay assay (VI, VII, X), nine of which were identified by mass spectrometry. The identified calcium-binding sperm proteins include three HSP70 chaperones, serum amyloid P-component, calreticulin, 80K-H protein, and CABYR, which are all discussed in detail below.

2DE immuno blotting was used to identify proteins catalogued in the database, which gradually matured from a simple mapping excersise to a valuable information resource. In some experiments immuno staining was precedeed by colloidal gold staining of the NC-membrane immobilized proteins.
This facilitated the exact positioning of specific antigens and their modified variants within the global pattern of 2DE separated human sperm proteins, as demonstrated for h-tekB1 in Figure 4.

The 2DE IB technique's ability to separate even complex mixtures of antigens at high resolution enabled us to identify and characterize previously unknown variants of sperm-specific proteins, including post-translational modified isoforms of PH-20 (Figure 7 in section 2G), SAGA-1 (Figure 8 in section 2E, (110, 111), SPAN-X/CTp11 (112), and CABYR (VII). In combination with mass spectrometry, 2D IB with human sera ultimately resulted in the identification and characterization of several major auto- and isoantigens within the human sperm proteome (see Chapter 3 below).

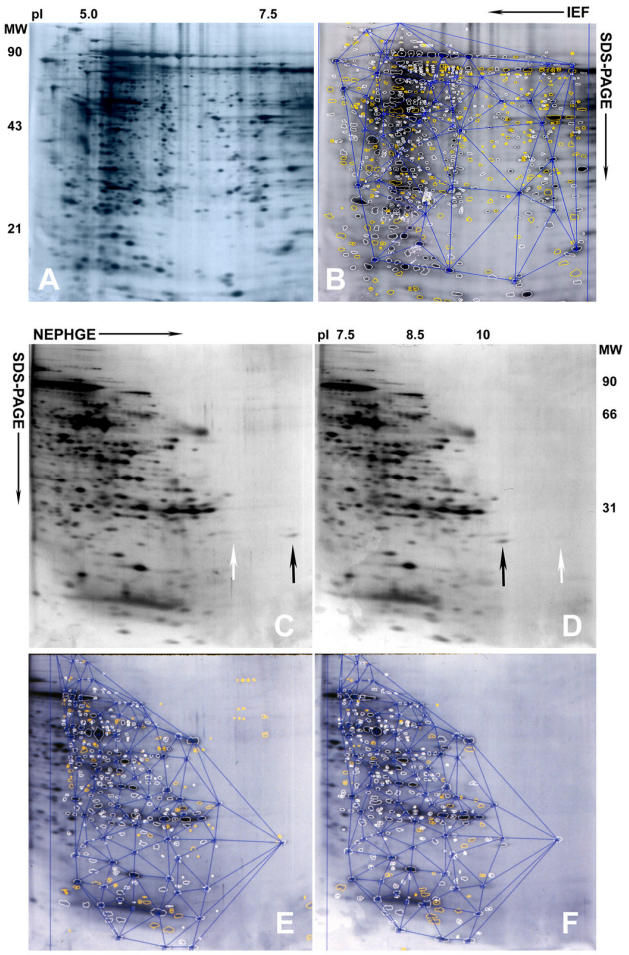

Figure 2
Computer-mediated analysis of silver stained IEF/PAGE gel (A & B) and NEPHGE/PAGE gels (C-F) of human sperm proteins solubilized in NP40/urea buffer.
The stained gels were scanned wet, digitised and analysed by the Bio Image "2D Analyzer" software, version 6.1 (Bio Image, Ann Arbor, MI). The images were acquired during the initial phase of the 2DE-based sperm proteome mapping program. C-F: Comparison of the protein profiles in NP40/urea extracts of sperm obtained from two healthy young men with normal semen parameters revealed minor interdonor proteome variations. The upward vertical arrows in C & D indicate a protein that appears to demonstrate different net-charges (i.e. degree of electronegative posttranslational modifications) in sperm obtained from the two donors (unpublished data). Note the inaccuracy of the automatically defined spot boundaries, which were achieved following optimal tuning of the spot annotation program.

4D. Cloning and characterization of human tektin B1 (IX)

By the mid-nineties mass spectrometry had replaced Edman degradation as the method of choice for protein microsequencing. By that time it was possible to obtain internal sequences from N-terminally blocked polypeptides by tandem mass spectrometry (ms/ms)(113), and continuous improvements in instrumentation and experimental strategies were preparing this increasingly versatile technology for its next major challenges, quantitative analysis of protein expression and characterization of post-translational modifications. The technology was thus at hand to determine the amino acid sequence of a given 2D gel protein spot, and if the obtained peptide sequences were unknown to deduce and synthesize a set of gene-specific oligonucleotides, which can be used to amplify and clone the cognate human testicular cDNA.

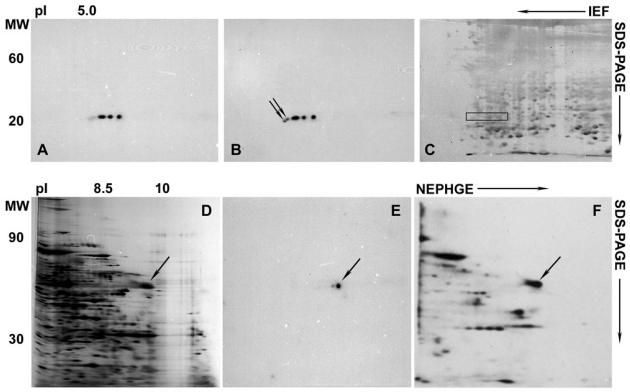

Figure 3
Nucleotide triphosphate-binding human sperm proteins demonstrated by radionucleotide overlay procedures following IEF/PAGE (A-C) and NEPHGE/PAGE (D-F) gel electrophoresis and electrotransfer to nitrocellulose membranes. A: Autoradiogram showing the small GTP-binding proteins detected in detergent/urea extracts from freshly ejaculated human sperm by the [α-^{32}P]GTP overlay assay. B: When sperm from the same mixture of Percoll purified donor sperm were incubated in a capacitation inducing medium for 6 hrs, [α-^{32}P]GTP-binding to two slightly more acidic proteins increased significantly (oblique downwards arrows), suggesting that in vitro capacitation alters these proteins GTP-binding capacity. C: Following autoradiography the NC-membrane used to detect [α-^{32}P]GTP-binding proteins in capacitated sperm was stained with gold colloids. The position of the GTP-binding proteins is indicated by a black rectangle. D: Neutral and basic human sperm proteins visualized by silver staining following NEPHGE/PAGE gel electrophoresis separation.
E: Two basic ATP-binding sperm proteins with pIs around 10 were detected by the [γ-^{32}P]ATP overlay procedure. Both 2DE protein spots appear to be labelled by the surface radioiodination procedure (F) and were found to bind lectins with affinity for terminal α-mannosyl residues (Figure 5), suggesting that the detected proteins might represent ATP-binding glycoproteins situated on the human sperm surface.
Preliminary mass spectrometry data suggested that the two basic ATP-binding sperm proteins might belong to the HSP-family of ATPase-regulated chaperones. However, the mass spectrometry analysis of the excised 2D gel spots was inconclusive as the tryptic digests also contained peptides that derived from other proteins in addition to several peptides from a HSP-family member.

One of the first 2D gel spots from which we obtained unknown amino acid sequences, was a 54 kDa protein with an isoelectric point (pI) of 5.3 (Figure 4). Eight reliable peptide sequences were acquired by tandem mass spectrometry. Two of the peptides were used to design optimised degenerate oligonucleotide primers, which were successful in producing DNA from human testis poly(A)+ mRNA. The RT-PCR-generated products were separated on agarose gels, stained with ethidium bromide, excised and subcloned into a pCR2.1-TOPO cloning vector. One of the resulting clones, consisting of a 173-bp insert, had both primers at either end of the RT-PCR-generated DNA segment and a productive reading frame that contained the translated amino acid sequences of the two peptides. The 173-bp-insert was then purified from the plasmid, radiolabelled with [α-^{32}P]dCTP, and used to isolate a full-length 1.5 kb clone (GenBank AF054910) from a human testicular cDNA library. The clone possessed a 1290-bp open reading frame (ORF) with a starting methionine codon at bp 128-130 and a stop codon at bp 1418-1420. Translation of the putative ORF revealed a 430-amino acid protein with a predicted molecular weight of 49.7 kDa. Computer analysis predicted a pI of 5.4, in agreement with the observed pI of the protein spot isolated from the 2D gel. Database searches revealed a marked resemblance of the full-length clone to sea urchin tektin B1 except at the N- and C-termini, where the sequences diverged. A BLOCKS search revealed all 4 of the tektin signatures in the sequence. We accordingly named the protein human tektin B1 (h-tekB1). Mice deficient in germ cell-specific tektin were shortly afterwards reported to exhibit male infertility and immobile-cilium syndrome due to impaired inner arm dynein function (114). The study demonstrated that tektin is essential for dynein arm integrity in the sperm flagella and that tektin deficiency debilitated sperm motility (114).

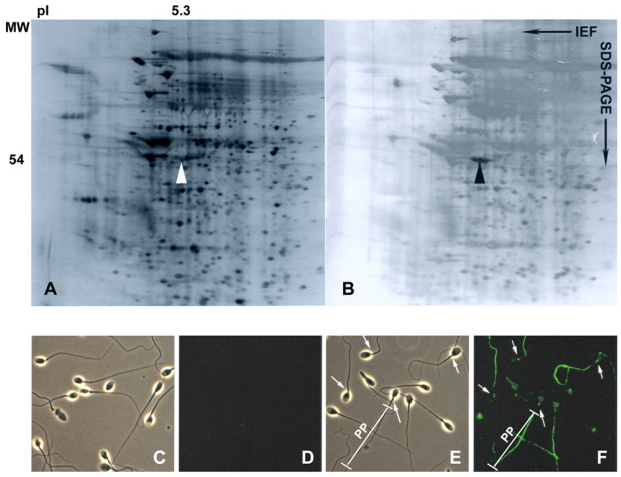

Figure 4
Analysis of the microtubule-associated protein tektin B1 in human sperm.
A: Area of silver stained IEF/PAGE gel of acidic human sperm proteins. The white arrow head indicates the protein spot that revealed the unknown amino acid sequences which led to the cloning of h-tekB1.
B: Antibody detection of h-tekB1 on a gold stained nitrocellulose membrane of IEF/PAGE separated sperm proteins. Immuno blotting with a rat antiserum raised against recombinant h-tekB1 protein revealed three tektin isoforms in human sperm. This finding is in accordance with the presence of numerous consensus kinase phosphorylation sites within the tektin B1 sequence, and suggests that human sperm tektin is regulated by modification of more than one phosphorylation site. Immunofluorescent studies localized h-tekB1 to the principal piece (PP) of the tail (bar) and the neck region (white arrows) in permeabilized human sperm (F). No staining was obtained with the pre-immune rat serum (D). C & E shows differential interference contrast (DIC) images corresponding to panels D & F, respectively.

The microtubule-associated protein h-tekB1 was the first human testicular cDNA we cloned and expressed based on unmatched amino acid sequence data obtained by ms/ms analysis of in-gel

tryptic digests. Although h-tekB1 turned out to be a cross-linker involved in the attachment of dynein to microtubules, which is expressed in cilia-bearing cells throughout the human body, and as such provided little novel insight into the regulation of sperm-specific functions, the cloning exercise generated valuable experience and validated our method for the design and employment of degenerate oligonucleotide primers. This approach was subsequently used to clone and characterize novel testis-specific sperm proteins, including FSP95 (III) and CABYR (VII), which demonstrated much more intriguing functional and immunological characteristics (see Chapter 4 below).

4E. Identification and characterization of surface expressed sperm proteins (I & II)

Detailed knowledge of the sperm surface molecules would be useful, not only to aid in understanding the complex processes of differentiation and maturation, but also because the plasma membrane is critical to sperm function. It is through their surfaces that sperm interact with their surroundings in the male and female tracts as they pass from the testis to the oviduct. Most important, the plasma membrane of the sperm contacts the egg investments, and the membrane overlying the equatorial segment of the acrosome is believed to be the initial site of fusion with the egg plasma membrane (8).

The composition and regulation of the plasma membrane (PM) of mammalian sperm have therefore been subjects of numerous studies, which have facilitated the identification and characterization of a variety of gamete surface molecules. The study of the sperm surface is complicated, however, by the organization of the plasma membrane into several distinctive domains, each with its own composition and function, by its complement of unique testis-specific proteins, which may be auto- or iso-antigenic in men and women, and by the addition of secretory proteins originating in the male sex accessory glands. As a consequence, the precise composition of the sperm plasma membrane, the identity of clinical relevant surface antigens, the molecular interactions that define domain specific functions, and the nature of the capacitation induced changes in membrane composition and activity still remain to be fully elucidated.

