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CARACTERIZAÇÃO BIOQUÍMICA E FUNCIONAL DO ANTÍGENO RECONHECIDO PELO ANTICORPO MONOCLONAL TRA 54 NO EPIDÍDIMO

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Biologia Celular e Estrutural, na área de Histologia.

Orientador: Prof. Dr. Luis Antonio Violin Dias Pereira

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SUMÁRIO

Resumo

O processo de fecundação em mamíferos depende de uma seqüência de eventos que culminam na ativação do oócito pelo espermatozóide. A diferenciação completa das células germinativas testiculares em células com capacidade fecundativa envolve testículos, epidídimos, ductos deferentes e trato reprodutor feminino. No epidídimo, a superfície dos espermatozóides pode sofrer diversas modificações, em um processo conhecido como maturação epididimária. Diversas proteínas sintetizadas e secretadas pelas células epiteliais do epidídimo serão posteriormente localizadas na superfície ou mesmo no interior da vesícula acrossômica do espermatozóide. A aquisição da capacidade de fertilização pelo espermatozóide tem sido correlacionada à esta nova organização das moléculas de membrana proporcionada durante o trânsito epididimário. A obtenção de anticorpos monoclonais que reconhecem os antígenos expressos pelas células germinativas testiculares durante seus processos contíguos de diferenciação, ou pelas células dos ductos pelos quais os espermatozóides transitam durante o processo de maturação tem constituído uma importante estratégia para permitir a geração de um mapa das moléculas que atuam na preparação do espermatozóide para a fecundação. O anticorpo monoclonal (Amc) TRA (*testicular germ cells immunized to rat - monoclonal antibody*) 54 reconhece um antígeno localizado em espermátides dos túbulos seminíferos de camundongos C57 BL/6 com idade superior a 24 dias pós-parto e também um antígeno expresso nas células epiteliais da cabeça do epidídimo e em espermatozóides da luz deste órgão. A molécula secretada percorre a luz do epidídimo no sentido ântero-posterior e, neste percurso, é incorporada pelos espermatozóides em trânsito. Com o intuito de contribuir para a compreensão dos complexos mecanismos que preparam o espermatozóide para a fecundação, o presente trabalho buscou identificar e reconhecer a estrutura protéica e a função biológica do antígeno reconhecido pelo Amc TRA 54 nas células epiteliais do epidídimo. No futuro, os resultados obtidos poderão contribuir com a geração de novas ferramentas clínicas para a solução de problemas relacionados à biologia da reprodução.

Abstract

In mammals, fertilization depends on a sequence of events that culminates in the activation of the oocyte by the spermatozoa. The complete differentiation of the testicular germ cells in cells with fertilization ability involves the testis, epididymis, vas deferens and female reproductive organs. In the epididymis, the membrane surface of the spermatozoa may be modified, through a process known as epididymal maturation. Several proteins synthesized and secreted by the epididymal epithelial cells can be further located on the spermatozoa membrane surface or even though inside its acrosomal vesicle. The acquisition of the fertilization ability by the spermatozoa has been related with a new molecular organization of the membrane provided during the epididymal transit. The production of monoclonal antibodies (mAb) that recognizes antigens exclusively expressed by testicular germ cells or by the cells of the ducts through the spermatozoa passes during its maturation has been considered an important approach to permit the elaboration of the molecular map involved in the spermatozoa preparation. The mAb TRA (testicular germ cells immunized to rat - monoclonal antibody) 54 recognizes an antigen located in spermatids of seminiferous tubules of C57 BL/6 mice more than 24 days old and also in a specific population of epithelial cells in epididymal caput and in the luminal spermatozoa. The synthesis and release of the epididymal antigen occur in an androgen-dependent manner and independent of the testicular expression. Released antigen moves down the epididymis and is further incorporated by luminal spermatozoa. This work was performed in order to comprehend the complex mechanisms that prepare spermatozoa for the fertilization process. The molecular structure of the epididymal protein and its biological functions were investigated and solved. In a near future, the obtained results can contribute with new clinical tools for solving problems in reproductive biology.

Capítulo I

Introdução

O processo de fecundação em mamíferos depende de uma seqüência de eventos que culminam na ativação do oócito pelo espermatozóide (LÉDARE et al, 1999). Este processo resulta na formação do zigoto, a partir do qual serão desenvolvidas duas populações celulares distintas, células somáticas e germinativas. A partir da diferenciação das células germinativas, são originados novos gametas com potencial para reiniciar o ciclo reprodutivo da espécie (McCARREY, 1993).

A gametogênese masculina ou espermatogênese consiste em um processo complexo, altamente eficiente e essencial para a propagação da vida, compreendendo uma seqüência de fenômenos celulares envolvendo divisões mitóticas, meióticas e processos morfogênicos de diferenciação (BRINSTER and NAGANO, 1998; SOFIKITIS et al, 2003). O processo de espermatogênese ocorre nos túbulos seminíferos do testículo e, para o homem, compreende um período de 64 dias, resultando na produção diária de aproximadamente 13 x 10^8 espermatozóides (OGAWA et al, 1997; OGAWA, 2001). Em mamíferos, a proliferação das espermatogônias e sua diferenciação em espermatozóides têm início na puberdade e se estende até a senescência, (JIANG and SHORT, 1998), sendo capaz de produzir, neste período, um número virtualmente ilimitado de gametas (DOBRINSKI, 2005), capazes de atingir o trato reprodutor e o gameta femininos com o objetivo de restabelecer a diploidia (SALING and LAKOSKI, 1985; YANAGUIMACHI, 2001).

Além do período gonadal, ocorrido no túbulo seminífero, o processo de diferenciação das células germinativas em espermatozóides envolve também um período extra e pós-gonadal, ocorrido no epidídimo, ducto deferente e trato reprodutor feminino (ORGEBIN-CRIST, 1967; DENIS, 1994). A regulação exata da diferenciação das células germinativas requer a expressão de genes em estágios específicos não somente nas células germinativas, mas também nas células somáticas testiculares e dos ductos pelos quais estas células transitam

(DENIS, 1994; TANAKA et al, 1994; KIRCHHOFF et al, 1998). Desta maneira, embora tenham completado o processo de meiose e adquirido muitas das estruturas essenciais para o desenvolvimento da capacidade de fecundação, os espermatozóides do fluido testicular são pouco móveis e incapazes de fecundar (HAMIL et al, 2000). A capacidade de fecundação só é adquirida depois da passagem pelos ductos eferentes, cabeça, corpo e cauda do epidídimo, bem como trato reprodutor feminino (DACHEUX et al, 1987).

Dentre os ductos pelos quais o espermatozóide passa até atingir o gameta feminino, o epidídimo é o responsável por providenciar um microambiente especializado no qual os espermatozóides desenvolvem importante grau de diferenciação funcional e antigênica. Além de transportar e armazenar os espermatozóides, o epidídimo induz alterações morfológicas e bioquímicas que permitem ao gameta potencializar a motilidade, estabilizar a cromatina e adquirir sítios de reconhecimento, ligação e fusão para as moléculas da zona pelúcida, bem como regulação indutiva para a reação acrossômica (ORGEBIN-CRIST, 1969; BOUÉ et al, 1994; MOORE, 1998; KAUNISTO et al, 1999).

A elaboração deste microambiente especializado se deve ao trabalho conjunto dos diferentes tipos celulares que compõem o epitélio epididimário ao longo de toda a extensão do órgão. O epidídimo é subdividido em três segmentos principais, cabeça, corpo e cauda, além de um segmento inicial anterior à cabeça (FLICKINGER et al, 1978; GOYAL, 1985; CUMMINS et al, 1986; CALVO et al, 1997; VICENTINI and ORSI, 1987; YEÜNG et al, 1994). A variação regional dos diferentes segmentos do epidídimo (segmento inicial, cabeça, corpo e cauda) indica que o epitélio celular ao longo do ducto possui diferentes funções e/ou estão sob diferentes fatores de regulação. Segundo Blanchard e Robaire (1997), dentre esses fatores encontram-se os andrógenos que atuam em diferentes regiões e em diferentes tipos de células epiteliais do ducto epididimário. Cinco tipos celulares distintos compõem o epitélio pseudo-estratificado cilíndrico do epidídimo: células principais, basais, apicais, claras e, por fim, células

halo (HAMILTON, 1975). Resumidamente, as células principais são as mais abundantes ao longo de todo o ducto epididimário e apresentam longos estereocílios relacionados com a secreção e absorção de proteínas (CUMMINS et al*,* 1986; FOUCHECOURT et al, 2003). As pequenas células basais, mais numerosas no segmento inicial e no corpo, atuam como possíveis elementos de estabilização e renovação do epitélio, bem como parecem atuar na produção de anti-oxidantes (CALVO et al, 1997). As células apicais são aparentadas às principais e parecem estar envolvidas no transporte e digestão de proteínas e carboidratos do lúmen (SYNTIN et al, 1999). As células halo correspondem a linfócitos intra-epiteliais e as células claras, igualmente distribuídas ao longo de todo o epitélio epididimário, apresentam funções ainda controversas, envolvendo o processo de endocitose ou a acidificação do fluido epididimário (FLICKINGER et al, 1978; YEÜNG et al, 1994; FLESCH and GADELLA, 2000; ROBAIRE et al, 2000; FRANÇA et al*,* 2005). Além do tipo celular, outras variações segmentares incluem a altura epitelial, que diminui da cabeça para a cauda do epidídimo, e os diâmetros tubular e luminal, que mostram comportamento oposto ao da altura epitelial.

O conjunto de eventos sofridos pelo espermatozóide durante a passagem pelo epidídimo é denominado maturação epididimária (DACHEUX et al, 1987). Durante a maturação epididimária, proteínas de superfície do espermatozóide podem ser alteradas, extraídas ou outras podem ser adicionadas, num processo complexo que resulta numa série de modificações essenciais e contribuem para a aquisição da capacidade de fecundação (FLESCH et al, 2000). A síntese e secreção de proteínas epididimárias é bem descrita em diferentes espécies, de camundongos a primatas (BOUÉ et al, 1995). Diversas proteínas sintetizadas pelas células epiteliais do epidídimo, inicialmente liberadas na luz deste órgão, parecem ser posteriormente localizadas na superfície do espermatozóide (VREEBURG et al, 1992; TULSIANI et al, 1993; EDDY and O'BRIEN, 1994; KIRCHHOFF and HALE, 1996; ARROTÉIA et al, 2004) ou mesmo no interior da vesícula acrossômica (COHEN et al, 2000). Algumas dessas proteínas parecem

ser envolvidas na ligação inicial do espermatozóide à zona pelúcida (BOUÉ et al, 1994) ou à membrana plasmática do oócito (SALING et al, 1985), bem como na posterior fusão de membranas entre oócito e espermatozóide (COHEN et al, 2000). Muitas destas proteínas secretadas pelas diferentes regiões do epidídimo correspondem a enzimas capazes de alterar as glicoproteínas de superfície do espermatozóide, sugerindo assim uma participação ativa do epidídimo na seqüência específica de modificação dos mesmos. A aquisição da capacidade de fecundação pelo espermatozóide tem sido correlacionada com a nova organização molecular adquirida pela membrana plasmática durante o fluxo do gameta a partir da cabeça em direção à cauda do epidídimo (JONES et al, 1985; BOUÉ et al, 1995; HAMIL et al, 2000; MATHUR et al, 2000). Interessantemente, muitos estudos têm relatado que o desenvolvimento e a manutenção da função do epidídimo estão sob regulação hormonal primária de andrógenos testiculares e seus metabólitos derivados (JONES and CONNEL, 1982; NITTA et al, 1993; McMAHON et al, 1995; SONEA et al, 1997; SYNTIN et al, 1999; ROBAIRE et al, 2000; PASTOR-SOLER et al, 2002). Várias das proteínas produzidas pela região da cabeça do epidídimo e posteriormente incorporadas pelo espermatozóide apresentam caráter andrógeno-dependente (TEZON et al, 1985; BOUÉ et al, 1995; ELLERMAN et al, 1998; ROBAIRE et al, 2000).

O conhecimento acerca das proteínas expressas pelas células germinativas testiculares durante os processos contíguos de diferenciação, bem como das proteínas expressas pelas células dos ductos pelos quais os espermatozóides transitam durante o amadurecimento, pode amplamente contribuir com o desenvolvimento de pesquisas com fins científicos e clínicos envolvendo a fecundação. Visto que muitas destas proteínas devem ser adquiridas pelo espermatozóide para sua maturação e, portanto, podem ser relevantes durante a fecundação, tais moléculas específicas poderiam ser utilizadas para o desenvolvimento de vacinas com potencial contraceptivo (NAZ et al, 1993; ELLERMAN et al, 1998), ou poderiam estar relacionadas à etiologia da infertilidade idiopática masculina (BOUÉ and SULLIVAN, 1996). A

obtenção de anticorpos monoclonais específicos para antígenos expressos durante o desenvolvimento dos espermatozóides, além de proporcionar ferramentas em potencial na investigação clínica da infertilidade, auto-imunidade e contracepção imunológica (FLORKE-GERLOFF et al, 1985; JASSIM and FESTENSTEIN, 1987; BOUÉ and SULLIVAN, 1996; ELLERMAN et al, 1998; PURI et al, 2000), têm constituído uma importante estratégia para permitir a geração de um mapa completo e integrado das moléculas que, em conjunto, atuam na preparação do espermatozóide para a fecundação (WATANABE et al, 1992; ENDERS and MAY II, 1994; TSUCHIDA et al, 1995; TANAKA et al, 1997; KIRCHHOFF et al, 1998; PEREIRA et al, 1998).

A série do anticorpo monoclonal (Amc) TRA (testicular germ cells immunized to rat - monoclonal antibody), utilizado no desenvolvimento do presente trabalho, foi obtida no Laboratório de Experimentação Animal do Instituto de Pesquisa de Doenças Microbiológicas da Universidade de Osaka, Japão, com o intuito de reconhecer os antígenos de maior interesse no estudo da diferenciação das células germinativas testiculares (PEREIRA et al, 1998). Os resultados preliminares mostraram que um dos anticorpos selecionados, posteriormente denominado Amc TRA 54, reconhecia um antígeno presente no acrossomo de espermátides do estágio 1 a 12 de camundongos C57 BL/6 com idade igual ou superior a 24 dias pós-parto (d.p.p.). Por análise de *Western blotting* foram identificados para este antígeno 3 complexos de bandas alongadas (pesos moleculares aproximados de 200 kDa, 190 kDa e 85 kDa) (PEREIRA et al, 1998). A análise imunohistoquímica de outros órgãos de camundongos C57 BL6 mostrou que algumas células epiteliais da cabeça do epidídimo também apresentavam o antígeno correspondente ao Amc TRA 54, e que, embora ausente em espermatozóides testiculares, forte imunomarcação podia ser verificada no conteúdo luminal do epidídimo.

Alguns parâmetros relacionados ao padrão de expressão do antígeno reconhecido pelo Amc TRA 54 nas células do epidídimo, como a ontogenia, o peso molecular e a influência de

hormônios e fatores testiculares foram previamente descritos (ARROTEIA, 2003; ARROTEIA et al, 2004). Analisados em conjunto, os dados indicaram que a molécula antigênica reconhecida pelo Amc TRA 54 é expressa por uma população limitada de células da região da cabeça do epidídimo e secretada para a luz do órgão de maneira andrógeno-dependente e independente em relação à expressão do antígeno nas células germinativas testiculares. A molécula em estudo percorre a luz do epidídimo no sentido ântero-posterior e, neste percurso, pode ser incorporada pelos espermatozóides em trânsito.

Com base nos dados acima e com o intuito de contribuir para a compreensão dos complexos mecanismos que preparam o espermatozóide para a fecundação, o presente trabalho buscou identificar e reconhecer a estrutura e a função biológica do antígeno reconhecido pelo Amc TRA 54 nas células epiteliais do epidídimo. O conjunto dos experimentos conduzidos no desenvolvimento deste trabalho será apresentado nos capítulos subseqüentes sob a forma de quatro artigos científicos (Capítulos II a V), os quais serão submetidos para publicação em revista especializada de âmbito internacional. Cada capítulo apresenta formatação específica, de acordo com as normas editoriais da revista a qual o artigo correspondente deverá ser publicado.

O primeiro artigo científico desta tese, constante do Capítulo II e intitulado "*Fate of the epidydimal antigen recognized by the monoclonal antibody TRA 54 in mouse*", compreende informações complementares ao trabalho inicial previamente publicado (ARROTEIA et el, 2004), e contempla a síntese e liberação do antígeno pelas células principais do epidídimo e a incorporação do mesmo à região acrossômica do espermatozóide contido no lúmen epididimário. Tais dados foram obtidos com auxílio de técnicas como imunoquímica em microscopia eletrônica de transmissão e SDS-PAGE seguido de *imunoblotting*.

O Capítulo III, intitulado "*The mouse TRA 54 epididymal antigen is also expressed in human male reproductive tract and remains at fertile sperm after capacitation and induced*

acrosome reaction", relata a presença do antígeno reconhecido pelo Amc TRA 54 em testículos e epidídimos humanos, com padrão de expressão comparável ao descrito para camundongos C57 BL/6. Interessantemente, os dados deste artigo indicam a presença do antígeno em estudo em espermatozóides de homens férteis, inclusive após os processos de capacitação e reação acrossômica, sugerindo fortemente a participação da molécula em estudo no processo de preparação do espermatozóide para o momento da fecundação.

Os estudos acerca da caracterização estrutural de molécula em estudo, incluindo a identificação de sua seqüência de aminoácidos, os domínios antigênicos e a estrutura tridimensional, estão contemplados no Capítulo IV, na forma do artigo intitulado "*Characterization of a new mouse epididymal antigen recognized by the monoclonal antibody TRA 54*". Adicionalmente, neste capítulo é também iniciada a caracterização funcional do mesmo antígeno. O artigo contemplado neste capítulo foi submetido à apreciação da revista científica internacional *The Journal of Biological Chemistry*.

O Capítulo V finda o conjunto de trabalhos desta tese com a apresentação do artigo intitulado "*The mouse epididymal TRA 54 antigen: a novel molecule adhered to luminal spermatozoa and required to oocyte-zona pellucida penetration before fertilization*", o qual aborda amplamante a função biológica desempenhada pelo antígeno reconhecido pelo Amc TRA 54 no processo de fecundação. A participação do antígeno nos processos de interação e passagem do espermatozóide pela zona pelúcida ou membrana plasmática do oócito foi investigada e identificada através de diversos ensaios *in vitro* realizados com gametas de camundongos C57 BL/6. Os dados deste artigo, correlacionados com os anteriormente apresentados, especialmente os descritos nos Capítulos II e IV, permitem a identificação e a elaboração de uma descrição completa e detalhada de uma das muitas moléculas que participam do complexo processo de fertilização. A presença da molécula em tecidos e espermatozóides humanos reforça a potencialidade da mesma neste processo e, portanto, a

configura como importante candidata a ferramenta para construção de imuno-contraceptivos masculinos ou ainda para terapias pró-fertilidade masculina.

Para finalizar o presente trabalho, o capítulo VI apresenta uma revisão intitulada "*The mammalian epididymis: a morpho-physiological overview*", a qual reporta um conjunto de informações acerca da estrutura, função e peculiaridades do desenvolvimento e morfo-fisiologia do epidídimo de mamíferos, fruto das numerosas pesquisas bibliográficas realizadas durante a execução deste trabalho.

O capítulo VII apresenta uma breve conclusão elaborada a partir dos resultados obtidos com o desenvolvimento deste estudo, os quais embasam perspectivas metodológicas futuras para novos estudos envolvendo antígenos como o reconhecido pelo Amc TRA 54. Em conjunts, os dados do presente e de futuros trabalhos poderão resultar em importantes ferramentas clínicas para a solução de problemas relacionados à fertilidade humana.

Completando os trabalhos desta tese, na seção ANEXO estão contempladas em detalhes as principais metodologias utilizada para o desenvolvimento deste trabalho.

Objetivos

Objetivo Geral

O objetivo maior do presente trabalho foi caracterizar o antígeno reconhecido pelo Amc TRA 54 nas células epiteliais do epidídimo, sob os aspectos funcional, bioquímico e estrutural, permitindo sua identificação e contribuindo para a geração de um mapa das moléculas essenciais as diferentes fases da fertilização em mamíferos.

Metas

1. Ampliar o conhecimento previamente estabelecido acerca do modelo de expressão do antígeno reconhecido pelo Amc TRA 54 no epidídimo de camundongos C57 BL/6, descrevendo os tipos celulares e a via biossintética envolvidos na secreção do mesmo; ampliar o conhecimento acerca da incorporação do antígeno em estudo pelo espermatozóide contido no lúmen epididimário;
2. Caracterizar a presença do referido antígeno na estrutura do espermatozóide de roedores e humanos em diferentes fases de maturação, sob os aspectos espacial e temporal;
3. Avaliar a influência do antígeno em estudo nas diferentes fases do processo de fertilização (camundongos), a saber: interação e passagem do espermatozóide pela zona pelúcida do oócito e interação e fusão das membranas do espermatozóide e oócito;
4. Isolar o antígeno sintetizado pelas células epiteliais do epidídimo e determinar sua seqüência de aminoácidos, possibilitando compará-la com seqüência de aminoácidos de peptídeos depositados em bancos de dados, visando a identificação da molécula;
5. Predizer, a partir das informações da seqüência de aminoácidos obtida, características bioquímicas e de conformação tri-dimensionais do antígeno em estudo, estabelecendo correlações entre a estrutura da molécula e a sua função durante o processo de fertilização.

Capítulo II

Fate of the Epididymal Antigen Recognized by the Monoclonal Antibody TRA 54 in Mouse

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Running head: Fate of epididymal antigen for mAb TRA 54

Keywords: Epididymis - Monoclonal Antibody - Imunoelectromicroscopy - Sperm Antigens.

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Abstract

The epididymis participates in the spermatozoa maturation prior to fertilization, but little is known about the role of this organ in modifying sperm. In a previous work, we described the expression pattern of the antigen recognized by the monoclonal antibody (mAb) TRA 54 in testicular germ cells and epididymal epithelial cells, and showed that its expression occurs independently in each organ. In the present study, the ultrastructural localization of the mAb TRA 54 antigen in epididymis was evaluated by transmission electron microscopy using immunogold labeling. Immunogold particles were found on the sperm surface and in the acrosome, suggesting that the epididymal antigen was released from the epididymal epithelium to the lumen and subsequently bound to the luminal spermatozoa. In addition, immunoblotting assays revealed that the antigen released into the epididymal lumen is adsorbed by the spermatozoa in a polarized way, overlaying the acrosomal segment previously to the acrosomal incorporation. These finding suggests that epididymal mAb TRA 54 antigen may play a role in the maturation of epididymal spermatozoa prior to fertilization.

1. Introduction

Fertilization in mammals depends on a sequence of events that culminates in the activation of an oocyte by sperm (Saling, 1996). Upon leaving the testis, mammalian spermatozoa move through the duct system formed by the vasa efferentia, epididymis, and vas deferens. The functions of the epididymis include the absorption of seminiferous fluid and the concentration, transport and storage of spermatozoa (Légare et al., 1999; Dacheux et al., 2005). The functional and antigenic modification of spermatozoa in the epididymal environment, a process known as epididymal maturation (Dacheux and Dacheux, 1987; Kaunisto et al., 1999; Mathur et al., 2000), involves changes in the profile of antigen expression, including the addition of new molecules to the surface of gametes (Jones et al., 1985; Toshimori et al., 1988; Vreeburg

et al., 1992; Tulsiani et al., 1993; Eddy and O'Brien, 1994; Kirchhoff and Hale, 1996; Kirchhoff, 1998).