To be able to address any of these questions we first had to define the complement of surface expressed proteins within the catalogued sperm proteome. Vectorial surface labelling with sulfonated N-hydroxysuccinimide biotin and IodoBead catalysed ^{125}I-incorporation were used to identify surface exposed proteins. Computer analysis of 2D gel autoradiograms showed that 181 proteins with a molecular mass between 5 and 150 kDa and pI ranging from pH 4 to 11, were accessible for radioiodination on the surface of fresh, Percoll-harvested human sperm. Chemiluminescent image analysis revealed 228 biotinylated sperm surface proteins with molecular masses between 5 and 120 kDa and pI ranging from pH 4 to pH 10. The 2DE patterns of surface labelled proteins were highly reproducible, although minor inter-donor variations were noted in the net-charge (pI) as well as in the relative concentration of some of the labelled proteins (I). Known intracellular proteins, including the cytosolic proteins valosin-containing protein, LDH-C4 and calreticulin, the cytoskeletal proteins tubulin and actin, and the intraacrosomal protein SP-10, were neither radioiodinated nor biotinylated. Conversely, the testis-specific isoform of angiotensin converting enzyme (tACE) a known ectoenzyme attached to the sperm plasma membrane (115, 116), the sperm specific GPI-anchored surface hyaluronidase PH-20 (117, 118) as well as known components of both cancer and gamete cell surfaces, including several members of the heat shock protein (HSP) superfamily (119-124) were all consistently labelled. Taken together these findings indicate that the employed procedures labelled surface exposed species.

98 human sperm surface proteins were found to be accessible for vectorial labelling with both radioiodine and biotin (I). However, several of the dual-labelled surface proteins were found to cluster in horizontal charge trains on 2D gels, suggesting that the actual number of distinct surface species may be lower, and that the net-charge of many sperm membrane proteins is altered by electro-negative modifications such as phosphorylation or glycosylation. The polymorphic testis specific CD52 analogue SAGA-1 typifies a sperm surface protein, whose electrophoretic mobility is affected by the composition of its N-linked carbohydrate sidechain (110, 111) (Figure 8).

The glycosylated nature of many sperm membrane proteins became evident when lectin blotting experiments were combined with vectorial surface labelling to identify the repertoire of glycoproteins expressed on the surface of human sperm. Several clusters of closely-migrating radioiodinated proteins were found to bind lectins (Figure 5), consistent with the contention that micro-heterogeneity in the charged moieties of a radiolabelled surface protein's oligosaccharide sidechain(s) produces a charge-train on 2D autoradiograms.

The study of radioiodinated sperm surface proteins unintentionally led to the discovery of a novel negative imaging technique for detection of stainable proteins on complex 2D autoradiograms (II). During a pre-holiday, last minute assembly of an autoradiography cassette, the X-ray film and the intensifying screen were incorrectly placed on each side of a NC-membrane containing 2DE separated radiolabelled proteins, of which one had been visualized by the chromogenic substrate DAB following antibody binding. The resulting autoradiogram demonstrated a clear negative image (shadow) corresponding to the position of the immunostained antigen. By comparing autoradiographic images generated from different arrangements of blotting membrane, intensifying screen and film, it was demonstrated that photons emitted from the intensifying screen are obstructed by a stained protein spot, resulting in the creation of a negative image on the X-ray film. The principle of action of the negative imaging method is illustrated in Figure 6. As the technique allows simultaneous detection of radiolabelled and immunolabelled and/or gold stained protein spots on a single 2D autoradiogram (Figure 5), and can be used with different radioisotopes, it is well suited to guide the selection of well-defined subsets of the proteome for microsequencing (II).

4F.Differential extraction and enrichment procedures

When 2DE separated surface labelled sperm proteins were targeted for ms/ms analysis it quickly became evident that many coomassie and silver stained 2D gel protein spots contained two or more different sperm proteins. Tryptic digests from protein spots consistently labelled with both biotin and radioiodine, could for example be dominated by peptides originating from proteins associated with the cytoskeleton of the flagellum, emphasizing the necessity for enrichment of preparations with sperm membrane proteins prior to electrophoresis. Temperature-induced partitioning of hydrophilic and hydrophobic proteins following TX-114 solubilisation, and the release of GPI-anchored membrane proteins by phosphatidylinositol-specific phospholipase C (PI-PLC) treatment, proved to be particularly resourceful and rewarding approaches.

Enrichment of hydrophobic proteins by TX-114 phase-partitioning prior to 2DE separation, enabled sufficient enrich-

ment of membrane proteins to achieve microsequencing data from several unknown sperm proteins (116). Some of these unmatched amino acid sequences were later used to design gene-specific oligonucleotide primers as described for h-tekB1 (section 2D), which resulted in the cloning of two novel human sperm proteins, SAMP14 and SAMP32. SAMP32 was found to contain a transmembrane domain in the carboxyl terminus and to be associated with the acrosomal membrane in
capacitated sperm (125), while SAMP14 was shown to be loosely associated with the plasma membrane, but firmly attached to the acrosomal membranes via a GPI-anchor (126), accentuating the efficiency of the TX114-based membrane protein enrichment procedure.

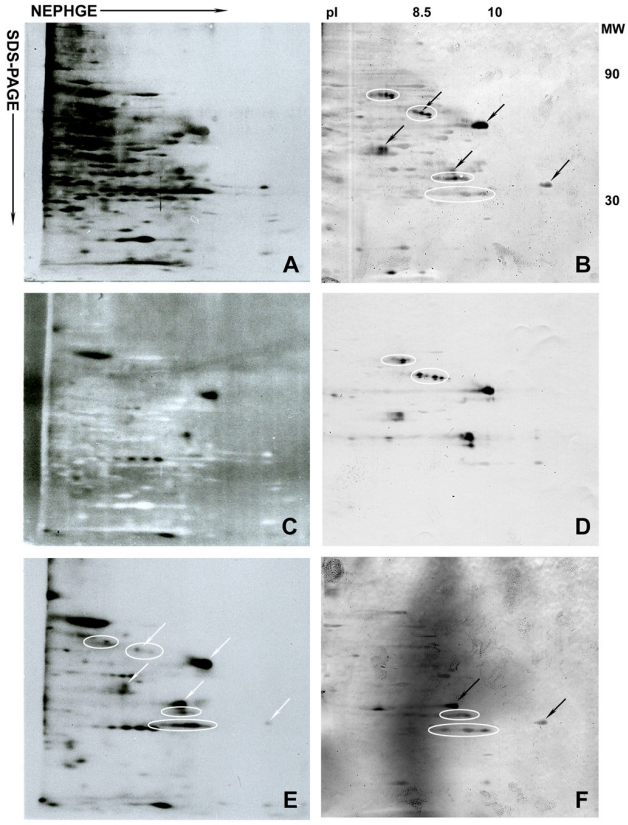

Figure 5
Analysis of non-ionic detergent/urea solubilized human sperm proteins following two-dimensional gel electrophoresis (NEPHGE/PAGE) and transfer to NC-membranes.
Proteins extracted from surface radiolabelled sperm were visualized by gold colloid staining (A), prior to detection of the 125I-labelled proteins by autoradiography (E). Autoradiography performed according to the principles of the negative imaging technique (II) revealed the exact positioning of surface labelled proteins within the global pattern of gold stained sperm proteins on the membrane (C). Human sperm glycoproteins were identified by the lectin blotting procedure. B: Affinity blot with HRP-conjugated concanavalin A lectin (ConA), which has affinity for terminal α-D-mannosyl and α-D-glycosyl residues. D: Blot with pisum sativum lectin (PSA) that has affinity for terminal α-D-mannosyl residues. F: Blot with solanum tuberosum lectin (STA) that, similar to wheat germ agglutinin (WGA), binds to oligomers of N-acetylglucosamine. Five surface exposed ConA-binding sperm glycoproteins are indicated by oblique arrows in panels B & E. Two of the proteins were also detected by STA (oblique arrows in F), suggesting that they contain multiple or complex carbohydrate side chains. Electrophoretic charge-trains of glycosylated sperm surface proteins are indicated by white ovals in panels B, D, E & F.

4G. Investigation of GPI-anchored sperm proteins
Glycosyl-phosphatidylinositol (GPI) anchors attach a wide variety of peripheral surface proteins to the outer leaflet of the cell membrane in a multiplicity of eukaryotic organisms from protozoa to man (127). Positioned at the C-terminus of mammalian proteins, the GPI anchor is a rather complex structure comprising a phosphoethanolamine linker, a conserved glycan core, and a phospholipid tail that anchors the protein within the outer leaflet of the plasma membrane lipid bilayer (128). More than one hundred different proteins are peripherally attached to eukaryotic plasma membranes in this fashion. GPI-anchors allow increased membrane protein mobility, packing of proteins and protein sorting, and GPI-anchored proteins (GPI-APs) have been implicated in important functions such as cell adhesion, cell-cell interactions, cell signalling and signal transduction (reviewed in 128-130). GPI-APs typically localize in lipid rafts, dynamic membrane microdomains enriched in selective lipids. The lipid rafts are formed by clustering of the cholesterols and sphingolipids of the outer leaflet of the plasma membrane into domains that float in the bilayer (131). The unique lipid composition of these membrane microdomains attracts a variety of membrane receptors, facilitating the assembly of multicomponent adhesion and signal transduction complexes (132). The cholesterol- and sphingolipid-rich rafts in the outer leaflet are connected to lipid domains in the inner leaflet of the bilayer where cytoskeletal components and signal tranducer molecules such as G-proteins and non-receptor tyrosine kinases are anchored at high density (131). Lipid microdomains thus create a dynamic membrane microenvironment where receptor molecules and their downstream effectors are recruited into close proximity, thus enabling the control of efficiency, intensity and specificity of signalling (132).

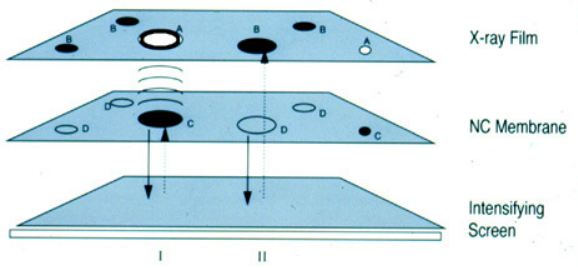

Figure 6
Principle of action of the negative imaging technique.
The negative imaging sandwich with intensifying screen and X-ray film placed on opposite sides of the NC-membrane containing stained proteins (left moist in a plastic sheet protector). Decaying radioactive isotopes in radiolabelled proteins immobilized on the NC-membrane emit β-particles in all directions, and those which hit the intensifying screen (downward arrows) generate photons. These photons must pass through the NC-membrane to reach the silver halide crystals in the film emulsion (dotted upward arrows). The subset of photons that hits the moist insoluble chromogenic reaction product or gold colloids is absorbed, giving rise to a negative shadow on the film (designated I). When the membrane immobilized protein is not stained the photons pass freely and no shadow image is generated on the film (II).

The content of lipids and their asymmetric distribution defines the fluidity and lateral diffusion properties of plasma membranes (133, 134). Differences in lipid content may thus be a critical factor in maintenance of the specific molecular composition of the plasma membrane in the distinct sperm surface domains by restricting the lateral diffusion of membrane components between adjacent domains (135, 136). Recent studies have demon-

strated the presence of lipid rafts in the plasma membrane of mammalian sperm (reviewed by Gadella et al. (135). More interesting, in vitro capacitation was shown to induce aggregation of sperm raft structures (135, 137), providing the first real insight into how molecular redistributions in the sperm plasma membrane may be regulated during the initial stages of capacitation (see also Chapter 4).

The mammalian sperm plasma membrane expresses both GPI-APs of testicular origin such as the sperm-specific hyaluronidase, PH-20 (117), and the CD52 variant, SAGA-1 (111), as well as a collection of GPI-anchored species acquired during post-testicular sperm maturation, including CD55, CD59 and CD73, which are also found on cells of the immune system (138-140). Native GPI-APs such as decay accelerating factor (DAF, CD55) and CD59 can spontaneously transfer from one cell to another in vivo (141, 142). Spontaneously released GPI-APs are inserted into the receiving cell membrane by simple exogenous addition, and appear to retain the same characteristics and functions as their endogenously expressed analogues (141-144). This may explain how GPI-APs, including CD52 and PH-20, transfer from epididymosomes onto the surface of the sperm plasma membrane (145, 146). It has also been suggested that GPI-anchored regulators of the complement cascade may play a role in protecting sperm from immune attack during transit in the male and female reproductive tracts (147).

Recent studies suggest that GPI-APs are involved in key stages of the fertilization process. PGAP1, a deacylase expressed in the endoplasmic reticulum, is responsible for the removal of the inositol-linked acyl chain from newly synthesized GPI-APs prior to their departure for the Golgi pathway (114). The transport of GPI-APs between the ER and the Golgi apparatus was severely reduced in PGAP1-defective CHO cells, indicating that remodelling of the lipid-anchor is important for the intracellular traffic of GPI-APs (114). While females from PGAP1-knockout mice were fertile, males were almost completely sterile due to the sperm's inability to attach to the zona pellucida (148). Sperm from PGAP1 KO mice showed elevated expression of surface associated GPI-APs such as CD52 and CD55 (148).