The alterations that occur in spermatozoa during epididymal maturation are essential for the success of fertilization (Yanagimachi, 1994). However, relatively little is known about the role of the epididymis and the modifications that occur in sperm during this process (Moore, 1998). The use of monoclonal antibodies (mAb) to study antigens expressed by cells of male reproductive organs has contributed to our understanding of the role of these proteins in the formation and maturation of functional sperm (Watanabe et al., 1992; Enders and May II, 1994; Tsuchida et al., 1995; Kirchhoff et al., 1998). The mAb TRA 54, a monoclonal antibody produced by immunizing rats with mouse testicular germ cells (testicular germ cell-immunized rats - monoclonal antibody), recognizes an antigen expressed by spermatids in the seminiferous tubules of C57 BL/6 mice older than 24 days (Pereira et al., 1998). Ventelä et al. (2003) provided an extensive description of the intracellular pathway of antigen production in testicular germ cells and showed that expression of the TRA 54 antigen started in the RE-Golgi system of early spermatids, with the antigen being incorporated into the acrosomal system of these cells during spermiogenesis.

In a previous report, we used immunolabeling to study the expression of the antigen recognized by TRA 54 in epithelial cells of mouse caput epididymis and showed that the epididymal antigen was expressed independently of the testicular germ cell antigen (Arrotéia et al., 2004). We also confirmed our previous observation that in seminiferous tubules the TRA 54 antigen was expressed in early spermatids but not in spermatozoa (Pereira et al., 1998). Interestingly, the TRA 54 antigen was detected in epididymal spermatozoa, suggesting that molecules with an epitope for mAb TRA 54 could be released from epithelial cells of the caput epididymis and could subsequently bind to the surface of the spermatozoa during the transit

inside of the epididymal duct (Arrotéia et al., 2004). However, so far, no description of the intracellular localization of this epididymal antigen was done to confirm this hypothesis.

In this study, we used immunohistochemistry, immunogold labeling and immunoblotting procedures in order to investigate the localization of the antigen recognized by mAb TRA 54 in mouse epididymal caput epithelium and luminal spermatozoa. Our results indicate a possible association between the expression of this antigen and its fate as a molecule involved in the epididymal maturation of spermatozoa prior to fertilization.

2. Materials and Methods

2.1. Animals

Male C57BL/6 mice were housed under standard conditions (12 light/dark cycle at 23°C), with free access to water and food, and were used at 10-12 weeks of age. Animal experiments were approved by the institutional Committee for Ethics in Animal Experimentation (CEEA/IB/UNICAMP, protocol number 590-1).

2.2 Preparation of testis and epididymis for immunohistochemistry with the mAb TRA 54

Mice were killed by cervical dislocation and testis and epididymis were immediately harvested, fixed in Bouin's solution and embedded in paraffin. Sections 5 µm thick were used for immunohistochemistry with the mAb TRA 54 as described elsewhere (Pereira et al., 1998; Arrotéia et a.l, 2004). Briefly, the sections were blocked with 20% normal goat serum and incubated overnight at 4° C with ascites fluid containing mAb TRA 54 (diluted 1:4000), followed by incubation for 1 hour with goat-anti-rat biotinylated secondary antibody (Dako A/S, Glostrup, Denmark) diluted 1:200 and then with Strept ABC kit (Dako A/S, Glostrup, Denmark) during 45 minutes. The reaction was developed by incubating the sections with hydrogen peroxide and diaminobenzidine (Sigma Aldrich, St Louis, MO). The sections were counterstained with

hematoxylin. In control experiments, the ascitic fluid containing mAb TRA 54 was omitted from the primary antibody incubation step.

2.3. Preparation of epididymal samples for immunogold labeling with the mAb TRA 54

Mice were anesthetized by intraperitoneal injection with ketamine (260 mg/kg) and xylazine (130 mg/kg) prior to cardiac perfusion fixation (15 min) with 30-40 ml of a solution containing 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. The epididymal caput was removed from each mouse and 300-500 µm thick slices were obtained in Tissue-Slicer (EMS, USA) and immersed in the same fixative solution for 2 h at 4°C. The slices were washed three times in 0.05 M Tris-HCl, pH 7.4, and dehydrated in a graded N-N-dymethylformamide series at 4ºC, embedded in LR-White resin (EMS, USA) and polymerized under UV radiation at -20°C for 48 h in freeze substitution unit (FSU-30, Balzers, Germany).

To the immunolabeling procedure, the ultrathin sections of LR-White embedded samples were collected on nickel grids, washed for 5 min in 0.05 M Tris-HCl, pH 7.4, and then treated with 1% bovine serum albumin (BSA type V, Sigma Chemical Co., St. Louis, MO) in 0.05 M Tris-HCl, pH 7.4, for 30 min. The grids were incubated overnight at 4°C with rat (mAb) TRA 54, diluted 1:3000 in 1%BSA, 0.1M TBS as primary antibody and after washed with 0.05 M Tris-HCl, pH 7.4.Then, the grids were incubated for 1 h with gold-conjugated (5 and 15 nm particles) goat anti-rat secondary antibody (Dako A/S, Glostrup, Denmark). After final wash in 0.05 M Tris-HCl, pH 7.4, the sections were stained with 2% uranyl acetate for 2 min and lead in citrate for 30 s, carbon coated and analysed with LEO 906 transmission electron microscope (LEO-Schot Zeiss, Germany) operated at 60 kV. In control experiments, the ascitic fluid containing mAb TRA 54 was omitted from the primary antibody incubation step.

2.4. Preparation of isolated spermatozoa for immunohistochemistry and immunolabeling with the mAb TRA 54

Epididymides collected from adult mice were carefully dissected and sperms were allowed to disperse in 200μL of PBS (Dulbecco's Phosphate Buffered Saline Solution, 1x, Irvine Scientific, Santa Ana, California) by cutting the epididymal cauda, at 37°C. The suspension of sperms was washed three times by centrifugation (200 x *g*) in PBS (10 min each). The pelleted spermatozoa were destined for immunohistochemistry or immunogold labeling analysis. For immunohistochemistry, pelleted spermatozoa were ressuspend in 50μL of PBS and 5μL samples were allowed to dry on the glass slide. The slides were fixed with iced methanol previously to the immunohistochemical procedure that was performed as described to testis and epididymal slices. Some slides were incubated for 30 min with 1% Triton-X-100 (Sigma Aldrich, St Louis, MO) diluted in PBS following the methanol treatment. For the immunogold labeling procedure, the pelleted spermatozoa obtained as describes above was ressuspended in fixative solution (4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) and processed for LR-White embedding. Immunolabeling procedure was performed as described to epididymal slices.

2.5. Preparation of testes, epididymis and isolated spermatozoa for SDS- PAGE and immunoblotting

Testes and epididymal caput collected from adult mice and isolated spermatozoa obtained as described above were ressuspend and homogenized in 10 mM Tris-HCl pH 7.4 containing 10 mM EDTA (Mallinckrodt, Paris, Kentucky), 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM sodium orthavonadate, 2 mM PMSF and 0.1 mg of aprotinin/ml (Sigma Aldrich, St Louis, MO). The homogenates of tissues and sperm were centrifuged (10.000 X g) and the protein concentration of the supernatants was determined using a Bradford protein

assay kit (Bio-Rad, Richmond, CA, USA). Aliquots of each homogenate (100μg per lane) were diluted in sample buffer to perform the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with 7.5% gels prepared according to Laemmli (1970). The proteins were transferred electrophoretically to polyvinylidenedifluoride (PVDF) membranes (Millipore, Bedford, MA, USA), which were then blocked with 5% low-fat dry milk during 1 hour and washed with 0.05% tween-Tris-buffer-saline (TBS-T). The membranes were subsequently incubated with mAb TRA 54 (diluted 1:2500 in TBS-T) overnight, washed in TBS-T, and finally incubated with peroxidase anti-rat IgG (Dako A/S, Glostrup, Denmark) during 1h. Immunoreactive bands were detected by incubating the membranes with 0.03% hydrogen peroxide and 0.05% diaminobenzidine in 50 mM Tris-HCl, pH 7.2.

2.6. Removal of the antigen from the isolated spermatozoa

To ensure that the secreted epididymal antigen could firstly adhere to spermatozoa surface and be further enclosed by the acrosome, spermatozoa directly recovered from epididymal cauda were repeatedly washed by centrifugation (400 x *g*; 10 min) and ressuspension in PBS (Liu et al., 1991). The samples supernatants were stored at each centrifugation step to further SDS-PAGE and immunoblotting analysis. The resuspension and washing procedures were repeated up to seven times. Furthermore to assess the extractability of the antigen incorporated to the spermatozoa, samples of the pelleted spermatozoa obtained after the last centrifugation were homogenized in 10 mM Tris-HCl pH 7.4 containing 0.5% of Igepal, 10mM Hepes, 0.15M NaCl and 0.2mM aprotinin (Sigma Aldrich, St Louis, MO) (Liu et al., 1991). The samples were sonicated at maximal speed (15 cycles; 8 sec) and centrifuged at 400 x *g* and 10.000 x *g* respectively (10 min each one). The obtained supernatants were used to SDS-PAGE and immunoblotting assays as described above.

3. Results

Immunohistochemical localization of the testicular and epididymal antigens

The expression pattern of the antigen recognized by the mAb TRA 54 in testis and epididymis was done according to the previous descriptions (Pereira et al., 1998; Ventelä et al 2003; Arroteia et al., 2004). Testis of adult mice showed a strong staining in the germinative epithelium of seminiferous tubules (Fig. 1a). No staining was found in sections used as negative controls (Fig. 1b). The positive cells were round (Fig. 1c) and elongated (Fig. 1d) spermatids, but no staining was observed in spermatozoa (Fig. 1e-f). Somatic cells as Sertoli and Leydig cells as well as spermatogonia were not stained. The epididymis showed strong specific staining in the whole epididymal extension, excepting the initial segment (Fig. 1g). The staining in the caput epithelial cells was seen in the supranuclear cytoplasm of the principal cells (Fig. h-i), but not in other epithelial cells as the basal and apical cells (Fig. i). In the corpus and caudal segment only the surface of the epithelia was stained (Fig. j). Luminal contents in all the epididymal segments were labeled (Fig. h-j).

Ultrastructural localization of the epididymal antigen

Imunolabeled sections assessed by transmission electron miscroscopy (TEM) revealed that staining was restricted to the principal cells of the caput epididymal epithelium and to stereocilia, except initial segment (Fig. 2a-g).

No gold particles were seen in basal or apical cells or in intraepithelial lymphocytes. The apical and supranuclear cytoplasm of the principal cells of the epididymal caput contained numerous large, dense granules of different sizes and densities, and the apical cytoplasm of these cells protruded into the lumen through long stereocilia (Fig. 2a). Gold particles in the principal cells were associated with the cisternae of the rough endoplasmic reticulum (RER) (Fig. 2b) and with flattened and distended portions of the Golgi complex (Fig. 2c,d). In the

supranuclear cytoplasm, vesicles of various sizes containing electron-dense and electron-lucent material showed the gold particles (Fig. 1e). Immunolabeling by mAb TRA 54 occurred throughout the entire length of the stereocilia (Fig. 1f-g). Pinocytic and coated vesicles, endosomes and other components of the endocytic apparatus, as well as mitochondria and the nuclear region were not labeled.

Immunohistochemical and ultrastructural localization of the spermatozoa antigen

Spermatozoa obtained from the epididymal lumen showed positive staining to the mAb TRA 54 in the acrosomal region (Fig. 3a-b). The same pattern of the antigen expression was found in samples that were previously treated with (Fig. 3a) or without (Fig 3b) the detergent Triton-X-100. No positive staining was found in flagellum or other region of the spermatozoa (Fig. 3a-b) neither in slides used as negative control (Fig. 3c). Immunolabeled sperm sections assessed by TEM showed a conspicuous staining to the mAb TRA 54 mainly in the plasma membrane of the sperm head and in the acrosomal region (Fig. 3d-e). No gold particles were seen in spermatozoan flagella (Fig. 3f) or in sections used as negative control (Fig. 3g).

Western blotting analyses of spermatozoa samples after the removing of the antigen by centrifugation and detergent extraction

An immunoreactive band complex of ~250 kDa was detected in testis, epididymis and isolated sperm (Fig. 4a). Immunoblot analysis of the samples of sperm obtained after washing steps by sequential centrifugation showed a decreasing in the band signal from the first to the seventh washing-step. The signal was amplified when the sample obtained in the seventh wash was extracted with the detergent Igepal (Fig. 4b).

4. Discussion

Mammalian spermatozoa acquire their capacity for fertilization through multiple and sequential interactions with proteins present in the epididymal luminal fluid (Castella et al., 2004). Several enzymes secreted from different epididymal regions into the luminal fluid contribute to the modifications of mammalian sperm surface and provide sperm maturation (Légaré et al., 1999; Hamil et al., 2000; Dacheux et al., 2005). Sperm maturation includes the acquisition of fertilization ability through multiple and sequential interactions with proteins present in the epididymal fluid (Castella et al., 2004).

In previous reports, a new antigen expressed in male mouse germ cells at spermatids stage but not by testicular spermatozoa was described by Pereira et al. (1998) and Ventella et al. (2003). This antigen was recognized by TRA54 monoclonal antibody, which also cross-reacted with epididymal epithelial cells (Arrotéia et al., 2004). Since the TRA 54 antigen was detected in spermatozoa present in the epididymal lumen, it was supposed that this antigen could be synthesized “de novo” and released from the epididymal epithelial cells to be incorporated by spermatozoa during their transit through the epididymis (Arrotéia et al., 2004).

Previous experiments performed in cryptorchid mice, as well as protocols designed to assess the recovery of the epididymal expression of TRA 54 antigen in castrated mice undergoing testosterone replacement indicated that the mAb TRA 54 antigen is secreted by epididymal epithelial cells in an androgen-dependent manner, in a process that is not influenced by the testicular germ cells (Arrotéia et al., 2004). As we have shown here, the TRA 54 antigen was detected only in the cytoplasm of the principal cells of the epididymal caput. These cells show morphological features consistent with active absorption and secretion of proteins(Dacheux et al., 2005). Our immunolabeling results strongly indicate that the antigen recognized by mAb TRA 54 is produced rather than absorbed by the principal cells of the epididymal caput. This conclusion is based on the finding that the antigen was not detected in

any intracellular component of the endocytic apparatus, such as pinocytic vesicles, apical coated vesicles, endosomes or lysosomes. In addition, the TRA 54 antigen was associated with cell components involved in the classic pathway of protein secretion in the principal cells, including the RER cisternae, Golgi complex and supranuclear apical vesicles located between the Golgi zone and apical membrane. Finally, the protruding stereocilia of these cells were strongly labeled, indicating secretion of the antigen from the apical region of the principal cells to the lumen.

The acrosome is a spermatic vesicle involved in exocytosis (Flesch and Gadella, 2002; Cohen et al., 2001; Murase et al., 2004). Interestingly, Pereira et al. (1998) and Ventelä et al. (2003) showed that the production of TRA 54 antigen in testicular germ cells involved the Golgi apparatus and the formation of the acrosomal vesicle. As shown here, the expression of the epididymal antigen recognized by mAb TRA 54 was also associated with the Golgi complex. Although the testicular and epididymal antigens recognized by mAb TRA 54 may be isoforms of the same protein (Arrotéia et al., 2004), they can both be classified as Golgi-derived molecules that share a common glycosidic epitope added by the trans-element of the Golgi complex.

It was demonstrated that the spermatozoa which do not express the TRA 54 antigen in seminiferous tubules can acquire this molecule as they passed through the epididymal lumen. The immunoblotting results obtained fof spermatozoa washed and extracted with detergent (Igepal) indicated that the antigen is found both as an easily removable extrinsic protein and as an acrosomal-integrated protein.

This data demonstrate that the antigen secreted by epididymal cells could be firstly adsorbed at the sperm membrane and further endocytosed to the acrosome. In addition, both permeated and not permeated sperm samples showed that the antigen was in an acrosomal location. This fact strongly suggests that the adsorption of the antigen occurs in a polarized way, overlaying the acrosomal segment previously to the acrosomal incorporation. Several reports

have described a number of molecules involved in sperm-egg fusion that are produced by the columnar epithelium of the epididymis and released into the epididymal lumen to be further bound in the spermatozoa surface (Boué et al., 1995; Légaré et al., 1999; Syntin and Cornwall, 1999; Cohen et al., 2000, 2001; Chu et al., 2000; Liu et al., 2000; Gaudreault et al., 2001; Pauls et al., 2003) or endocytosed in the spermatozoa acrosomal region during epididymal maturation (Myles and Primakoff, 1984; Nonaka et al., 2003). Many of these proteins are also expressed in testicular germ cells (Cornwall and Hann, 1995; Syntin and Cornwall, 1999; Gaudreault et al., 2001; Pauls et al., 2003) as occurs with the antigen recognized by the mAb TRA 54. A new molecule with an expression pattern similar to that of TRA 54 antigen, SED1, was identified and recognized as an important molecule involved in mice fertilization (Ensslin and Shur, 2003). In addition, infertility in mice and humans has been associated with the absence of epididymal antigens (Boué and Sullivan, 1996; Sipilä et al., 2002).

Although the role of the TRA 54 antigen in the fertilization was not stablished, circumstantial evidences indicate that this molecule antigen may contribute to the maturation of spermatozoa providing them with molecules needed for fertilization.

The isolation and physico-chemical characterization of the TRA 54 antigen, as well as the investigation of its function in oocyte penetration, will strongly contribute to our understanding about the epididymal function in the fertilization process.

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Figure Legends

Fig. 1 – Immunohistochemical staining of mouse testicular (a-f) and epididymal (g-j) cross-sections using the mAb TRA 54. Testis showed a strong staining in the germinative epithelium of seminiferous tubules (a) that was not found in sections used as negative controls (b). The positive testicular cells were round (c) and elongated spermatids (d). Leydig cells, Sertoli cells (c; arrow), spermatogonia (c; arrowhead) and spermatozoa (e-f; arrows) were not stained. The epididymis showed strong specific staining in whole epididymal extension (g), except initial segment (g, IS). Positive staining was verified in the supranuclear cytoplasm of principal cells in the epididymal caput (h-i), but not in basal (h; arrowhead) or apical i; arrow) cells. Only the surface of the epithelia were stained In the corpus and caudal segment (j). Epididymal luminal contents were strongly labelled (h-j; asterisk). Bar: a-b: 100 μm; c-d: 32 μm; e-f: 10 μm; g: 1150 μm; h: 20 μm; i: 10 μm; j: 20 μm.

Fig. 2. Transmission electron microscopy (TEM) of epithelial cells of the epididymal caput immunolabeled with mAb TRA 54. (a) low magnification of the principal cells and luminal spermatozoa. (PC: principal cell; GR: Golgi region; asterisk: multivesicular bodies; AP: apical region of the cytoplasm; ST: stereocilia extending from the apical cytoplasm to the epididymal lumen; arrowhead: luminal spermatozoa); (b) Immunolabeling (gold particles) was restricted to the principal cells and associated with the cisternae of the rough endoplasmic reticulum (RER); (c-d) flattened (arrowhead) and distended sacules of the Golgi complex (GS) showing

immunogold particles; (e) apical vesicles in the supranuclear cytoplasm showed immunogold particles; (f) immunolabeling by mAb TRA 54 was seen throughout the entire length of the stereocilia (AP: apical region of the cytoplasm of a principal cell); (g) high magnification showing immunolabeling associated with stereocilia. Diameter of gold particles: 15 nm. Bar: a: 1.0 μm; b-c: 0.4 μm; d: 0.1 μm; e: 0.3 μm; f: 1 μm; g: 0.02 μm.

Fig. 3. Immunolocalization of the antigen recognized by mAb TRA 54 assessed by immunochemical staining (a-c) and immunogold labeling (d-g). Images a-c were obtained by *diferential interference contrast* (DIC) and images d-g were obtained by transmission electron microscopy (TEM). Positive staining was found on the acrosomal region of the spermatozoa head in samples previously treated with (a) or without (b) Triton-X-100 (arrows). No positive staining was found in flagellum (a-b) neither in the slides used as negative control (c). Transmission electron microscopy (TEM) of immunolabeled sperm sections showed gold particles both in plasma membrane and inside the acrosome (d-e; N: nucleus). f: the spermatozoan mid-piece (MP) and flagellum (F) showed no immunogold particles. Negative control (g) showed no immunolabeling by mAb TRA 54 (N: nucleus; A: acrosome). Diameter of gold particles: 15 nm. Bar: a-c: 3 μm; d-g: 0.2 μm.

Fig. 4. Western blot analysis of testis, epididymis and isolated spermatozoa with the mAb TRA 54. The positions of the molecular mass marker are indicated on the left (250 kDa). Control immunoblot without TRA 54 showed no immunoreactive band (cont). a: testis (te), epididymal caput (epi), sperm isolated from epididymal cauda (sp). An immunoreactive band complex of ~250 kDa was detected all samples (a). Immunoblot analysis of the sperm samples obtained from sequential centrifugation-washing steps (b, 1-7) showed a decreasing band signal from the first to the seventh washing-step sample. The signal was sensibly amplified when the seventh washed sample was extracted with the detergent Igepal (b, Ig).