Phosphatidylinositol-specific phospholipase C (PI-PLC), which catalyses the hydrolysis of phosphatidylinositol to generate 1,2-diacylglycerol and myo-inositol 1,2-cyclic phosphate provides a useful tool for releasing peripheral proteins from their lipid anchorage. However, PI-PLC only cleaves lipids containing the myo-inositol group, whereas GPI-APs with a fatty acid acylation of the inositol ring are resistant to PI-PLC cleavage (128).

In order to use PI-PLC to investigate the expression of GPI-APs on the human sperm surface and to study the involvement of PI-PLC sensitive surface proteins in gamete interaction, a procedure for hydrolysis of phosphatidylinositol-anchors on the surface of intact cells was established and optimized by monitoring the release of the known PI-PLC sensitive GPI-anchored sperm hyaluronidase PH-20 and the appearance of surface biotinylated sperm proteins in the culture medium from PI-PLC treated sperm. The existence of two groups of 53 kDa PH-20 isoforms in human sperm, one of which is resistant to PI-PLC treatment, was demonstrated by these pilot experiments (Figure 7).

PH-20 has previously been localized over the entire surface of sperm from several mammalian species where it is attached via a GPI-anchor and in the acrosomal compartment, where it is bound to the inner acrosomal membrane (149, 150). In accordance with this dual localization of PH-20, the basic, surface radioiodinated PH-20 forms in human sperm were found to be PI-PLC-sensitive, while the unlabelled acidic PH-20 forms were resistant to PI-PLC treatment (Figure 7). This suggests that the acidic PH-20 forms represent the human homologue to the intra-acrosomal macaque PH-20 form thought to be involved in the penetration of zona pellucida (150, 151).

The newly gained experience with GPI-AP analysis was next applied to the study of a polymorphic sperm-specific analogue of CD52, a GPI-anchored lymphocyte differentiation marker implicated in signal transduction. The sperm antigen was identified with a monoclonal antibody S19, which had previously been shown to agglutinate and immobilize human sperm, and to inhibit mouse *in vitro* fertilization (152).

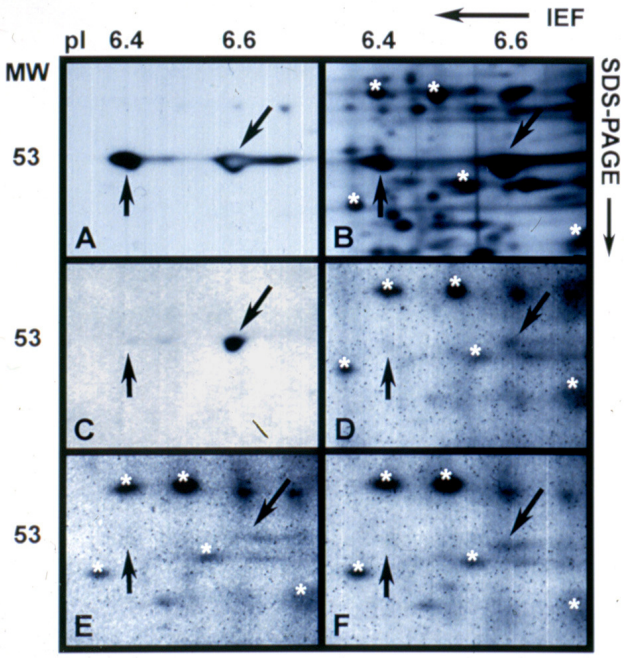

Figure 7
Investigation of the testis-specific, GPI-anchored hyaluronidase PH-20 in human sperm.
In order to optimise the PI-PLC treatment procedure, the temporal release of surface biotinylated sperm proteins was monitored by avidin blotting. Treatment with 3 units of PI-PLC from bacillus cereus (Molecular Probes) per ml for 20 min was found to be optimal for cleavage of the glycolipid anchors in 10^8/ml human sperm, as no more surface biotinylated proteins were released by prolonged treatment with this amount of enzyme, while reduced enzyme concentrations required longer treatment periods to complete the release of PI-PLC sensitive membrane proteins into the culture medium (data not shown).
2DE-IB of human sperm with a polyclonal, monospecific rabbit antiserum against PH-20 revealed two major groups of PH-20 antigens with MW of 53 kDa, each comprised of two or more closely migrating isoforms (A). The position
of the PH-20 antigens within the pattern of silver stainable sperm proteins is indicated on the enlarged gel area shown in figure B.
While PI-PLC treatment of intact Percoll-harvested sperm released the majority of the basic PH-20 from the sperm plasma membrane, only traces of the acidic PH-20 forms were retrieved from the medium (C).
PI-PLC only cleaves lipids containing the myo-inositol group, whereas GPI-APs with a fatty acid acylation of the inositol ring are more or less resistant to PI-PLC cleavage.
2DE autoradiograms of radioiodinated sperm surface proteins were next analysed to determine whether the acidic PH-20 forms are accessible for PI-PLC treatment on the surface of intact sperm or if their resistance to PI-PLC might be due to the lack of a GPI-anchor or acylation of the anchorage structure. Panels D-F shows the enlarged gel area on autoradiograms obtained from three separate surface labelling experiments. While the PI-PLC sensitive basic PH-20 forms were consistently and strongly labelled

with ^{125}I on the human sperm surface, the amount of ^{125}I-isotopes incorporated into the PI-PLC resistant acidic PH-20 forms barely allowed their detection by autoradiography (D-F). This suggests that the acidic PH-20 forms represent the human homologue to the intraacrosomal hyaluronidase form found in other primates (Yudin et al., 1999).
The unpublished data presented in this figure contradicts our previous report that both the acidic and basic PH-20 forms are accessible for biotin labelling on the sperm surface. What was considered an indication for surface localization of all 53 kDa PH-20 antigens in the earlier study (I) was based on computer comparison analysis, which showed co-migration of the PH-20 antigens on 2DE immuno blots and biotinylated protein spots on 2DE avidin blot images. However, at that time, experiments that would have validated the findings, such as immuno precipitation followed by avidin blotting or avidin-mediated purification of biotinylated proteins followed by immuno blotting, were not performed, and we later discovered that several surface labelled 2D protein spots contained more than one protein. Thus, it cannot be excluded that the reported biotinylation of PH-20 forms actually reflects the detection of a distinct group of co-migrating biotin-labelled sperm proteins.

The S19 mAb bound a carbohydrate epitope present on the 15-25 kDa polymorphic glycoprotein SAGA-1 (110). 7 charge variants each consisting of 3-5 mass variants of SAGA-1 were resolved on high resolution IEF/PAGE gels, in which the acidic gel area had been expanded (Figure 8). Edman degradation analysis of immunopurified SAGA-1 glycoprotein showed that the core peptide is identical to the sequence of CD52. Sperm SAGA-1 was shown to be sensitive to PI-PLC treatment (Figure 8), and immunochemical studies revealed N-linked carbohydrate epitopes present on sperm CD52 that are absent on lymphocyte CD52, indicating that the sperm-specific variant is generated by differential glycosylation (111). SAGA-1 localized over all the surface domains in human sperm, and is one of only a few known sperm surface isoantigens implicated in immunological infertility in humans (153).

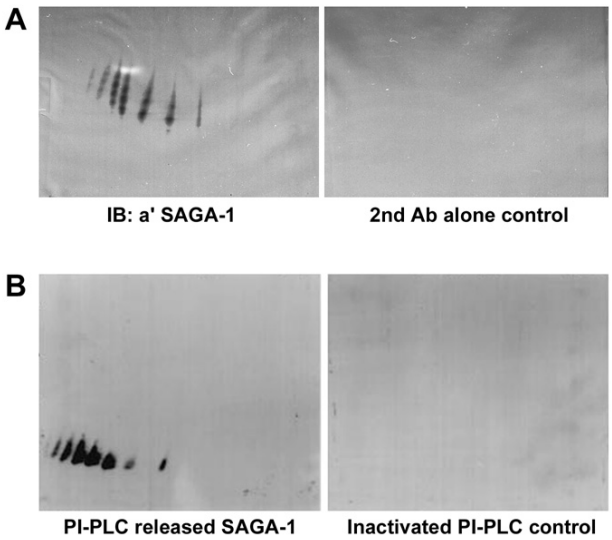

Figure 8
2DE immuno blot analysis of SAGA-1, the sperm-specific analogue of CD52.
A: Immuno staining with the S19 monoclonal mouse antibody revealed 7 charge variants of the SAGA-1 antigen, each consisting of 3-5 mass variants in human sperm (left). No signals were detected on blots incubated with secondary antibody alone (right). Enlarged areas of IEF/PAGE gels where the content of acidic ampholines had been increased to expand the coverage of very acidic sperm proteins are shown.
B: PI-PLC treatment of whole, motile sperm released the seven charge variants of SAGA-1, indicating that all are attached to the human sperm surface by GPI-anchors (left). No SAGA-1 antigens were detected in the medium from sperm treated with heat-inactivated phospholipase (right).

To study the entirety of PI-PLC sensitive GPI-APs on the surface of human sperm, percoll harvested sperm were surface labelled with biotin before being resuspended in Ham's F-10 medium containing PI-PLC. After 20 min, sperm and particulates were pelleted by centrifugation, and the cell-free medium was dialyzed against distilled water after the addition of protease inhibitors. The dialyzed solution was concentrated by lyophilization and the proteins were reconstituted in a detergent/urea buffer. Following 2DE and electrotransfer to nitrocellulose membranes, the biotinylated sperm proteins released by phospholipase treatment were visualized by avidin blotting. PI-PLC treatment of intact human sperm caused the release of five major and a several minor clusters of biotinylated sperm surface proteins into the culture medium (Figure 9). The most acidic low molecular weight group of sperm surface proteins released by PI-PLC, includes the epididymal glycovariant of CD52, SAGA-1 (indicated by a star in Figures 9A & 9D).

By 1998 multiple studies had implicated GPI-anchored sperm proteins in several key prefertilization events as well as in the fertilization process itself. The first indications for the involvement of GPI-APs in fertilization came from the identification and characterization of a GPI-anchored egg receptor in sea urchin sperm (154, 155). Antibody blocking assays have since implicated both PH-20 (150) and SAGA-1 (156) in binding of mammalian sperm to the zona pellucida, and the monoclonal antibody against human SAGA1, S19, was shown to influence the sperm penetration assay (152). Moreover, immunization of male and female guinea pigs with the GPI-anchored hyaluronidase PH-20 was found to provide 100% effective contraception for all experimental animals (157).

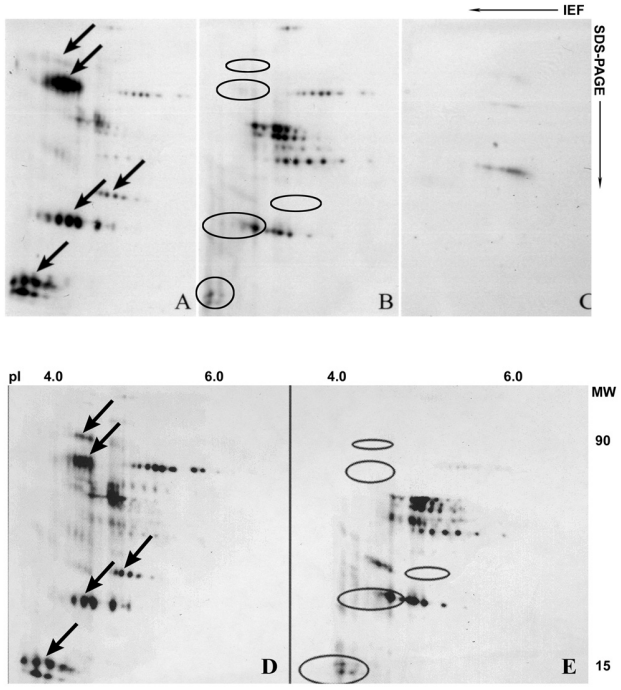

Figure 9
Global analysis of PI-PLC-sensitive GPI-anchored human sperm surface proteins.
A: Surface biotinylated proteins from PI-PLC treated sperm were retrieved from the culture medium and detected by avidin blotting following

IEF/PAGE separation. PI-PLC sensitive GPI-anchored proteins are indicated by oblique arrows.
B: Several groups of biotinylated proteins were spontaneously released from the surface when sperm were left in a PI-PLC free medium for a similar length of time.
C: The position of biotin labelled PI-PLC demonstrated by 2DE avidin blotting.
D: Biotinylated proteins released from PI-PLC treated sperm were detected by 2DE avidin blotting. The sperm preparation used in D & E was obtained from a second donor.
E: Spontaneous release of biotin-labelled surface proteins from donor 2 sperm.
Note the similarity between the clusters of PI-PLC sensitive and spontaneous released surface proteins detected in the samples from the two different sperm donors.
The composition of the reconstitution buffer was: 2% (w/v) octyl-β-D-glucopyranoside (OBG), 9 M urea, 100mM dithiothreitol, 2% ampholines (pH 3,5-10) and the protease inhibitors PMSF (2 mM), EDTA (5 mM), TLCK (3 mg/ml), pepstatin A (1.5 μM), and leupeptin (2 μM).