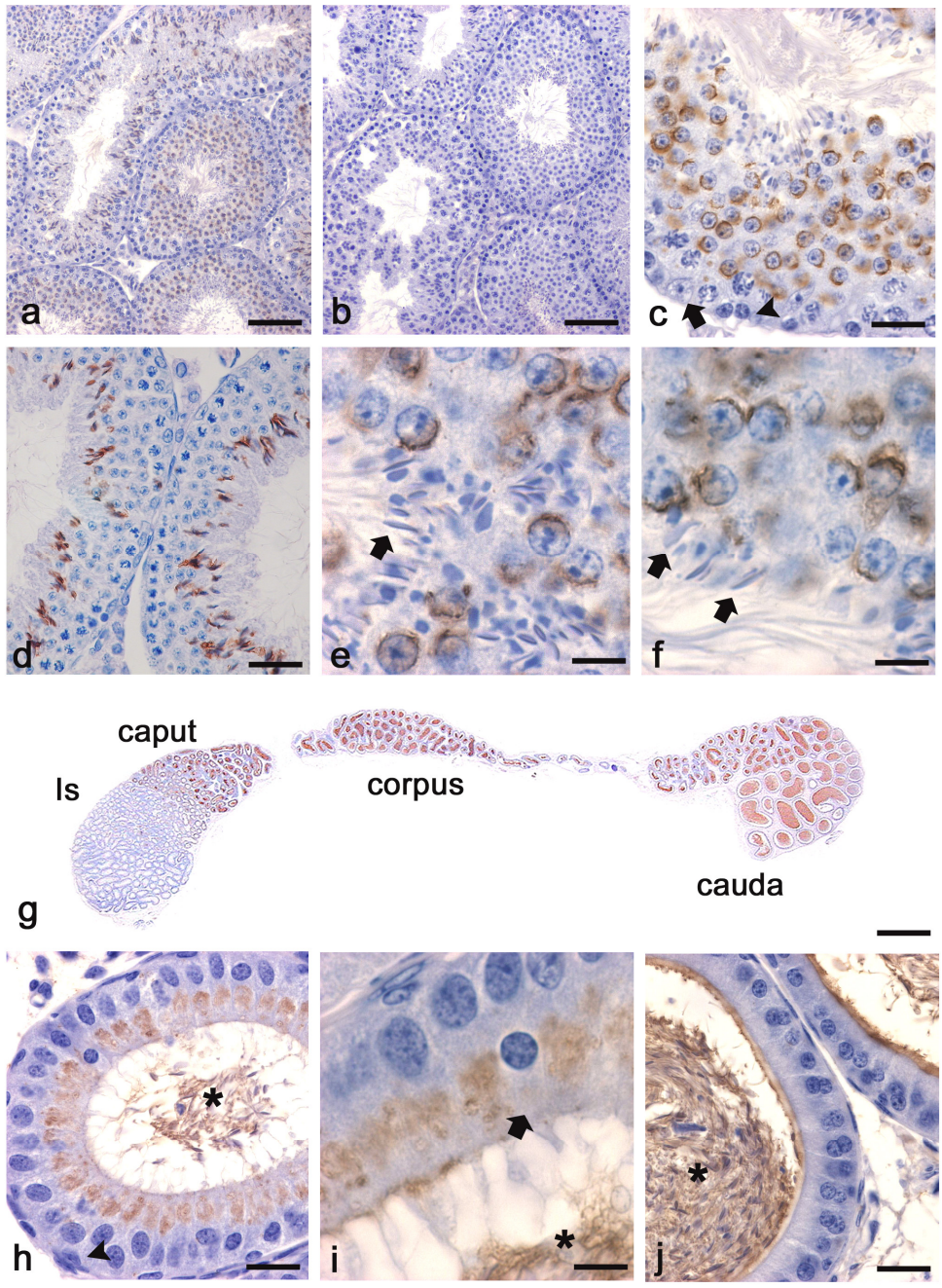

Figure 1

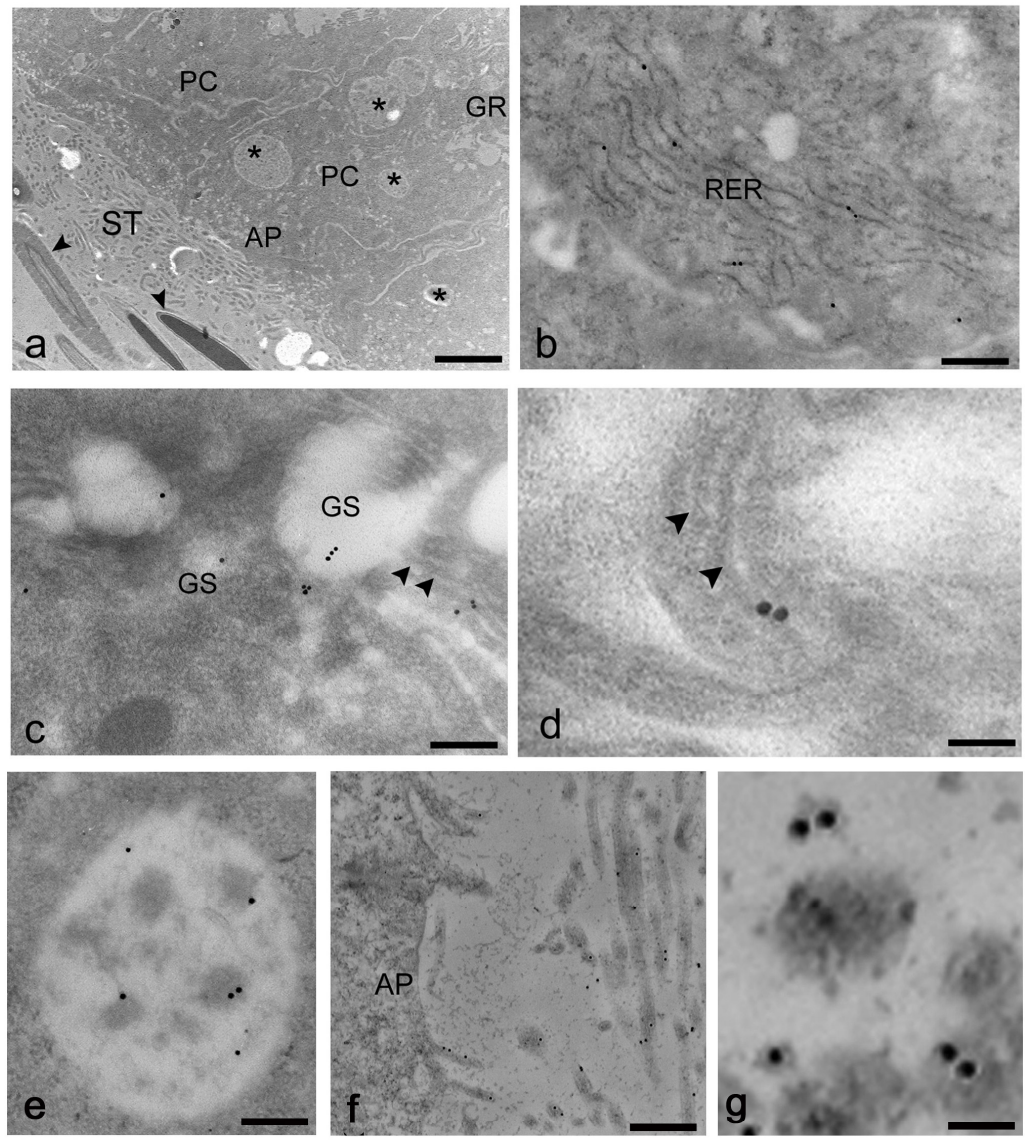

Figure 2

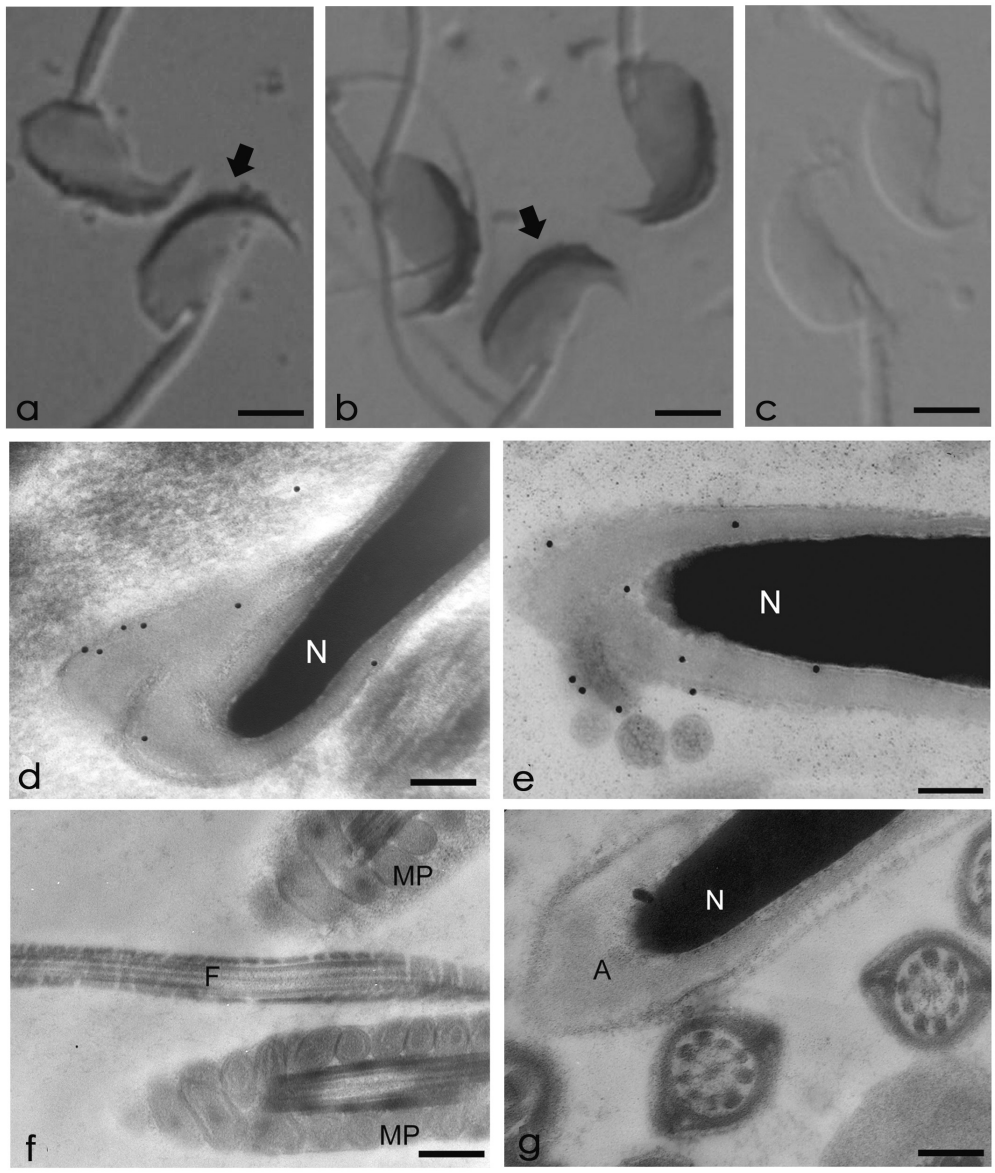

Figure 3

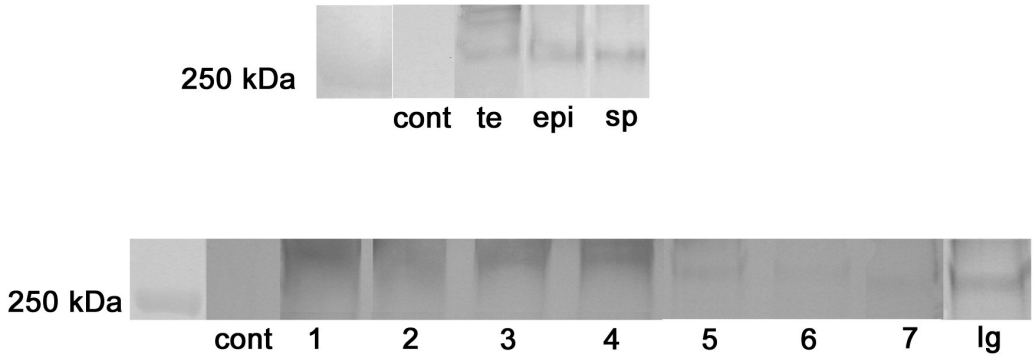

Figure 4

The mouse TRA 54 epididymal antigen is also expressed in human male reproductive tract and remains at fertile sperm after capacitation and induced acrosome reaction

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Running title: Human expression of the epididymal TRA 54 antigen.

Abstract

BACKGROUND: Spermatozoa in testicular fluid are known to be not able to fertilize eggs, and the epididymis is known to participate in the sperm maturation leading fertilization. In a previous study, we have characterized the expression of a new molecule recognized by the monoclonal antibody (mAb) TRA 54 in the mouse testicular germ cells and epidydimal epithelial cells. The epididymal antigen is produced by the mouse epididymal epithelial cells, released into lumen and; it is designed to bind on the luminal spermatozoa. The aim of this work is investigating the pattern of this antigen expression in human epididymal and spermatzoa. METHOD: The presence of the antigen was investigated by using immunocitochemical and immunobloting analysis of human testis, epididymis and ejaculated, capacitated and acrosome-reacted spermatozoa. RESULTS: The expression pattern and the molecular antigenic bands of the human antigen were similar to those found in mouse samples. Ejaculated and capacitated sperms showed the antigen at the post-equatorial region of the sperm head, and the location of the antigen was found to be changed during the acrosome reaction. CONCLUSION: We conclude that the epididymal mAb TRA 54 antigen could be engaged in the human epididymal sperm maturation for capacitate the human spermatozoa for fertilization.

*Key words***:** acrosome-reaction, epididymis, fertilization, sperm antigens.

Introduction

Fertilization in mammals depends on a sequence of events that culminates in the activation of an oocyte by sperm (Saling, 1996). In mammals, a collection of physiological processes allows the sperm and egg to approach each other in the female reproductive tract (Lin *et al*., 1994). Mammalian sperm leaving the testis are unable to fertilize eggs (Myles and Primakoff, 1984; Légaré *et al*., 1999), but it is made competent for fertilization after the epididymal maturation, after capacitation in female reproductive tract and after acrosome reaction (Myles and Primakoff, 1984; Saling and Lakoski, 1985; Okabe *et al*., 1990; Larson and Miller, 1999; Cohen *et al*., 2000; Flesch and Gadella, 2000; Ramalho-Santos *et al*., 2002; Dacheux *et al.,* 2005). In the male reproductive tract, the epididymis is responsible for creating a special microenvironment within its lumen that helps transform immotile, immature testicular spermatozoa into fully-competent fertile cells (Jones, 2004). This microenvironment provides essential modification of the sperm antigen profile and contributes to the success of the fertilization (Jones *et al*., 1985; Toshimori *et al*., 1988; Vreeburg *et al*., 1992; Tulsiani *et al*., 1993; Kirchhoff and Hale, 1996; Kirchhoff, 1998; Kaunisto *et al*., 1999, Mathur *et al.,* 2000).

The study of the antigens expressed in the male reproductive tract by using monoclonal antibodies (mAb) has contributed to our understanding of their role in sperm preparation leading fertilization (Enders and May II, 1994; Tsuchida *et al*., 1995; Kirchhoff *et al*., 1998). The mAb TRA 54 (mouse testicular germ cells immunized to rat - monoclonal antibody) recognizes an antigen expressed in haploid testis germ cells and in the caput epithelial epididymal cells of C57 BL/6 mice older than 24 days (Pereira *et al*., 1998; Arrotéia *et al*., 2004). In previous study (Arrotéia *et al*., 2004), we reported that the epididymal antigen molecule is released by epithelial cell in mouse epididymal caput in an androgen-dependent manner, and seems to bind on the sperm head surface during the moving down of the sperms through the epididymis. A large number of antigens expressed by epididymal epithelial cells have been reported by others to

adhere to luminal spermatozoa in mice (Eberspaecher *et al.*, 1995), rats (Rochwerger *et al.*, 1992; Cohen *et al.*, 2000), hamsters (Echeverría *et al.*, 1982; Robitaille *et al.*, 1991) and humans (Légaré *et al.,* 1999; Cohen *et al.*, 2001). Many of these proteins and glycoproteins are known to participate in the sperm egg adhesion and fusion and show homologous counterparts shared by more than one mammal species (Lin *et al.* 1994, Ellerman *et al.*, 2002; Nonaka *et al.*, 2003).

The aim of this study was investigating if the mAb TRA 54 antigen is also expressed in fertile human male reproductive tract and sperms besides C57BL/6 mice. We also investigated the dynamics of the antigen localization after the sperm capacitation and induced acrosome reaction. Furthermore, we verified if the human molecule recognized by the mAb TRA 54 antigen differed in molecular weight from the antigenic molecule expressed in mouse male reproductive tract.

Material and methods

Preparation of Monoclonal Antibody

The monoclonal antibody was produced as described by Pereira *et al.* (1998).

Subject

Fertile men were invited to participate in the study from Familiar Planning Program of the Women Healthy Assistential Center (CAISM) at UNICAMP. Patients participated on a voluntary basis.

Human follicular fluid was obtained from follicular punction from women under assisted reproductive techniques. Human testis and epididymis samples were obtained during necrology examination from 20-40 years old man dead by traumatic accident (sudden death), at the Legal Medical Institute of Campinas, SP, Brazil.

This study was approved by the Medical Ethics Research Commit of the UNICAMP (protocol # 610/2003) and was performed in accordance to the guidelines found in the Declaration of Helsinki.

Collection and preparation of the samples

Fragments of human testis and epididymis were fixed in Bouin's solution during 12-24 hours and paraffin-embedded for immunohistochemistry.

Human spermatozoa were obtained by masturbation in a sterile plastic dish. Semen was liquefied during 30 minutes at 37°C in atmosphere with 5% CO_2 (carbonic dioxide). Seminal plasma was removed by centrifugation (400 X g, 10 minutes) with PBS (Dulbecco's Phosphate Buffer Salt Solution – Gibco). Motile spermatozoa were selected by a swim up technique using 4 ml of Human Tubal Fluid (HTF, Irvine Scientific) supplemented with 35mg/ml Bovine Albumin Serum (BSA, fraction V, Sigma Aldrich, St Louis, MO). After 1h of incubation under 37°C in 5% CO_2 atmosphere, 3ml of the supernatant that contained the motile spermatozoa were carefully removed. The spermatozoa recovered after swim-up were capacitated by incubation for 20h in a culture stove at 37°C in a 5% CO_2 atmosphere. Capacitated sperms were pelleted by centrifugation in PBS (400 X g, 10 minutes). In order to induce the acrosome reaction, capacitated sperm were ressuspended in 20% v/v of human follicular fluid for 20 minutes (Calvo *et al.*, 1989; Bahamondes *et al.*, 2003). After the incubation, the suspension of sperms was washed three times in PBS by centrifugation (400 X g, 10 minutes). For immuncytochemical analysis, 5μL samples of the ejaculated, capacitated and acrosome reacted spermatozoa were allowed to dry on glass slides. The samples were fixed with iced methanol for 30 minutes before the immunoassaying.

Immunocytochemisty assays

Testis and epididymal sections and spermatozoa in the three categories (freshly ejaculated, capacitated and induced-acrosome reacted) were rendered to immunocyitochemistry with mAb TRA 54 according to previously reported (Pereira *et al.,* 1998; Arrotéia *et al.,* 2004). Non-specific sites were blocked with 20% normal goat serum. Sections were incubated overnight at 4° C with ascites fluid containing mAb TRA 54 (diluted 1:4000), followed by incubation for 1 hour with goat-anti-rat biotinylated secondary antibody (Dako A/S, Glostrup, Denmark) diluted 1:200 and then with Strept ABC kit (Dako A/S, Glostrup, Denmark) during 45 minutes. The reaction was developed by incubating the sections with hydrogen peroxide and diaminobenzidine (Sigma Aldrich, St Louis, MO). Tissues sections were counterstained with hematoxylin. In control experiments, the ascitic fluid containing mAb TRA 54 was omitted from the primary antibody incubation step.

To detect the acrosomal status in the spermatozoa samples, prepared immunostained slides were washed in PBS and stained by incubation with lectin FITC labeled from *Pisum sativum* (FITC-PSA, 40μg/ml), for 30 minutes protected from light (Mendoza *et al*., 1992; Tesarik *et al*., 1993; Yovich *et al*., 1994, Bahamondes *et al*., 2003). After incubation, the slides were washed in PBS and covered in Vectashield Mounting Medium for Fluorescence (Vector Laboratories Inc, Burlingame, CA). This protocol provides double-labeled spermatozoa (with both mAb TRA 54 and FITC-PNA) to studying the location of the antigen recognized by the mAb TRA 54 and the spermatozoa acrosomal status simultaneously. Evaluation of the double-labeled spermatozoa was assessed under two different conditions: antigen localization was available by differential interference contrast (DIC) microscopy, whereas the acrosomal status of the immunolabeled sperm was available by fluorescence microscopy (filter with 494-blue excitation, 520 emission, 510-514 barrier).

The association between the distribution of the different pattern of the antigen expression and the acrosomal status of spermatozoa was assessed by Pearson's Chi-square test. Significance level was a priori established as $\alpha = 0.05$.

Effect of the mAb TRA 54 on the acrosome-reaction

Capacitated sperm obtained as described above were separated in 4 samples (I, II, III and IV). Samples II and IV were incubated with the ascites fluid containing mAb TRA 54 (diluted 1:400 in HTF) for 30 min at 37°C in a 5% CO_2 atmosphere, followed by two centrifugation cycles with PBS. Samples III and IV were centrifuged and then incubated with 20% v/v of human follicular fluid for 20 min at 37°C in a 5% CO_2. Sample I (control sample) was not incubated with neither antibody nor follicular fluid. After the incubations, all samples were washed in two centrifugation cycles with PBS. Aliquots of 5µL of each sample were allowed to dry on glass slides and fixed with iced methanol. The spermatozoa acrosomal status was detected by labeling with FITC-PSA as described above. The percentage of acrosome-reacted spermatozoa (AR %) in each sample was determined by two countings of the number of the acrosome-reacted sperm within 200 scored spermatozoa. The assay was repeated several times. Differences of the acrosomal-reaction rates among the four samples (I, II, III, IV) were investigated using an one-factor analysis of variance (ANOVA) followed by Tukey pairwise comparisons. Significance level was a priori established as $\alpha = 0.05$.

Immunoblotting

Freshly collected human testis and ejaculated spermatozoa were homogenized in 10 mM Tris-HCl pH 7.4 containing 10 mM EDTA (Mallinckrodt, Paris, Kentucky), 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM sodium orthavonadate, 2 mM Phenylmethylsulfonylfluoride (PMSF) and 0.1 mg of aprotinin/ml (Sigma Aldrich, St Louis, MO).

Tissues and spermatozoa homogenates were centrifuged (10.000 X g) and the protein concentration of the supernatants was determined using a Bradford protein assay kit (Bio-Rad, Richmond, CA, USA). Aliquots of each homogenate (100µg per lane) were diluted in sample buffer to perform the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with 7,5% gels prepared according to Laemmli (1970). The proteins were transferred electrophoretically to polyvinylidenedifluoride (PVDF) membranes (Millipore, Bedford, MA, USA), which were then blocked with 5% low-fat dry milk during 1 hour and washed with 0,05% tween-Tris-buffer-saline (TBS-T). The membranes were subsequently incubated with mAb TRA 54 (diluted 1:2500 in TBS-T) overnight, washed in TBS-T and incubated with peroxidase anti-rat IgG (Dako A/S, Glostrup, Denmark) during 1h. Immunoreactive bands were detected by incubating the membranes with 0.03% hydrogen peroxide and 0.05% diaminobenzidine in 50 mM Tris-HCl, pH 7.2.

Washing and Igepal extraction of the antigen from ejaculated sperm

In order to investigate the easiness of the antigen removal from the sperm surface, ejaculated spermatozoa collected as described above were repeatedly centrifuged at 400g and ressuspended in PBS (Liu *et al.,* 1991). Spermatozoa supernatants were stored in each centrifugation step to further SDS-PAGE and immunoblotting analysis. The centrifugation and supernatant storage procedures were repeated up to seven times. Furthermore, to assess the extractability of the antigen from the spermatozoa membrane (Liu *et al.,* 1991), the pelleted sperm were homogenized in 10 mM Tris-HCl pH 7.4 containing 0,5% of Igepal, 10mM Hepes, 0,15M NaCl (Merck) and 0,2mM aprotinin (Sigma Aldrich, St Louis, MO) (Liu *et al*., 1991). The samples were sonicated at maximal speed (15 cycles of 8 seconds) followed by centrifugation at 400 x g and later at 10.000 X g for 10 min at 4^{o}C. Obtained supernatants were destined both to SDS-PAGE and immunoblotting analysis as described earlier.

Results

Antigen expression in human testis and epididymis

The antigen recognized by the mAb TRA 54 was expressed in both human testis and epididymis. Testis control reactions without TRA 54 showed no positive labeling (Figure 1a). Specific positive reaction was seen on the acrosome of spermatocytes and spermatids inside of seminiferous tubules (Figure 1b-c), as well as in postacrosomal region of the testicular sperm (Figure 1d).

Epididymal control reactions without TRA 54 showed no positive labeling (Figure 1e). No mAb TRA 54 positive staining was seen in the initial segment of the caput epididymis, but specific positive staining in the caput epithelial cells was seen in the supranuclear cytoplasm of the epithelial cells (Figure 1f-g). Both in corpus and caudal segments only the surface of the epithelia was stained (Figure 1h). Luminal contents and spermatozoa present in all segments were labeled with the mAb TRA 54 (Figure 1f-h).

Location of the antigen on human spermatozoa

Fresh spermatozoa, capacitated and acrosome-reacted spermatozoa showed positive labeling to the mAb TRA 54 on the spermatozoa surface. The staining was extended from the equatorial and post-equatorial segments to the middle-piece of the spermatozoa (Figures 2a-c). Control section did not show the staining (Figure 2d). The anterior region of the sperm head and the flagella were not stained (Fig 2a-c.)

Isolated spermatozoa in the three category (freshly ejaculated, capacitated and acrosome-reaction-induced) were simultaneously analyzed for both staining pattern with the mAb TRA 54 and for the acrossomal status with PSA-FITC labeling (Fig. 2e-f, g-h, i-j, k-l). The parameters used to identify the spermatozoa acrossomal status were previously published (Morales *et al.,* 1986; Mendoza *et al*., 1992). Spermatozoa showing uniform fluorescence over

the anterior head were classified as live and intact, not-reacted spermatozoa (Figure 2e). Spermatozoa barely showing fluorescence on the anterior portion of the head and a clear dark green band over the postacrosomal region were classified as live and acrosome-reacted sperm (Figure 2g). Spermatozoa with undetectable or disorganized fluorescence over the whole surface were classified as dead and degenerated spermatozoa (Figure 2i, k).