4H. Demonstration of GPI-anchored proteins involved in the fertilization process (V & VI)

Heterologous sperm are readily incorporated into zona pellucida-free hamster oocytes (158). Due to this promiscuity, which is not seen with zona pellucida-free eggs from other rodents (159), ZP-free hamster egg have frequently been used to assess the fertilization capacity of human sperm (160). In order to determine whether the polymorphic complement of PI-PLC sensitive membrane proteins resolved in Figure 9 might be involved in the final stages of fertilization, we next examined how PI-PLC treatment of human sperm affected their ability to bind to and fuse with hamster oocytes.

While PI-PLC treatment of human sperm significantly increased their ability to bind to zona pellucida-free hamster oocytes, it did not alter the ability of sperm to fuse with the oolemma (IV). One explanation for the increased binding of PI-PLC treated human sperm to the hamster oolemma could be that a GPI-anchored decapacitation factor is removed from the sperm surface, unmasking or enabling the assembly of specific receptor molecules/complexes required for sperm-oolemma binding. A recent study indeed identified a decapacitation factor receptor in mouse sperm as the GPI-anchored protein phosphatidylethanolamine-binding protein 1 (161). A similar existence of GPI-AP receptors for decapacitation factors on human sperm would thus explain the elevated oolemma-binding of PI-PLC treated human sperm.

While treatment of human sperm with PI-PLC did not affect sperm-egg fusion, treatment of ZP-free hamster oocytes with PI-PLC was found to block their ability to bind and fuse with untreated human sperm (IV). This significant finding encouraged us to continue the study of GPI-anchored gamete proteins role in fertilization in a more appropriate in vitro fertilization model, where both gametes originate from the same species, namely mice (V).

When both mouse sperm and zona-intact oocytes were pretreated with a highly purified preparation of PI-PLC and coincubated, there were no significant effects on sperm-zona pellucida binding however, fertilization was reduced from 59.6% (control group treated with heat inactivated PI-PLC) to 2.8% (PI-PLC treatment group). PI-PLC treatment of mouse sperm alone had no significant effect on sperm-egg binding or fusion. The discrepancy between this finding and the increased binding of PI-PLC treated human sperm to ZP-free hamster oocytes previously observed, may be due to specie-specific variations in the surface expression of GPI-APs or may be caused by differences in plasma membrane 'maturity' between the two sperm populations. While the first study used ejaculated human sperm, the second study used mouse sperm collected from the cauda epididymidis. Similar to what was found with hamster oocytes, PI-PLC treatment of mouse oocytes significantly reduced their ability to bind and fuse with untreated mouse sperm (Figure 10), and the inhibitory effects were shown to be PI-PLC dose-dependent.

The involvement of oolemma-attached GPI-APs in sperm binding was later confirmed in a study by Alfieri and colleagues, who found that oocyte-specific GPI-AP knockout female mice have severely reduced fertility (162). As the mouse model investigation had the best prospects of being accepted for publication in a well-regarded journal, we submitted this study first.

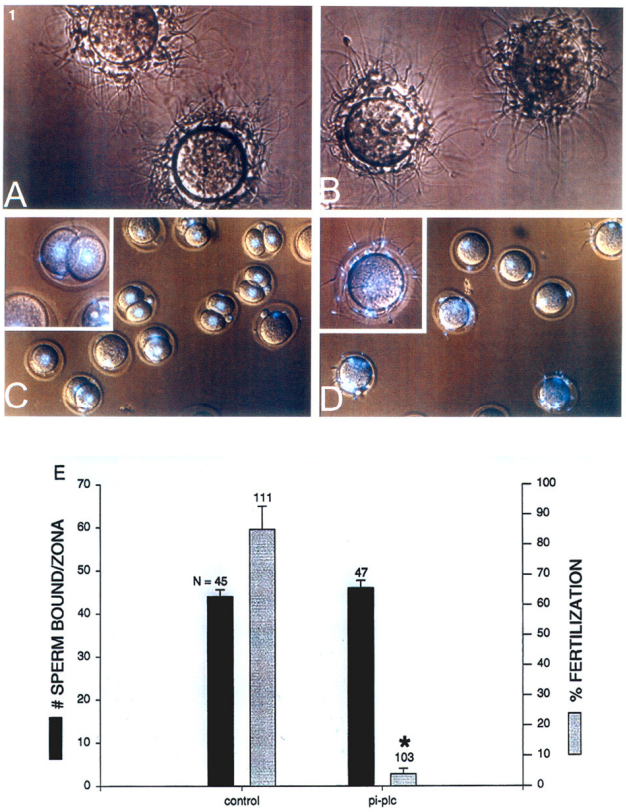

Figure 10

Evidence for the involvement of GPI-anchored proteins in fertilization. PI-PLC treatment of mouse gametes has no effect on sperm-zona pellucida binding, however, fertilization of zona-intact oocytes is blocked. No difference in sperm–zona pellucida binding was noted between the control group treated with inactivated PI-PLC (A) and the PI-PLC treated group (B). To evaluate the effects of PI-PLC on fertilization, the oocytes were washed following 2 h of gamete coincubation and incubated overnight. Following a 24-h incubation, a significant decrease in the number of fertilized oocytes (as determined by cleavage) was noted in the treatment group (D) compared to the control group (C). In (C) and (D), zygotes were treated with 1μM Hoechst 33342 for 10 min and washed to visualize sperm which accumulated in the perivitelline space of the PI-PLC treated group (Fig. D, inset). Images for (A) and (B) (200x) were recorded using phase contrast. Images for (C) and (D) (200x, insets 400x) were recorded using combined phase-contrast and fluorescence microscopy. Results are shown quantitatively in the histogram (E). Bars represent means +/- the standard deviation of three individual experiments. N, total number of oocytes per group. *P lower than or equal to 0.05 (Student's T test).

Treatment of sperm with PI-PLC prior to coincubation with zona-free oocytes was subsequently shown to have no effect on sperm-egg binding and fusion, whereas treatment of eggs with PI-PLC inhibited sperm-egg binding and fusion (V).

In order to identify the GPI-anchored oolemma proteins involved in fertilization, egg surface proteins were labelled with biotin and the PI-PLC released species detected by avidin blotting. PI-PLC treatment of ZP-free mouse oocytes released two polymorphic clusters of biotinylated proteins with approximate molecular weights of 70 kDa (pI 5) and 35-45-kDa (pI 5.5) into the culture medium. The two groups of GPI-APs were clearly visible on 2D avidin blots of oocytes treated with heat inactivated PI-PLC but were absent from images of PI-PLC treated egg surfaces. The identity of these oolemma GPI-APs remains to be determined, but preliminary data suggest that the 70 kDa forms consists of GPI-anchored variants of the ZP3-glycoprotein, which is best known for its role as the sperm receptor of the egg coat (163). Interactions between sperm and the zona pellucida glycoprotein ZP3 have been shown to trigger the acrosome reaction in mammalian sperm (reviewed in 164 & 165). Several molecules, including PH-20 previously implicated in ZP-binding are present on the inner acrosome membrane, which becomes exposed on the anterior face of the sperm head upon acrosomal exocytosis. It is thus tempting to speculate that, following the penetration of the zona pellucida, such surface exposed ZP3-binding proteins may also facilitate the binding of sperm to GPI-anchored ZP3 variants on the oolemma surface.

5. Humoral immunity to sperm antigens

5A. Introduction

In order to better define the role of ASA in infertility, it is necessary to identify and characterize their cognate sperm surface antigens. A comprehensive understanding of the composition of the sperm plasma membrane and its content of sperm-specific antigens with key roles in sperm function and/or fertilization is not only imperative for the development of new diagnostic procedures that can distinguish fertility-reducing ASA from those found in fertile individuals, but is also likely to facilitate the design of sperm-based contraceptive vaccines. The development of a safe contraceptive vaccine has long been regarded as a valuable alternative to the presently available methods of contraception, and antisperm antibody-mediated immunoinfertility provides a naturally occurring model that indicates how a sperm-based contraceptive vaccine might work in humans (166).

In addition, emerging evidence for the involvement of cancer-testis antigens (CTA) in tumorigenesis (167), along with an increasing understanding of their biological roles and antigenicity, suggest that development of CTA-based cancer vaccines also will be the focus of much attention in the coming years.

Finally, the reactivity of ASA in infertile sera that have been shown to block a particular sperm function (e.g. zona pellucida-binding) may be used to identify the sperm surface antigens involved in the implementation of the antibody impaired activity, as demonstrated in section 3B below.

5B. Methods utilized in the study of antisperm antibodies and their cognate antigens

The first publication of data indicating a correlation between the concentration of agglutinating antisperm antibodies in sera from infertile men and their fertilization capacity (34) accelerated the development of new methods (and optimization of old techniques) for the detection of antisperm antibodies (ASA) in serum and genital tract secretions. Most of the resulting semi-quantitative assays were designed to measure functionally important aspects of ASA, such as their ability to immobilize sperm due to agglutination or complement-dependent lysis, and their interference with cervical mucus penetration, zona pellucida-binding/penetration or the capability of human sperm to fertilize zona-free hamster eggs, which was achieved with varying degrees of success (168, 169). Clinically relevant sperm auto- and iso-antigens must naturally be situated on the external face of the plasma membrane or exposed on the inner acrosomal membrane following the acrosome reaction. The immunobead binding test (170) and the sperm immobilization test (SIT)(171) both have relatively low incidences of false-positive tests and are arguably the best available qualitative assays for detection of antibodies bound to the surface of living sperm. Although the immunobead binding test is well suited to distinguish between ASA of different immunoglobulin classes (48), the gross antibody binding measured by the assay provides little information regarding the functional and prognostic relevance of the detected ASA. While the sperm immobilization test, which measures the capability of ASA to bind complement and destroy the sperm membrane, provides a far more reliable estimate of the clinical relevance of ASA (171, 172), the assay merely detects complement binding isotypes of ASA and sperm immobilization only occurs if the majority of the tail is coated with complement fixing antibodies (53). Thus the major limitations of both assays are their inability to distinguish the antigenic specificities of ASA and to provide accurate, specific quantitation of clinically relevant ASA.

The substantial number of different sperm functions and gamete interactions which may potentially be affected by ASA to reduce fertility indicates that several different sperm surface antigens or groups of antigens might be involved in immunosubfertility. The high incidence of ASA in the fertile population further implies that ASA from infertile patients target dissimilar sperm antigens, clusters of antigens, or antigenic epitopes, or possess distinct antigen-binding characteristics. Identification and characterization of sperm surface antigens (or epitopes) that are related to specific sperm functions, recognized by ASA from infertile patients, and distinct from “the common household antigens” detected by sera from fertile individuals, would make it relatively simple to establish new antigen-specific quantitative assays (e.g. microarrays). The development of function- and/or epitope-related ASA assays suitable for large scale serum analysis, would not only improve diagnostic/prognostic capacity and accuracy, but also ensure that the significance of ASA in infertility could be established once and for all.

The immuno blotting technique appears especially well-suited to investigate ASA reactivity as it combines complex antigen analysis with consecutive target purification compatible with mass spectrometry analysis and readily can be performed with prefractionated samples. The only obvious restriction is that the sperm antigens are exposed to denaturing conditions during electrophoresis. This drawback might be circumvented by the use of native gel electrophoresis at the expense of sample resolution.

Numerous studies have successfully employed immuno blotting for the analysis of sperm antigens since the technique was first used to compare antisperm antibody reactivities in sera from immunoinfertile patients and fertile controls in the mid-eighties (92, 173). We have used immuno blots from unidirectional SDS-PAGE gels (174) as well as from two-dimensional IEF/PAGE gels (97) to investigate the humoral immune response to sperm antigens in vasectomized men. These studies showed that the increased resolution of the protein antigens achieved by the addi-

tion of a second separational dimension was critical in order to achieve a detailed description of qualitative changes in serum antibody binding specificity following surgical sterilization.

We therefore routinely employed the 2DE IB technique to investigate and compare ASA reactivity in specimens from fertile and infertile individuals in subsequent studies. However, although one report revealed a possible correlation between 6 specific sperm surface antigens and ASA reactivity in infertile sera with high immunobead binding titers (175), and another study identified a distinct subset of sperm surface antigens specific recognized by ASA in sera from infertile women with high titers of sperm immobilizing antibodies (42), the molecular identity and functional characteristics of the identified antigen candidates remain to be determined.

5C. Immunogenicity of heat shock proteins on the human sperm surface (IX)

In a benchmark study, Clarke and colleagues demonstrated that preincubation of donor sperm with female sera containing high titers of anti-sperm antibodies caused significant inhibition of in vitro fertilization in humans (55). One of the six anti-sperm positive sera examined in the study was found to completely block IVF. Absorption with protein A abolished the serum's ability to inhibit sperm-zona binding and block IVF. The female serum (# 629) was later shown to react with a prominent 65 kDa band in western blots of SDS-PAGE separated human sperm proteins (176).