Simultaneously to the acrosomal status definition, four patterns of the antigen expression were identified after spermatozoa immunostaining with the mAb TRA 54. Spermatozoa classified in pattern 1 (p1) showed weak detectable or undetectable positive labeling (Figure 2f, h). Spermatozoa in pattern 2 (p2) showed restrict positive staining on the post-equatorial segment of the spermatozoa head (Figure 2f, h). Spermatozoa in the pattern 3 (p3) showed positive labeling on the middle-piece region besides the post-equatorial segment (Figure 2f, h). Spermatozoa in the pattern 4 (p4) showed disperse or disorganized labeling on any spermatozoa region (Figure 2j, l). Statistical analyses showed that there is a significant association between the different patterns of the antigen expression and the spermatozoa acrossomal status (χ^2 = 152,316; *df* = 6; *P* <0,0001, Table 1). The contingency analysis (Figure 3) indicated that the antigen expression patterns 1 and 2 were more frequent in the acrosome intact spermatozoa. Antigenic expression pattern 3 was the most frequent pattern associated with acrosome-reacted spermatozoa. Antigenic pattern 4 was more frequent in dead/degenerated spermatozoa.

Effect of the mAb TRA 54 on the induced acrosome-reaction

Follicular fluid significantly increased the acrosomal reaction rates (*F*=5,877, *df*=3; *P*=0.0048). Tukey pairwise comparisons are shown in Figure 4. Presence of the mAb TRA 54 (sample II) did not alter the basal acrosome reaction rates (sample I), but the addition of the follicular fluid (samples III and IV) increased the acrosome reaction rates. No differences were

observed in the induced acrosomal reaction rates between samples pre-incubated (sample III) or not pre-incubated (sample IV) with the mAb TRA 54.

Molecular identity of the antigen

Immunoblot analysis of human testis extracts showed large immunoreactive bands of 260, 200, 115 and 90 kDa (Figure 5a). Immunoreactive bands of 260 and 200 kDa were also detected in extracts from human isolated spermatozoa (Figure 5a).

Removal of antigen from the spermatozoa surface and Igepal extraction

Immunoblot analysis with the mAb TRA 54 showed the complex immunoreactive band of ~ 190 – 260 kDa in all samples obtained from spermatozoa after repeated washing, centrifugation and ressuspension (Figure 5b). The same pattern of the antigenic bands distribution was verified to both supernatant and pelleted spermatozoa obtained after the Igepal extraction of the membrane proteins (Figure 5b).

DISCUSSION

For fertilization to occur, the ability of sperm to reach the egg is a critical event of sperm egg adhesion and fusion. After the gametes approach each other, the spermatozoa must have means to penetrate the structures surrounding the egg in order to reach, and untimely fuse with the egg plasma membrane (Lin *et al.*, 1994).

The epididymal maturation is a collective term to the set of alteration undergone by spermatozoa in epididymal lumen, resulting in essential modifications of several antigens profiles that provide the success of fertilization (Jones *et al.*, 1985; Toshimori *et al.*, 1988; Vreeburg *et al.*, 1992; Tulsiani *et al.*, 1993; Kirchhoff and Hale, 1996; Kirchhoff, 1998; Kaunisto

et al., 1999, Mathur *et al.,* 2000; Yoshinaga and Toshimori, 2003). It is known that several androgen-dependent proteins produced by epididymis are further incorporated by the spermatozoa (Tezon *et al.*, 1985; Boué *et al.*, 1995; Ellerman *et al.*, 1998; Liu *et al.*, 2000) and could participate in the spermatozoa binding to the egg zona pellucida (Boué *et al.*, 1994) or membrane (Saling *et al.*, 1985; Naz *et al.*, 1992; Flesch and Gadella, 2000, Ensslin and Shur, 2003).

In a recent study (Arrotéia *et al.*, 2004) we have characterized the new mouse epididymal expression pattern of an antigen previously identified in germ cells by mAb TRA 54 (Pereira *et al.*, 1998; Ventellä *et al.*, 2003). Previous results (unpublished data) demonstrated that the antigen recognized by the mAb TRA 54 is a molecule synthesized and released by the epididymal epithelia in a classical pathway of protein secretion. The molecule released in epididymal lumen seems to bind on spermatozoa moving down through the epididymal duct, suggesting a critical role in one or more events of the fertilization process including oocyte recognition and penetration. In this study we investigated the pattern of the expression and the behavior of the antigen recognized by the mAb TRA 54 in human male reproductive tract and spermatozoa.

The mAb TRA 54 antigen was expressed in human testicular germ cells and in human caput epididymal epithelial cells in a pattern similar to the one described to C57BL6 mice testis and epididymis (Arrotéia *et al.*, 2004). As in mouse organs, the antigen recognized by the mAb TRA 54 was found in acrosomal vesicles of spermatides and in the apical cytoplasm of the caput principal cells. Furthermore, the immunoblot analysis showed that the molecules recognized by mAb TRA 54 in human testis were similar to the molecules expressed in mouse counterpart. As verified in mouse model, the presence of various immuno-reactive bands may indicate that mAb TRA 54 recognizes a common epitope of different isoforms of the same molecule modified by different-sized carbohydrate chains. Altogether, the immunohistochemical and immunoblot

analyses strongly suggest that the mAb TRA 54 antigen seems to be an inter-specific glycoprotein expressed in a conserved way in the male reproductive tract of mammals including the human being. Beyond the intrinsic importance of this affirmation, these findings might suggest that the human antigen recognized by the mAb TRA 54 can assume a crucial role in the spermatozoa epididymal maturation and also oocyte fertilization. Cases of mice and human infertility are reported to be associated with absence of one or more epididymal antigens (Boué and Sullivan, 1996; Sipila *et al*., 2002).

We reported somewhere else (Arrotéia *et al*., 2004) that the antigen released by mouse epididymis is incorporated by the acrosomal region of the spermatozoa moving down in the epididymal duct. Interestingly, this study shows that the antigen recognized by the mAb TRA 54 was shown to be adhered not to the acrosomal region of the human spermatozoa head but to the surface membrane overlaying the equatorial and post-equatorial segments. In addition, results of the spermatozoa washing and membrane proteins Igepal extraction procedures showed that the antigen could both be absorbed at the spermatozoa surface as an extrinsic membrane protein and also to be integrated to the spermatozoa equatorial segment membrane as an intrinsic molecule. Interestingly, it is the spermatozoa head equatorial region that provides a molecular machinery to introduce the spermatozoa into the oocyte by fusing with the oocyte membrane (Flesch and Gadella, 2000; Manandar and Toshimori, 2001; Ramalho-Santos *et al*., 2002). The location of the antigen recognized by the mAb TRA 54 on the spermatozoa head equatorial region clearly indicates that this antigen could be involved in the human fertilization process. Furthermore, besides dead or degenerated spermatozoa seem to weaken the studied antigen, the *in vitro* capacitated spermatozoa are able to keep the molecule on the gamete surface. Since the capacitation process leads spermatozoa to the exocytic acrosome reaction, and since that the acrosome reaction leads the spermatozoa to contact the oocyte membrane (Myles and Primakoff, 1984; Flesch and Gadella, 2000), the maintenance of the studied antigen

on the human capacitated and acrosome reacted spermatozoa constitute important evidence that this molecule could be involved in the sperm-egg fusion process.

The immunolabeling analysis with the mAb TRA 54 in induced acrosome-reacted sperms showed that the antigen moved from post-acrosomal and equatorial segments of the spermatozoa head to the spermatozoa midpiece both during and after the acrosome reaction. These results are in agreement with previous work demonstrating that the spermatozoa surface antigens could change their location from a initial to a new position pattern as a response to the capacitation and to the acrosome reaction processes (Myles and Primakoff, 1984; Myles *et al.,* 1987; Rochwerger and Cuasnicu,1992; Tanii *et al*., 1995; Cowan *et al.,* 2001). Furthermore, other researchers report that spermatozoa membrane molecules can be dislocated through the membrane because of the exerted function or the spermatozoa maturation degree, resulting in a new complex redistribution of these molecules in the spermatozoa surface (Saxena *et al*., 1986; Myles and Primakoff, 1984; Myles *et al*., 1987; Villarroya and Scholler, 1987; Batova *et al*., 1998; Lassare *et al*., 2003). It is known that during the acrosome reaction the outer acrosomal membrane fuses with the overlaying plasmatic membrane resulting in a large surface alteration that allows the spermatozoa to cross zona pellucida and bind/fuse with the egg membrane (Flesch and Gadella, 2000). In this way, the subsistence of the antigen recognized by the mAb TRA 54 on the spermatozoa surface after the acrosome reaction strongly supports the hypothesis that this antigen could be involved to the complex mechanisms that allow the spermatozoa to fertilize an oocyte. Once the antigen was demonstrated not to be involved in the triggering of the acrosome reaction process itself (Table 1, Figure 4), it could be supposed that the antigen could participate in the fertilization process as a protective glycoprotein of the essential fertilizing receptors located at the post-equatorial region. This hypothesis allows us to suggest that acrosome reaction could move the antigen from an anterior to a final or posterior position on the spermatozoa head, resulting in a critical exposition of oocyte receptors located at

the spermatozoa post-equatorial region, leading in the last analysis to the outcome of the fertilization process.

Concluding remarks

The findings obtained from this work indicate that the human antigen recognized by the mAb TRA 54 shows the same pattern of expression previous related to mouse testis and epididymis. In the same way, the studied antigen was demonstrated to be an inter-specific conserved glycoprotein produced and released by mammal's epididymis to later adhere to spermatozoa membrane moving down from epididymal caput to the corpus and cauda regions during the maturation process. The maintenance of the antigen in capacitated and acrosome-reacted spermatozoa strongly suggests that the molecule could participate in local events that result in fertilization of the egg by sperm. The antigen was found to move from the equatorial segment to the midpiece of the human spermatozoa surface, as a result of membrane alterations provided by the acrosome reaction process. The moving of the antigen at the final steps of the spermatozoa preparation leading fertilization together with the final position of the antigen seem indicate that the molecule could be involved in the initial protection and further exposition of the spermatozoa receptors to the oocyte membrane. Further investigation involving the presence of the studied antigen in spermatozoa obtained from infertile donors could contribute to the evaluation of the molecule biological function in the fertilization process. Although, the human epididymal antigen recognized by the mAb TRA 54 seems to play an important role in epididymal function related to spermatozoa maturation and capability of fertilization.

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Table I – Mean values (%) of association among the four antigen expression patterns (p1, p2, p3 and p4) and the three spermatozoa categories (intact, acrosome-reacted and dead/degenerated). χ^2 = 152,316; df = 6; P <0,0001.

	Pattern 1	**Pattern 2**	**Pattern 3**	**Pattern 4**	**Total**
Intact acrosome	17,32% (n=146)	14,12% (n=119)	10,56% (n=89)	0,24% (n=2)	42,23% (n=356)
Acrosome-reacted	6,29% (n=53)	7,83% (n=66)	8,07% (n=68)	1,07% (n=9)	23,25% (n=196)
Dead spermatozoa	11,86% (n=100)	4,86% (n=41)	8,54% (n=72)	9,25% (n=78)	34,52% (n=291)
Total	35,47% (n=299)	26,81% (n=226)	27,16% (229)	10,56% (n=89)	100% (n=843)

Figure Legends

Figure 1. Immunohistochemical staining with mAb TRA 54 in cross-section of human testis (a-d) and epididymis (e-h). Negative control reactions did not show positive labeling (a, e). Positive labeling was found in acrosome of spermatids and in postacrosomal region of the testicular spermatozoa (b-d). Supranuclear cytoplasm of the epididymal caput epithelial cells showed specific positive staining (f, g), whereas in the corpus and caudal segment only the surface of the epithelia were stained (h). Luminal contents and sperm present in all the segments were labeled with the mAb TRA 54 (e-h). Scale bar - a: 100 μm; b: 40 μm; c: 32μm; d: 20μm; e-f: 40 μm; g: 32μm, insert: 10 μm; h: 40 μm, insert: 20 μm.

Figure 2. Evaluation of the human sperm was performed under both differential interference contrast (DIC) (antigen expression) and fluorescence microscopy (acrosome PSA-FITC labeling). Positive labeling with the mAb TRA 54 was found on human spermatozoa from ejaculated (a), in vitro capacitated (b) and induced acrosome reaction (c) samples. Negative control reactions did not show the positive labeling (d). The labeling was observed in the equatorial/post-equatorial (arrows) or in the middle piece (b, c) spermatozoa regions. The figures e-f, g-h, i-j and k-l shows the same spermatozoa observed under fluorescence (e, g, i, k) or diferential interference contrast (DIC) (f, h, j, l). Spermatozoa were classified in three categories according the acrosome: intact spermatozoa (e), acrosome-reacted spermatozoa (g) and degenerated or dead spermatozoa (i, k). Four patterns of the antigen expression were observed in all spermatozoa samples: the pattern 1 was pointed by large arrows (f, h); the pattern 2 by short arrows (f, h), the pattern 3 by arrowheads (f, h) and the pattern 4 by long arrows (j, l). Scale bar: a-c: 5μm; d: 6μm; e-j: 15μm; inserts: 6μm; k-l: 10μm.