When we used serum 629 to stain 2D blots of human sperm proteins, the prominent 65 kDa surface antigen detected by the serum's IgG antibodies was found to consist of the testis-specific HSP70 family members, HSPA2 and HSPA1L. Six additional sperm antigens or charge-trains of similar sized antigens were detected by serum 629. Three of these groups of sperm antigens with MW of 90-93 kDa, 84 kDa and 78-79 kDa, were found to be accessible for vectorial surface labelling with biotin (IX).

Interestingly, these three high MW groups of serum 629-reactive sperm surface antigens were also recognised by a polyclonal rabbit antiserum raised against the most abundant form of the testis-specific HSPA2 antigen. This suggests that all the serum 629 reactive human sperm surface isoantigens either belong to or are closely related to the HSP70 family of chaperones.

This observation instigated us to look for mechanisms by which such anti-HSP immunity might be induced. HSPs are immunodominant antigens in numerous microbial pathogens (177) and both humoral and cellular immunity against microbial HSPs have been shown to cross-react with human HSPs (178). There is a high prevalence of asymptomatic persistent genital tract infections among infertile couples (179), and Chlamydia trachomatis (ct) serum antibodies in men correlate with reduced chances of achieving pregnancy (180), but no correlations between serum IgG against ctHSP60 in the male partners and pregnancy rates were detected (180).

An antiserum raised against a linear epitope in Chlamydia HSP70 was successfully used to immunoprecipitate sperm surface HSPA2, HSPA1L, and the two groups of HSPA2 cross-reactive surface antigens with molecular weights of 78 kDa and 90 kDa, which were also recognized by serum 629. These results demonstrated molecular cross-reactivity between Chlamydia trachomatis HSP70 protein (ctHSP70) and HSP70-like antigens on the human sperm surface which had been indirectly implicated in ZP-interaction and fertilization through their recognition by the IVF-blocking serum 629.

Taken together these findings imply an association between genital tract infection, immunity to HSP70 and reproductive failure. The study additionally demonstrates that the constituency of HSP chaperones on the human sperm surface is far more diverse, abundant and immunogenically cross-reactive than previously recognized, and suggests that plasma membrane-associated chaperones serve multiple functions in human sperm, some of which appear to be critical for sperm-ZP interaction and fertilization.

6. Capacitation induced changes in the sperm proteome

6A. The molecular basis for capacitation

In mammalian sperm the capacitation process has been shown to involve a series of concerted changes in ion fluxes, phospholipid metabolism, cAMP levels, protein phosphorylation, and plasma membrane composition, that trigger increases in cytosolic pH and $[Ca^{2+}]_i$, and hyperpolarization of the membrane potential Em (reviewed by Darszon et al., 181).

Capacitation takes place when sperm are exposed to a series of poorly defined molecular stimuli during their ascent of the female reproductive tract. The capacitation process is believed to begin with changes in the molecular composition of the plasma membrane. While adjustments in the sperm membrane lipid composition and rearrangement of its microdomains increases the membrane's fluidity characteristics (135, 136), alterations in protein composition include the loss or removal of surface-associated inhibitory compounds termed 'decapacitation factors' (182). Decapacitation factors (DFs) originate from the secretions of both the epididymis and the accessory sex glands. The first DF was identified by Fraser, who demonstrated that its removal from mouse sperm by gentle centrifugation enhanced the fertilization capacity, while reintroduction of the DF to capacitated sperm suspensions rapidly, but reversibly, inhibited their fertility ability (183). The DF was later shown to be a 40 kDa glycoprotein (184), which attaches to the mouse sperm surface through interaction with a GPI-anchored membrane protein (185). Other putative decapacitation factors have since been identified, including cysteine-rich secretory protein 1, phosphatidylethanolamine-binding protein 1, and decapacitation factor 10 (161, 186).

Recent studies have demonstrated that disordering of the fatty acid chains of phospholipids, as well as distorted phospholipid asymmetry in the sperm plasma membrane, occur simultaneously with changes in the distribution/mobility of lipid raft-associated proteins and alterations in the protein composition of membrane microdomains during in vitro capacitation (135, 136). In vitro capacitation in the presence of albumin and bicarbonate was shown to cause depletion of cholesterol from the membrane while at the same time inducing aggregation of lipid rafts in the sperm head plasma membrane (187). Bicarbonate mediates scrambling of the membrane amino phospholipids, which is essential to allow cholesterol efflux and clustering of lipid rafts. Notably, capacitation-induced cholesterol removal was found to be restricted to the non-raft membrane fraction of the sperm plasma membrane, leaving the reorganized, enlarged lipid rafts rich in cholesterol (136). Changes in the sperm plasma membrane lipid composition, bilayer asymmetry and microdomain organization will simultaneously alter the distribution of phospholipid interacting, raft-associated, and GPI-anchored sperm proteins. This lipid-mediated redistribution of membrane proteins is thought to facilitate the establishment of new signalling complexes among raft-associated proteins, initiating signalling pathways that further promote capacitation (135, 188). The redistri-

bution of sperm membrane proteins during capacitation may also serve to ensure that key multiprotein adhesion and signal transduction complexes of importance for interaction with the oocyte or its investments are assembled and positioned at the correct surface localization (136).

Capacitation can be accomplished in vitro using caudal epididymal or ejaculated sperm incubated in defined media containing a protein source such as albumin, $NaHCO_3^-$, Ca^{2+} and energy substrates such as glucose, pyruvate or lactate (189). Conditions conducive to in vitro capacitation result in increased tyrosine phosphorylation of proteins in both mouse (190) and human sperm (191, 192). The removal of BSA, $NaHCO_3^-$, or Ca^{2+} from the capacitation media conversely prevents the occurrence of both tyrosine phosphorylation and capacitation in mouse sperm (190). Two of the substrates for the capacitation-activated tyrosine kinases to be identified initially were AKAP4 (originally called AKAP82 or Fsc1) and AKAP3 (originally called FSP95 or AKAP110), two members of the A kinase anchoring protein family. Both phosphoproteins are localized in the fibrous sheath, a cytoskeletal structure unique to the principal piece of the mammalian sperm flagellum. The regulation of these and other sperm proteins by tyrosine phosphorylation is discussed in more detail below.

Variations in the amplitude and frequency of intracellular calcium oscillations and a gradual increase in the mean cytosolic free calcium ($[Ca^{2+}]_i$) concentration during capacitation are essential for spermatozoa to attain motility hyperactivation, a tail motion characterized by increased flagellar bend amplitude and beat asymmetry (8, 23). Sperm never initiate hyperactivated motility in Ca^{2+}-free or Ca^{2+}-deficient media (193). If hyperactivated in calcium containing media and transferred to calcium free media none are hyperactive 30 to 60 min later, but hyperactivation can be restored by addition of 2 mM calcium. Demembranated sperm have been used as models to ascertain that calcium can increase flagellar bend amplitude (194, 195). Measurements of intracellular calcium using a calcium sensitive fluorescent dye, Indo-1, showed that $[Ca^{2+}]_i$ is increased in hyperactivated sperm in both the head and tail, and that $[Ca^{2+}]_i$ oscillates with each flagellar bend, indicating a direct relationship between intracellular calcium regulation and hyperactivation (22).

Both adenosine 3',5'-cyclic monophosphate (cAMP) and calcium are involved in the regulation of sperm motility (196) and the cytosolic level of cAMP increases during capacitation (197). Pharmacological stimulants, which elevate intracellular cAMP such as phosphodiesterase inhibitors, caffein and pentoxifylline, have been shown to enhance sperm hyperactivated motility (198), increase the ability of sperm to penetrate the cervical mucus and bind to homologous zona pellucida (199, 200), thus increasing fertilization capacity (201). Calcium/calmodulin is an activator of both mammalian sperm adenylate cyclase (sAC) (202, 203) and cyclic nucleotide phosphodiesterase (204).

Numerous in vivo and in vitro studies have shown that bicarbonate (HCO_3^-) plays important roles in controlling sperm motility (205-213). The transmembrane movement of HCO_3^- has been associated with the increase in intracellular pH, the hyperpolarization of the sperm plasma membrane potential, and the enhanced cAMP anabolism, which have been shown to occur during the initial stages of capacitation (206, 211, 214-217). The enhanced synthesis of cAMP is mediated by a testis-specific , HCO_3^- -sensitive adenylyl cyclase, known as sAC (205, 218, 219). The HCO_3^- -dependent raise in cAMP production leads to the activation of protein kinase A (PKA) and, ultimately, increased protein tyrosine phosphorylation and the progression of the capacitation process towards the induction of hyperactivated motility and the acrosome reaction (191, 213, 220). Inward fluxes of HCO_3^- may thus influence sperm motility/capacitation directly through the induction of sAC-activity or indirectly via a rise in intracellular pH and the plasma membrane potential.

cAMP agonists have been shown to accelerate tyrosine phosphorylation of sperm proteins, while antagonists of PKA inhibit tyrosine phosphorylation and capacitation (220-223). Taken together these studies suggest that the activity of non-receptor tyrosine kinases in sperm at least in part is under the control of a sAC-dependent cAMP/PKA-regulated pathway, which is activated by elevated cytosolic levels of calcium and HCO_3^- anions.

The membrane protein(s) responsible for the transmembrane movement of HCO_3^- in mammalian sperm remained unknown until 1993, when a study by Parkkila et al. (224) showed that an anion exchanger detected in human testis and associated with the sperm plasma membrane is related to the major erythrocyte HCO_3^-/Cl^- exchanger AE1, also known as Band 3. The sperm protein was localized to the equatorial segment of both human and rat sperm by immuno fluorescence staining with antibodies raised against synthetic C-terminal peptides found in AE1 and its close homolog AE2 (224).

2D immuno blotting with a monospecific rabbit antiserum raised against the erythrocyte anion exchanger Band 3 revealed a polymorphic group of cross-reactive human sperm proteins with approximate molecular weights of 110 kDa. Moreover, we found that the cross-reactive sperm antigens had similar electrophoretic mobility as a group of neutral integral membrane proteins, which were radiolabelled with the hydrophobic photoactivatable reagent 3 (trifluoromethyl)-3-(m-[^{125}I]iodophenyl) diazirine (TID) (Figure 11), in accordance with the membrane spanning nature of the putative sperm HCO_3^- transporter (Naaby-Hansen, S. and Bjerrum, O.J., unpublished data).

Consistent with these findings, the cross-reactive human sperm anion transporter was later identified as the AE2 isoform of HCO_3^-/Cl^- antiporters, which is a close homolog of erythrocyte Band 3 (225). More recent studies of mouse sperm suggest that other channel types, including Na^+/HCO_3^- and $Na^+/K^+/Cl^-$ co-transporters, also participate in the regulation of anion fluxes during the capacitation process (226, 227).

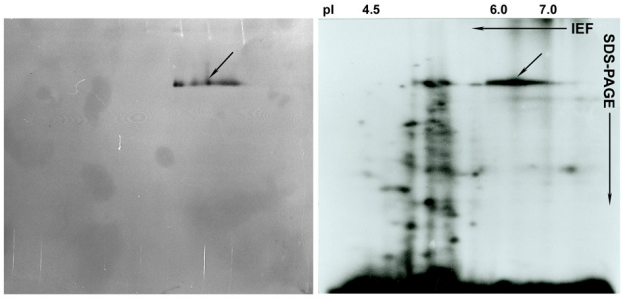

Figure 11
Human sperm proteins that are immunologically cross-reactive with the major erythrocyte anion exchanger Band 3 (AE1) as demonstrated by 2DE immuno blotting.
2D immuno blotting with a monospecific rabbit antiserum raised against the erythrocyte anion exchanger Band 3 revealed a polymorphic group of cross-reactive human sperm proteins with approximate molecular weights of 110 kDa (left). 2DE autoradiogram of human sperm proteins radiolabelled with the hydrophobic photoactivatable reagent 3 (trifluoromethyl)-3-(m-[^{125}I]iodophenyl) diazirine (right). Note that the cluster of band 3 cross-reactive sperm antigens had similar electrophoretic mobility as a group of TID-labelled membrane proteins [indicated by an arrow in the right image] (Naaby-Hansen, S. and Bjerrum, O.J., unpublished data).

6B. Cloning and characterization of FSP95 a novel phosphotyrosine regulated AKAP in the sperm flagellum (III)

Although the nature and integration of the regulatory mechanisms that control various aspects of the capacitation process are gradually emerging, the identity of the associated signal transducers and the plethora of effector molecules they trigger still remain to be defined.

We therefore instigated a 2DE-based proteomics study to identify tyrosine phosphorylated proteins in the human sperm and investigate their regulation during in vitro capacitation. 2D immuno blotting with monoclonal antibodies specifically directed against tyrosine phosphorylated residues was used to identify tyrosine phosphorylated proteins in fresh sperm, and to investigate phosphotyrosine regulation in sperm that had been exposed to capacitation conductive conditions for varying lengths of time. 2D IB analysis revealed increases in both the number and staining intensity of tyrosine-phosphorylated proteins with the duration of the period of capacitation (VII). The pattern and intensity of tyrosine phosphorylated proteins were shown to differ with the composition of the capacitation medium. Tyrosine phosphorylation was abolished in a concentration-dependent manner when sperm were capacitated in the presence of the tyrosine kinase inhibitor genistein. The immuno-detection of tyrosine phosphorylation was significantly reduced if the detergent solubilized proteins were treated with phosphatase prior to electrophoretic separation. Taken together, these findings indicate that the antibody-based procedure used for detection of phosphorylated sperm proteins following in vitro capacitation was valid and specific. The phospho-regulated sperm proteins were next targeted for mass spectrometry analysis. The first tyrosine phosphorylated protein we identified in this fashion was valosin-containing protein (VCP), a 90 kDa protein with a pI of 5.2. The intensity of the immuno staining with the anti-phosphotyrosine mAb RC20 was strongly increased in VCP from sperm that had been exposed to a capacitation inducing medium for 6 hr (Figure 12). 2D IB with a monoclonal antibody against VCP showed appearance of additional acidic antigen forms of VCP in sperm that had undergone in vitro capacitation compared to the VCP isoforms detected in fresh sperm (Figure 12), supporting the contention that capacitation induces tyrosine phosphorylation in human sperm VCP (VII) and indicating that more than one amino acid are modified.

Activation of T-lymphocytes has previously been shown to result in a tyrosine phosphorylation induced relocalization of VCP (228). A similar change in the subcellular localization of human sperm VCP following capacitation has been reported. VCP was found to relocate from the neck region to the anterior head region of human sperm after overnight capacitation (229). Due to these findings and the role of VCP as a chaperone, involved in membrane fusion events in other biological systems, it was suggested that the phospho-regulation of human sperm VCP might be a link between capacitation and the acrosome reaction (229).

However, immuno fluorescence studies of VCP in human sperm, we performed several years prior to the publication of the cited report, failed to show capacitation induced relocation of VCP to the acrosomal region of the sperm head. In contrast, our IF experiments using the same mAb as employed in the study by Ficarro et al., demonstrated VCP localization in the anterior head region of fresh, percoll harvested human sperm (Figure 12), and the number of sperm which stained positive for VCP in the neck region increased steadily during the first 6 hr of in vitro capacitation (Figure 12). This discrepancy indicates that further studies are necessary to determine the exact localization of VCP and to define its involvement in the capacitation of human sperm.

The strongly tyrosine phosphorylated protein migrating at 95 kDa, just above VCP, on 2D gels of human sperm proteins (Figure 12) was found to be a major auto- and iso-antigen frequently recognized in immuno blot analysis with sera from infertile men and women. Tandem mass spectrometry analysis (ms/ms) of tryptic digests of the 95 kDa protein produced sequencing data from 18 peptides (5-18 aa), none of which matched any known protein (present in the GenBank database). An 8 amino acid sequence was used to design a completely degenerate inosine containing primer which was successfully used to amplify a 1.0 kb piece of cDNA by 3'RACE PCR from human testicular Marathon ready cDNA (Clonetech). The full length cDNA contained a 2559-bp ORF encoding a 653 amino acid (aa) protein, which we later named Fibrous Sheath Protein 95 (FSP95) (III). Comparison of the deduced FSP95 sequence to the GenBank database in the summer of 1998, using BLAST and FASTA, revealed that human sperm FSP95 had closest amino acid similarity to a mouse sperm fibrous sheath AKAP, precursor of mouse AKAP82 (identity 33.6%, similarity 42.5%) and to the homologous human pro-hAKAP82 (identity 32.4%, similarity 39.4%). The expression of FSP95 mRNA was found to be testis-specific. A protein kinase A RII binding domain was identified between amino acids 124 and 141 of FSP95 (Vijayaraghavan et al., 1999). Immunoelectron microscopy identified the FSP95 antigen in association with the ribs of the fibrous sheath, which is believed to be involved in defining the shape of the flagellar beat (230). FSP95 was the second fibrous sheath protein to be cloned, sequenced and localized in human sperm. FSP95 and the identical protein AKAP 110 (the sequence of which was deposited in the GenBank September 1998, one month after the sequence of FSP95 had been deposited) have since become known under the common name AKAP3. AKAP3 is regulated by both serine- and tyrosine-phosphorylations (229), and was recently shown to associate with phosphodiesterase (PDE), which along with adenylyl cyclase defines the cytosolic level of cAMP (231).

The RII dimerization/docking (R2D2) domain of protein kinase A's regulatory subunit binds to the short (approx. 18 aa) amphipathic helix region of AKAPs (232). This interaction enables AKAPs to target protein kinase A (PKA) to specific substrates and distinct subcellular compartments, providing spatial and temporal specificity in the mediation of biological effects controlled by the cAMP-PKA pathway (Reviewed by Pidoux and Tasken, 231). However, in addition to scaffolding PKA, PDE and protein phosphatases, AKAPs also bind to a group of four proteins that share homology to the RII dimerization/docking (R2D2) domain. The R2D2 proteins function in the regulation of flagella and cilia independent of PKA activity and unlike RII do not bind cAMP (232). R2D2 proteins (ropporin, SP17, CABYR and ASP) are expressed at high levels in both testis and sperm, and mutants lacking R2D2 proteins exhibit abnormal sperm motility (233). The multiple phosphorylation sites involved in the regulation of AKAP3 during capacitation, the diversity of interaction partners implicated in the regulation of motile organelles (Figure 13), and the localization to the motion defining structures in the flagellum, indicate that AKAP3 is critically involved in the regulation of sperm motility and imply that its modification by tyrosine phosphorylation during capacitation marks a shift in flagellar activity/motion.

6C. Co-localization of the inositol 1,4,5-triphosphate receptor and calreticulin in the human sperm

Increased calcium oscillation frequency and a gradual raise in $[Ca^{2+}]_i$ in the sperm flagellum have been shown to initiate motility hyperactivation (23, 193). However, the nature of the initiating stimuli, the identity and regulation of the involved calcium binding proteins, and the flagellar-specific effector molecules they control, have remained more or less elusive. We therefore employed a ^{45}Ca-overlay assay to address the identity and regulation of calcium binding proteins in human sperm.

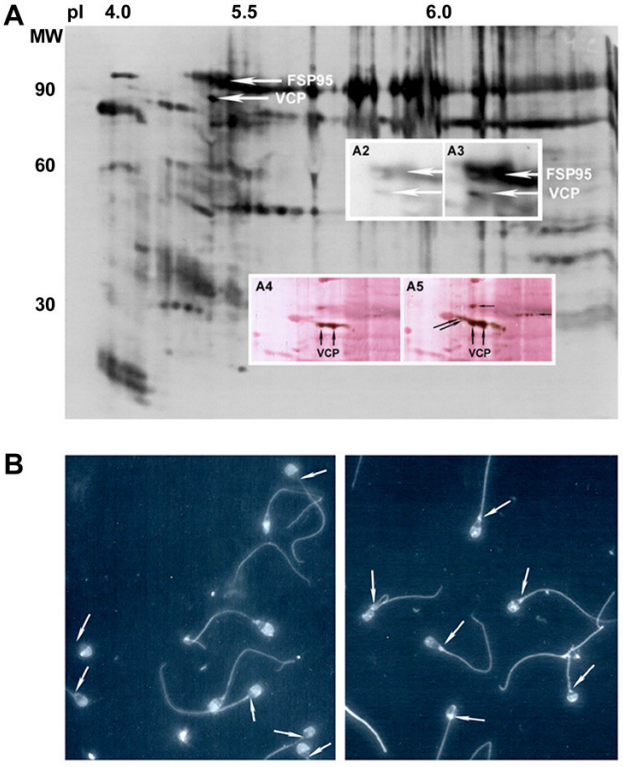

Figure 12
Analysis of valosin-containing protein (VCP) in the human sperm.
A: Tyrosine phosphorylated proteins in sperm that had been subjected to a capacitation-inducing medium for 6 hrs were demonstrated by immuno blotting with the monoclonal antibody RC20. The positions of VCP and FSP95 are indicated by horizontal arrows. In vitro capacitation significantly increased tyrosine phosphorylation of both proteins (A3) compared to the levels detected in fresh sperm (A2). Immuno staining with a monoclonal antibody against VCP revealed the existence of two major 90 kDa VCP forms in fresh sperm (A4) and the appearance of at least two additional VCP antigen forms following in vitro capacitation (upward oblique arrows in A5). The NC-membranes used in A4 & A5 were stained with colloidal gold prior to antibody incubation and subsequent chromogenic detection.
B: Localization of VCP in fresh human sperm (left) and in sperm subjected to a capacitation-inducing medium for 6 hrs (right), demonstrated by immuno fluorescence staining of permeabilized cells. The arrows in the left image indicate sperm without VCP concentrations in the neck region. In contrast, VCP localized to this region in most of the in vitro capacitated sperm (arrows in right image).

The first evidence for the existence of mobilizable stores of intracellular Ca^{2+} in mammalian sperm arose from studies employing thapsigargin, a specific inhibitor of the endoplasmic reticulum Ca^{2+} -ATPase Ca2+-pump. This pump moves cytosolic calcium back to the endoplasmic lumen and its inhibition leads to an increase in the free cytosolic Ca^{2+} concentration in other cell types. Meizel and Turner (234) demonstrated that the acrosome reaction in capacitated human sperm could be initiated by treatment with thapsigargin and suggested the existence of thapsigargin-sensitive intracellular Ca^{2+} stores either in the cytoplasmic droplets, the nucleus or the acrosome. Spungin and Breitbart (235) confirmed this finding with bovine sperm and showed that the outer acrosomal membrane indeed contains a thapsigargin-sensitive Ca^{2+} pump. Our demonstration of the major calcium storage protein calreticulin (CRT) in the acrosome and its concentration in the equatorial segment strongly supports the existence of a mobilizable store of calcium in the human sperm acrosome (VI). The localization of the IP3R to the same vesicle suggests that Ca^{2+} may be mobilized from this store at least partly via the IP3 pathway and subsequently pumped back via the above mentioned thapsigargin-sensitive ATPase.

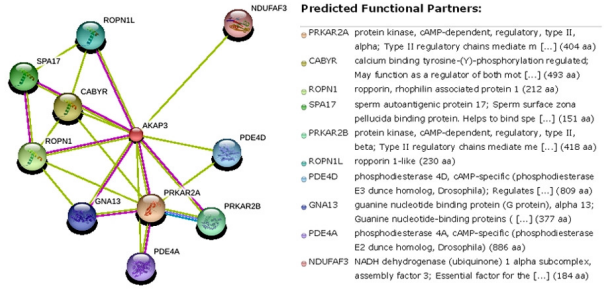

Figure 13
Schematic representation of the AKAP3 interactome as currently pre - sented in the protein-protein interaction database; STRING 9.0

The existence of two or more regulatory stores for intracellular calcium in sperm, one for the tail and one for the head, was first suggested by fluorimetric studies using calcium indicators such as Indo-1 and Fura-2. Cytoplasmic Ca^{2+} -oscillations in sperm are significantly more rapid than oscillations detected in other cell types (22). Intracellular Ca^{2+} is elevated in hyperactivated sperm and a correlation between Ca^{2+} -oscillation frequency and flagellar-bending frequency has been demonstrated in the proximal flagellar midpiece (23). Intracellular Ca^{2+} reaches two different elevated levels in intact moving hamster sperm, first with the achievement of hyperactivation and second upon completion of the acrosome reaction (23). Moreover, the increase of intracellular Ca^{2+} was greater in the midpiece than in the head of hyperactivated sperm, while the reverse was true for acrosome reacted sperm (23). The cited studies were almost all performed in rodent sperm, and at this time only the acrosome had been identified as a calcium store in mouse and rat sperm, leaving the localization of the putative second storage site unsolved.

The first calcium binding human sperm protein we identified by a combination of Edman degradation and mass spectrometry was calreticulin (CRT), migrating at a MW of 60.5 kDa and a pI of 4.2. CRT had previously been localized to the acrosome of rat sperm (236). CRT, the major calcium sequestrating protein in the sarcoplasmic reticulum of skeletal muscles and the major calcium binding protein in the ER of non-muscle cells, had recently been implicated in interactions with the inositol 1,4,5-triphosphate receptor (IP_3R) in Xenopus oocytes (237). Since IP3 serves as a second messenger in mammalian sperm, we postulated that co-localization of CRT and IP_3R to intracellular structures in human sperm would be indicative of the presence of an IP3-mobilizable calcium store. Double immunolabelling experiments demonstrated co-localization of CRT and IP_3R in the acrosome, the equatorial segment, and in vesicular structures in the cytoplasmic droplets of the neck region (Figure 14). Electron microscopic immunogold labelling localized CRT to the equatorial segment of

acrosome- reacted sperm and to membrane-enclosed vesicles within the cytoplasmic droplets of both acrosome-intact and acrosome-reacted human sperm, providing evidence for the existence of more than one intracellular Ca^{2+} -storage site in human sperm. Mouse sperm showed acrosome-restricted localization of CRT (Figure 14) similar to that reported for rat sperm (236), indicating that intracellular calcium is differentially regulated (or at least are sequestrated and released from different stores) in human and rodent sperm.

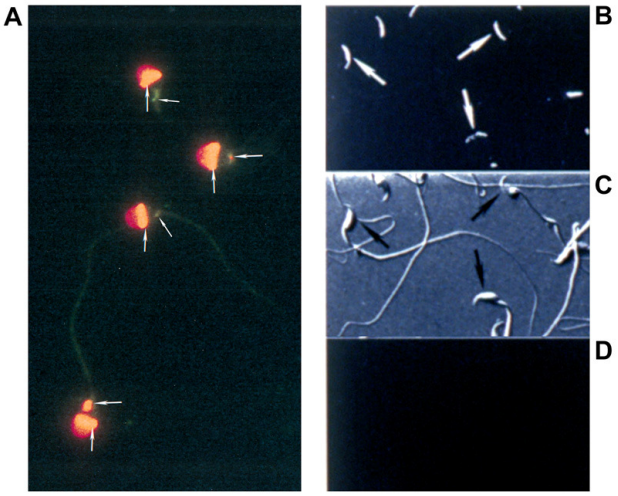

Figure 14
Localization of calreticulin in human and mouse sperm by immuno fluorescence microscopy.
A: Co-localization of calreticulin (CRT) and inositol triphosphate receptor (IP_3R) demonstrated by dual fluorescence staining. Co-localization was observed over the acrosomal cap, in the equatorial segment, and in vesicular structures in the neck region of human sperm (yellow staining). In mouse sperm the localization of CRT appears to be restricted to the acrosomal compartment (white arrows in B). Panel C shows the corresponding DIC image with arrows. No signals were detected in secondary antibody alone control experiments (D). Human and mouse sperm are shown at different magnifications.

The co-localization of CRT and IP_3R in membrane-enclosed structures in the neck of the human sperm added a new dimension to the comprehension of spatio-temporal calcium regulation in human sperm. A similar experimental approach was later used to identify an intracellular calcium store at the base of the head in bull sperm, which was indeed shown to be involved in the regulation of hyperactivated motility (238). This finding supports the notion that the membrane-enclosed calcium stores identified in the neck of human sperm are involved in the regulation of flagellar calcium waves/oscillations.

The putative molecular link between IP_3R-controlled calcium-store depletion and TRP-channel-mediated capacitative calcium entry, presented by the recent discovery of 80K-H protein in human sperm, is discussed below.

6D. Identification and characterization of calcium binding proteins associated with the human sperm plasma membrane (X)
This study combined vectorial radiolabelling with the ^{45}Ca-overlay assay and mass spectrometry analysis, to identify calcium-binding proteins situated on the surface of human sperm. Nine calcium-binding 2D gel protein spots were identified on coomassie stained preparative gels by computer-aided image analysis, five of which were found to be accessible to Iodo-Bead catalysed ^{125}I-labelling on the surface of intact, motile sperm.

An abundant calcium-binding surface protein with MW of 26.5 kDa and a pI of 5.2 was identified and excised from stained 2D gels and PVDF membranes. The protein was identified as serum amyloid P-component (SAP) by a combination of mass spectrometry and Edman degradation analysis. Computer comparison and densitometry analysis identified SAP as the surface labelled sperm constituent that bound relatively most ^{45}Ca-isotope in the overlay assay. Immunofluorescence staining of intact, motile cells demonstrated SAP binding over the neck, midpiece and tail regions, consistent with the mixed agglutination pattern obtained with antiserum against SAP (X). Recent studies suggest that SAP can act as an opsonin (239-242) and facilitate the uptake of apoptotic cells by direct interaction with the Fcγ-receptors on macrophages (243, 244). Binding of SAP and other members of the innate immune system to the asymmetric pattern of phospholipids found on apoptotic cells is also thought to have important immunomodulatory effects on the ingesting phagocyte, triggering it to release anti-inflammatory cytokines rather than to produce inflammatory cytokines, thereby collaborating in T-cell suppression and the maintenance of tolerance (245-247).

SAP binding and stabilization of cellular debris and soluble immune complexes thus appear to facilitate their subsequent clearance by phagocytes (248, 249). This is noteworthy, because mammalian spermatozoa are removed from the female genital tract via phagocytosis, mediated mainly by invading leukocytes and macrophages (250-252). SAP binds DNA and chromatin with high affinity and avidity (253), and it has been proposed that chaperone-like binding and stabilization of nuclear macromolecule antigens by SAP protect them from proteolysis and prevent subsequent spread of immunogenic degradation products (254).

Based on these findings, it is tempting to speculate that SAP participates in a molecular mechanism that facilitates the disposal of sperm remnants from the female genital tract, while at the same time ensuring that repetitive clearance of isoantigenic sperm and their cargo of super-coiled DNA by professional phagocytes occur without triggering severe inflammatory or antinuclear autoimmune responses (X).

A second ^{45}Ca-binding protein with a molecular weight of 80 kDa and a pI of 4 was identified as the 80K-H protein by microsequencing. 80K-H is a multifunctional Ca^{2+} -sensor originally identified as a substrate for PKC (255). While the calcium-binding opsonin SAP and the three calcium-binding HSP70 chaperones HYOU1, HSPA5 and HSPA2 are known constituents of the human sperm plasma membrane, this was the first demonstration of the 80K-H protein in a mammalian sperm. 80K-H has been associated with the regulation of intracellular signalling downstream of both the fibroblast growth factor receptor (256, 257) and the advanced glycosylation end products receptor (258), and it has been implicated in the regulation of protein translocation (259). 80K-H interacts with PKCζ and munc18c to induce glucose transporter 4 translocation to the plasma membrane (260). A recent study suggests that 80K-H additionally participates in the regulation of IP_3-induced calcium release through interaction with the cytoplasmic tail of IP_3-receptors (261). Finally, 80K-H has been shown to interact with and to regulate the activity of the epithelial TRP channel V5 (TRPV5) (262). The plasma membrane density and activity of TRPV5 channels are regulated via changes in their extracellular glycosylation status (263). Processing of specific N-linked carbohydrate sidechains from the ectodomain of TRPV5 channels appears to entrap them in the plasma membrane, resulting in increased Ca^{2+} influx (264, 265). This is noteworthy, as

previous studies have implied that 80K-H acts as a regulatory subunit of α-glucosidase II, an N-linked glycan-processing enzyme (266, 267).

Glycoproteins in the egg's extracellular coat, the zona pellucida, are the physiological agonists for the acrosome reaction. Zona pellucida (ZP)-binding generates a biphasic calcium response in sperm, which is currently thought to involve at least three separate yet sequentially-linked Ca^{2+} channels (268, 269). Activation of the ZP-receptor leads to a transient influx of calcium through T-type voltage-dependent calcium channels in the plasma membrane that are thought to be released from inactivation by the capacitation-induced hyperpolarization of the membrane potential (270). This brief (<500ms) initial elevation of $[Ca^{2+}]_i$ to micromolar levels activates the Ca^{2+} -sensitive phospholipase PLCδ, causing the generation of diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3), and consumption of the PM positioned substrate phosphatidylinositol biphosphate (PIP_2) (268, 271). The increased production of IP3 leads to the emptying of IP_3-receptor regulated intracellular Ca^{2+}-stores previously thought only to be situated in the acrosomal compartment (272-274). Similar to what happens in somatic cells, the depletion of Ca^{2+} from internal stores is thought to activate store-operated channels (SOC) in the sperm plasma membrane causing a sustained elevation in $[Ca^{2+}]_i$ (272, 274). Increases in calcium, cAMP and small G protein activities act together to set in motion the SNARE machinery (soluble N-ethylmaleimide-sensitive factor attachment protein receptor), which is required for the fusion between the outer acrosomal membrane and the overlying plasma membrane (268).
Several studies suggest that store-operated calcium channels in mammalian sperm belong to the transient receptor potential (TRP)-family of cation channels, whose members are closely related to the TRP gene expressed in Drosophilia photoreceptors (275). Five members of the TRP channel family have been detected in mammalian sperm (271, 274, 276), of which 4 localize to the head of the human sperm (268). More important, maitotoxin, which induces Ca^{2+} -uptake through its action on TRP channels, is the most potent inducer of the acrosome reaction in mouse sperm aside from ZP (277). TRPC2 has been proposed to participate in the sustained Ca^{2+} -influx triggered by ZP3 in mouse sperm (274), but appears to be a pseudogene in humans (277, 278). TRPC channels can form heteromultimers (279), and it is likely that the store-operated Ca^{2+} entry pathway in sperm involves several family members, which can at least partly substitute for each others, as TRPC2 null mice are fertile (280, 281). Several TRP channel regulating molecules have been identified, including STIM (282), junctate (283), PIP_2 (284), enkurin (285), and 80K-H (262), of which enkurin and junctate have been demonstrated in mouse sperm (283, 285).

Previous studies have indicated a major role for PKC in the upregulation of cytosolic calcium levels prior to the AR in human sperm (286-290), and it has been suggested that PKC participates in the opening of store-operated calcium channels in the sperm plasma membrane (291, 292). However, the molecular mechanism by which PKC controls capacitative calcium entry has remained elusive. Identification of the PKC substrate 80K-H in the human sperm proteome thus defines the first putative effector molecule which directly links PKC to both the regulation of IP3R-controlled intracellular calcium stores and to the opening of store-operated calcium channels in the plasma membrane. The presence and phospho-regulation of sperm 80K-H support the notion that store-operated calcium channels in human sperm belong to the TRP channel superfamily, and suggest that PKC might increase and sustain Ca^{2+} -influx prior to the AR through 80K-H-mediated upregulation and stabilization of active TRP channels in the sperm plasma membrane.

6E. Cloning and characterization of CABYR, a novel calcium binding tyrosine-phosphorylation regulated fibrous sheath protein involved in capacitation (VII)

In this study phosphoprotein analysis was combined with the ^{45}Ca-overlay assay and mass spectrometry to identify proteins at the intersections between the calcium and protein tyrosine kinase signal transduction pathways in human sperm.

The most prominent species identified by computer-aided comparison of ^{45}Ca-autoradiograms and 2DE immunoblots of tyrosine phosphorylated sperm proteins, was a polymorphic 86 kDa acidic protein with pI of 4. Mass spectrometry analysis of tryptic gel digests revealed five peptides whose sequences did not match any known sequences in the databases. Six variants of the protein, which we named CABYR (calcium-binding tyrosine-phosphorylation regulated), containing two coding regions (CR-A & CR-B) were cloned from human testis cDNA libraries, including five variants with alternative splice deletions (VII). The transcription of CABYR CR-A was found to be testis specific, while small amounts of minor splice CABYR variants are found in motile cilia of normal human bronchus and fallopian tubes (232).

A domain highly homologous with the A-kinase anchoring protein (AKAP)-binding dimerization/docking domains (R2D2) of the regulatory subunits of protein kinase A (PKA) is present in the N-terminus (aa 12-48) of CR-A in four CABYR variants. Five motifs containing the SH3 domain interacting consensus sequence P-X-X-P are present in CR-A and three in CR-B, providing structural modules for interactions with other proteins. CABYR also possesses an extensive proline-rich extension-like domain, which was recently shown to overlap several glycogen synthase kinase 3β-binding and dimerization domains (293). A putative EF-hand-like motif was identified in CR-A at aa 197-209. The calcium binding capacity was later shown to be restricted to this sequence in CABYR coding region A (294). More interesting, CABYR' calcium-binding capacity was shown to be positively regulated by capacitation-induced tyrosine phosphorylation (VII).

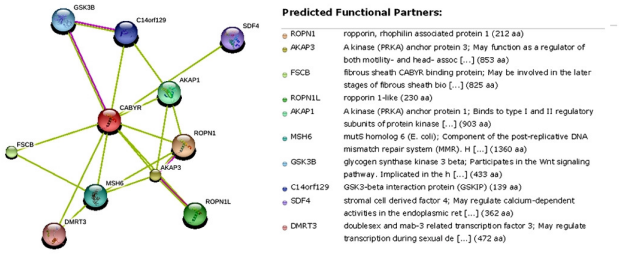

Figure 15
Schematic representation of the CABYR interactome as currently presented in the protein-protein interaction database; STRING 9.0

Indirect immunofluorescence microscopy with antibodies raised against recombinant CABYR showed staining of the entire length of the principal piece of methanol fixed sperm. Immunocytochemical electron microscopy showed gold particles distributed over the fibrous sheath compartment, including the surface of the longitudinal columns and ribs. Immunofluorescence localization of CABYR in the human testis showed staining of round and elongating spermatids in the seminiferous epithelium and testicular

spermatozoa within the lumen of the tubules, indicative of a postmeiotic pattern of expression of the CABYR gene.

CABYR interacts with a diverse array of proteins via its SH3-, R2D2-, and proline-rich extensin-like domains. Some of CABYR interaction partners are shown in the interactome presented in Figure 15 (STRING 9.0), and other associations are given below. CABYR possesses putative motifs for self-assembly, and unpublished data from our laboratory, achieved by diagonal gel electrophoresis (native gel electrophoresis followed by electrophoresis under denaturing conditions) followed by immuno blotting with anti-CABYR antiserum (Figure 16), suggest that the high molecular weight calcium-binding forms of CABYR are generated by homo- and hetero-oligomerization of lower molecular weight splice variants, which upon posttranslational modification (including phosphorylation) are assembled into high molecular weight complexes.

CABYR thus appears to have a role in the regulation of calcium sequestration and episodic release, and to form a high order scaffold with other R2D2-proteins and testis-specific A kinase anchoring proteins that serve to integrate cAMP/PKA, Rho and calcium signalling in the sperm flagellum. CABYR is finally believed to act as a Ca2+-shuttle and/or scaffold for enzyme complexes that mediate glycolysis-driven energy generation in the distal flagellum during hyperactivated motility.

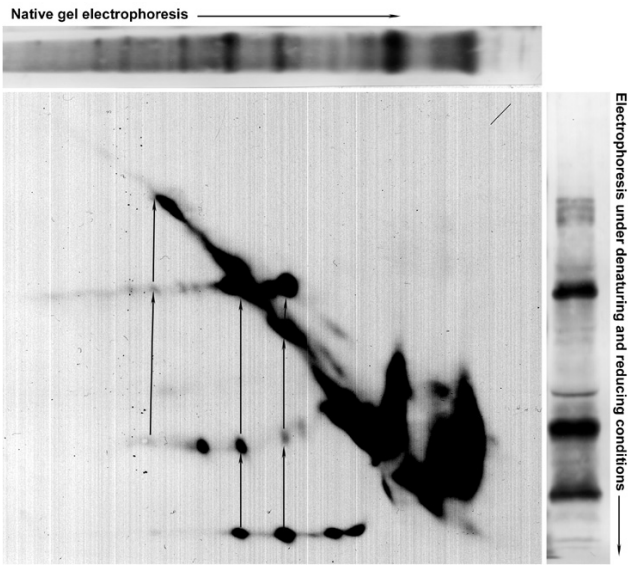

Figure 16
Oligomerization of CABYR variants demonstrated by immuno blotting following diagonal gel electrophoresis of sperm proteins solubilized in a non-reducing, non-ionic detergent buffer. Vertical arrows indicate heterodimerization of low molecular weight CABYR variants.

6F. CABYR is a cancer-testis antigen expressed in a variety of solid tumours

CABYR expression has recently been demonstrated in a variety of cancers, including brain (293), lung (295), and head and neck squamous cell cancers (Herr et al., unpublished data). However, while CABYR's roles during spermatogenesis and in the sperm fibrous sheath structure are emerging, its function in cancer cells still remains to established.

We are currently studying CABYR's role during malignant transformation and evaluating its diagnostic and prognostic potentials. The data presented below have been presented at the CIMT–meeting in Mainz, May 2010, and at the HUPO –meeting in Geneva, 2011.

In this study, we combined immunoprecipitation procedures with mass spectrometry and western blot analysis to define the molecular interactions of CABYR in lung adenocarcinoma cell lines. l Butyrate-induced protein 1, a protein tyrosine phosphatase and the regulatory subunit of serine/threonine-protein phosphatase 2A, both co-precipitated with CABYR. These putative interactions are in accordance with our previous demonstration of phospho-modified serine- and threonine-residues in testicular CABYR, as well as the contention that the calcium-binding capacity of CABYR is regulated by tyrosine phosphorylation.

More notable, several enzymes involved in the regulation of glycolysis, including phosphofructokinase, pyruvate kinase, fructose-biphosphate aldolase, malate dehydrogenase, and the testis-specific subunit of lactate dehydrogenase, as well as hypoxia-inducible factor prolyl hydroxylase 3 and ADP/ATP translocase 1, also co-purified with CABYR from A549 cells.

Taken together, these results suggest that CABYR associates in high order, multiprotein complexes that mediate anaerobic glycolysis-driven energy generation in A549 adenocarcinoma cells, a finding in accord with CABYR's localization to the fibrous sheath structure in sperm where elements of the glycolytic cascade and nucleotide carriers also are found. Our immuno histochemical analysis revealed that distinct islets of CABYR positive cells are frequently localized within the malignant regions of lung tumours. Based on the CABYR interactome, it is thus likely that the high mitotic activity seen within such CABYR positive areas reflects the growth advantage gained by cancer cells in which energy production has been adapted to the hypoxic conditions of the tumour environment. We therefore hypothesize that CABYR expression confers improved survivability to lung cancer cells and is an indicator of aggressive tumour growth.

7. Summary & future perspectives

The major objectives of the research program; 1) to provide new insight into the regulation of sperm function, 2) to identify and characterize sperm antigens implicated in immuno-subfertility, and 3) cloning and characterization of new testis-specific sperm proteins, were all successfully achieved.

The initial phase of the study (1993-1995) established the most detailed map of the human sperm proteome of its time. For the first time both acidic, neutral and basic human sperm proteins were analysed in a single study (I). The majority of the function-targeted analytical approaches employed had never previuosly been used for the study of mammalian gametes. Two methods for vectorial labeling of surface exposed sperm proteins were employed, and the techniques were combined with membrane protein enrichment procedures such as TX114 phase partitioning and PI-PLC treatment, for the analysis of the human sperm surface arcitecture and regulation.

The analysis of human sperm plasma membrane proteins resulted in the following 1) Characterization of previously unknown variants of epididymis-specific, GPI-anchored proteins in the human sperm, including the PH-20 hyaluronidase and the glycovariant of CD52, SAGA-1, which represents one of the few known sperm-specific surface isoantigens implicated in immunoinfertility. 2) Identification of human sperm surface antigens recognized by human serum antisperm antibodies, which had previously

been shown to inhibit sperm-zona pellucida binding and block human in vitro fertilization. These sperm surface antigens were shown to be immunogenically cross-reactive with the testis-specific chaperone HSPA2 and Chlamydia trachomatis HSP70, indicating the existence of an association between genital tract infection, immunity to HSP70 and reproductive failure (IX). 3) Identification of five calcium binding proteins associated with the human sperm surface, of which SAP and 80K-H protein proved particularly interesting. The demonstration of the calcium-sensing PKC substrate 80K-H in human sperm supports the contention that store-operated calcium channels (SOCs) in mammalian sperm belong to the transient receptor potential-family, and provides a strong candidate for the elusive signalling links between PKC and SOCs in the human sperm plasma membrane, and between these SOCs and IP_3R-regulated intracellular Ca^{2+} -stores (X). 4) A candidate participant in the removal of sperm remnants from the female genital tract was identified when the calcium-binding opsonin SAP was shown to be associated with the human sperm surface (X). 5) Analysis of radioiodinated sperm surface proteins led to the development of a new imaging technique that enables simultaneous analysis of radiolabelled and chromogenically or gold stained proteins in a single autoradiographic image (II). 6) Treatment of mouse and hamster oocytes with PI-PLC was shown to significantly reduce their ability to bind and fuse with sperm, while treatment of fresh human or caudal epididymal mouse sperm had no negative effect on their ability to bind to and fuse with the oocyte plasma membrane. These two in vitro fertilization studies thus revealed the existence of PI-PLC sensitive GPI-anchored proteins on the oolemma surface with essential role(s) in sperm binding and fusion (V & VI). The increased binding of PI-PLC treated human sperm to untreated hamster oocytes, supports the notion that GPI-anchored proteins also participate in 'decapacitation' events at the human sperm surface. This finding also implies that the heterogenic complement of PI-PLC-sensitive GPI-APs at the human sperm plasma membrane must have accomplished any "adhesive" role(s) prior to or at the time of ZP-penetration.

Our study of calcium binding and tyrosine phosphorylated sperm protein regulation during in vitro capacitation resulted in the cloning and characterization of two previously unknown tyrosine-phosphorylated proteins associated with the fibrous sheath structure in the tail of human sperm (III & VII). Both testis-specific phosphoproteins are involved in the regulation of sperm motility. One of the proteins belong to the A kinase anchoring protein-family (AKAP3), while the other protein, which gains calcium-binding capacity when phosphorylated during capacitation (CABYR), is an AKAP-interacting R2D2 protein. Characterization of these proteins in our and other laboratories has provided valuable new insight into the regulation of sperm motility and energy generation in the mammalian sperm flagellum.

Calreticulin was identified as a major calcium-binding protein in human sperm, and investigation of its subcellular localization resulted in the discovery of membrane-enclosed vesicular structures in the neck of human sperm, where calreticulin was shown to co-localize with the inositol 1,4,5-triphosphate receptor, a key regulator of intracellular Ca^{2+} -stores in somatic cells (VI). This indication for the existence of other intracellular, IP_3-sensitive Ca^{2+} -stores in human sperm, in addition to the previously known acrosomal storage site, presented a new dimension to our comprehension of the spatio-temporal regulation of capacitative calcium entry in human sperm.

Thus, despite the limitations in the dynamic range of 2DE and its inherent restrictions in the resolution of high molecular weight and very hydrophobic proteins, our employment of the technique for sperm proteome analysis in 1994 was proven to be a good choice; given that the human genome project remained to be finalized, our approach occurred in time to enable the discovery of new genes.

The long list of exciting results generated by the huge number of 2D gel and mass spectrometry -based proteomics studies of mammalian gametes that have been published by different research laboratories over the past five years further validates the technique's suitability for the study of gamete proteomes. Today several hundred human sperm proteins have been identified and mapped following 2D gel electrophoresis, and the 2DE methodology is likely to remain a valuable component of the analytical toolbox that will be employed for fertilization studies in the foreseeable future. New developments in mass spectrometry-based methodologies have propelled dynamic proteome studies forward, simplifying, among others, the examination of posttranslational protein modifications and quantitative protein analysis. The drastic advances in analytical capacity, along with the steady growth in available bioinformatics data and the increasing number of easily accessible data-mining programs, imply that our current understanding of the molecular networks that regulate gamete-specific functions and the fertilization process will be significantly improved in the near future.

The sperm proteome research program, which I pioneered in 1994 is still ongoing in Professor Herr's laboratory at University of Virginia, and has so far led to the cloning and characterization of 8 novel testis-specific proteins (III, VII, VIII, 125, 126, 296-298). Some of these sperm-specific proteins have been identified as cancer-testis antigens in different types of cancers.

Emerging evidence indicating that reactivation of normally silent germline gene-expression programmes confers some of the central characteristics of malignancy to tumours (reviewed by Simpson et al., 167) emphasizes the importance of future research in reproductive biology, in particular delineation of the regulatory networks that control gene expression during gametogenesis and the post-translational regulation and molecular function of cancer-testis antigens. Advanced understanding of the gamete specific proteins, including their biological roles, interactive regulation and immunogenicity will likely define their diagnostic/prognostic values as cancer-markers, identify the best-suited candidates for cancer vaccines, and perhaps even facilitate identification of new therapeutic targets or combinatorial treatment regimes.

CABYR is among the most promising of the new cancer-testis antigens identified in the sperm proteome research program. Due to its role as a highly interactive, phospho-regulated scaffolding component with a putative involvement in anaerobic glycolysis-driven energy generation and its immunogenicity in cancer patients, CABYR has the potential of becoming a useful marker for energy metabolism in tumour cells and emerges as a cancer vaccinogen candidate. However, further studies are required before CABYR' efficiency as a cancer biomarker and/or vaccinogen can be accurately evaluated.

SUMMARY

This is a review of ten previously published studies of the human sperm proteome. Proteins expressed on the sperm cell surface were identified and characterized by a combination of vectorial labelling with radioiodine and biotin, PI-PLC treatment, two-dimensional gel electrophoresis, immuno and lectin blotting

procedures, affinity overlay assays with radioactive nucleotide triphosphates and 45Ca, and mass spectrometry analysis. Examination of capacitation-induced modifications of the human sperm proteome led to the cloning and characterisation of two new phospho-regulated cancer-testis antigens, which we named Fibrous Sheath Protein 95 (FSP95) and CABYR (calcium-binding tyrosine phosphorylation regulated).
A protein kinase A RII binding domain is present between amino acids 124 and 141 identifying FSP95 (now commonly known as AKAP3) as a member of the A kinase anchoring protein-family which provides spatial and temporal specificity to the cAMP-PKA pathway. In addition to scaffolding PKA, PDE and protein phosphatases, AKAPs also bind to a group of four proteins that share homology to the RII dimerization/docking (R2D2) domain of PKA' regulatory subunit.
CABYR, which is one of these four proteins, also interacts with a diverse array of signal tranducers via its SH3-, R2D2-, and proline-rich extension-like domains. AKAP3 and CABYR appear to associate in high molecular weight multi-protein complexes, which regulate the flagella' energy supply and movements. Diagonal gel electrophoresis experiments suggest that the high molecular weight signal-integrating scaffold partly is established by homo- and hetero-oligomerization of lower molecular weight splice variants of CABYR. The putative role of CABYR in lung cancer cells is finally discussed

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