Figure 3. Mean values of the association among the four antigen expression patterns (p1, p2, p3 and p4) and the three spermatozoa categories (intact, acrosome-reacted and dead or degenerated). Black areas: pattern 1; gray areas: pattern 2; white areas, pattern 3 and hachured areas: pattern 4. The pattern 3 was more frequent in the spermatozoa acrosome-reacted, and the pattern 4 was more frequent in dead spermatozoa category. The pattern 4 was absent in the intact spermatozoa, which showed a higher frequency of the pattern 1.

Figure 4. Effect of the mAb TRA 54 on the human spermatozoa acrosome reaction. Mean values Tukey pairwise comparisons of acrosome reaction rates in spermatozoa (groups I, II, III, IV). Differences between the media were given by Tukey test of multiple comparisons following the one-factor analysis of variance. Means are significantly different if showing different letter. No differences were observed in the induced acrosomal reaction rates between samples pre-incubated (sample III) or not pre-incubated (sample IV) with the mAb TRA 54.

Figure 5. Western blot analysis with mAb TRA 54. **a.** Protein samples of testis (Hte) and ejaculated sperm (esp) were electrophoresed and transferred to the membrane filter and immunoblotted with mAB TRA54. Cont: negative control to the immunereaction. Immunoreactive bands of ~260, 200, 115 and 90 kDa were detected in testis extract whereas bands of ~260 and 200 kDa were detected in spermatozoa extracts. Positions of molecular weight markers were at the left margin. **b.** Protein samples of ejaculated spermatozoa after 1 to 7 washes (1-7) and after Igepal extraction following the seven previous washes. Igs: Igepal sample, supernatant; Igp: Igepal sample, sperm pelleted. Proteins were immunoblotted with mAB TRA54. Cont: negative control to the immunereaction. Immunoreactive bands of ~260 and 200 kDa were detected in all the washes extracts. Positions of molecular weight markers were at the left margin.

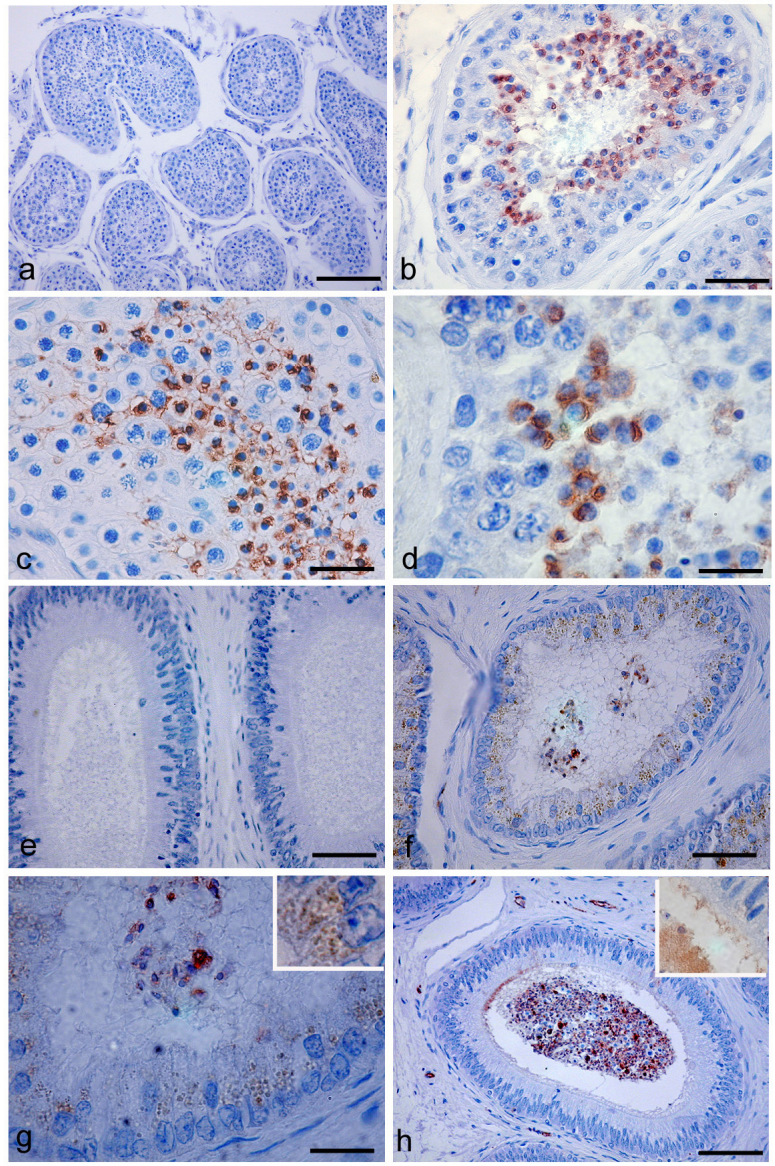


Figure 1

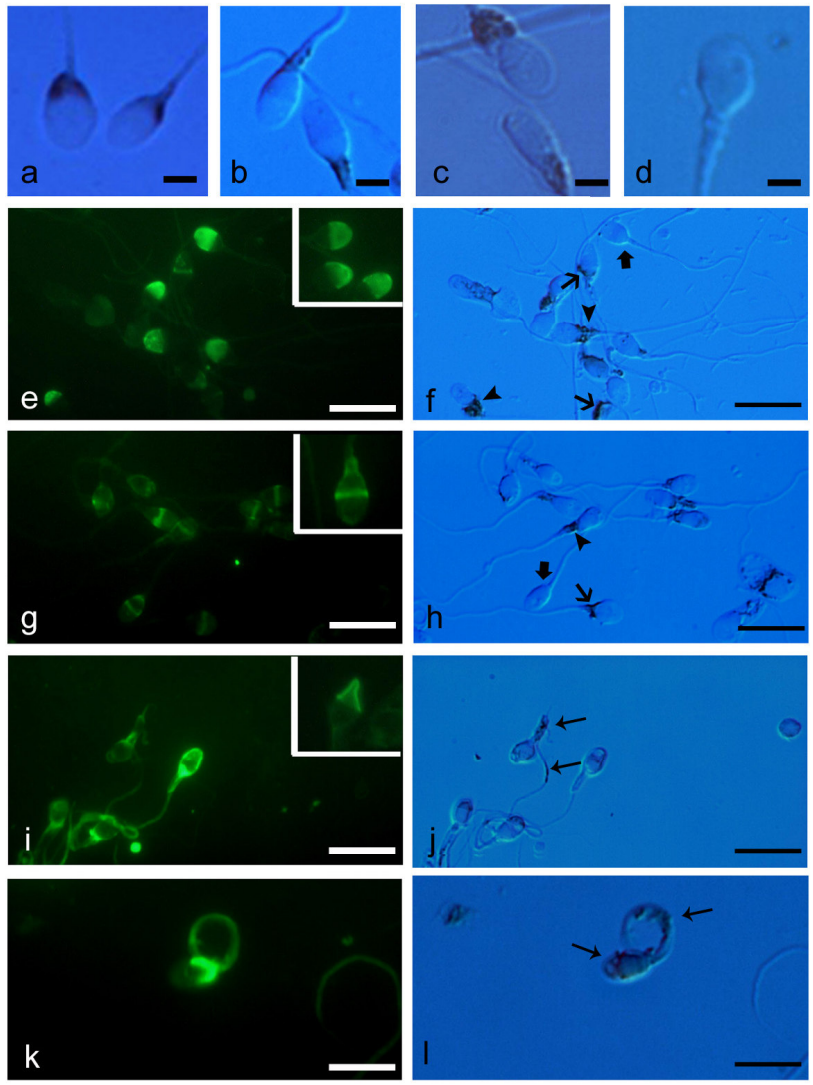


Figure 2

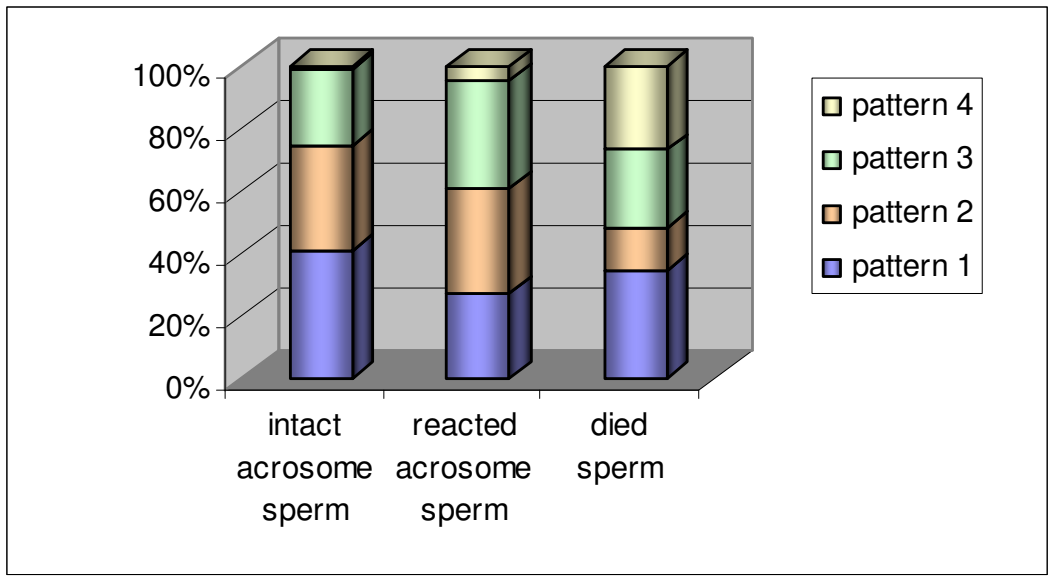


Figure 3

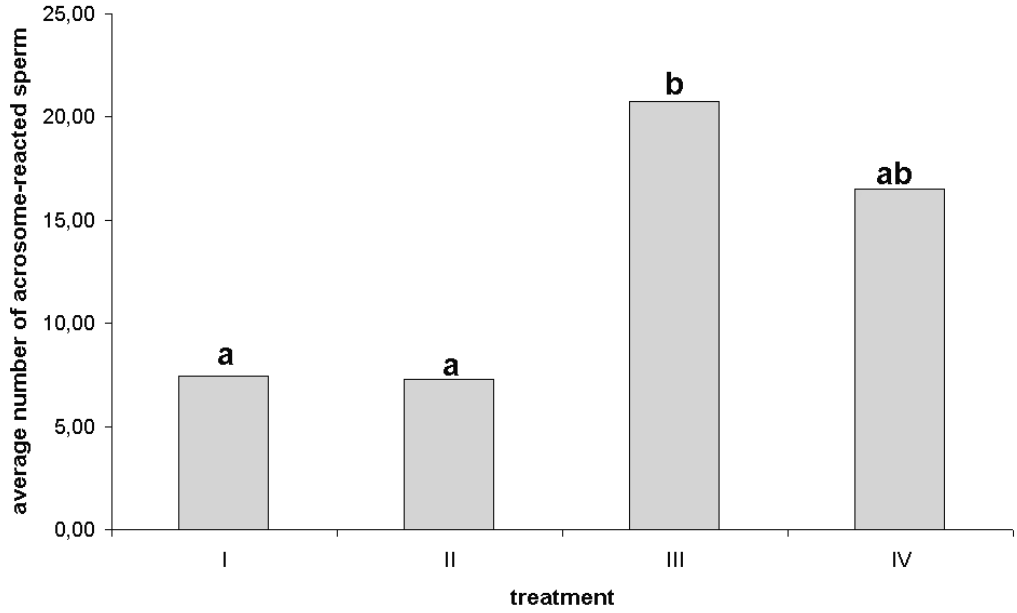
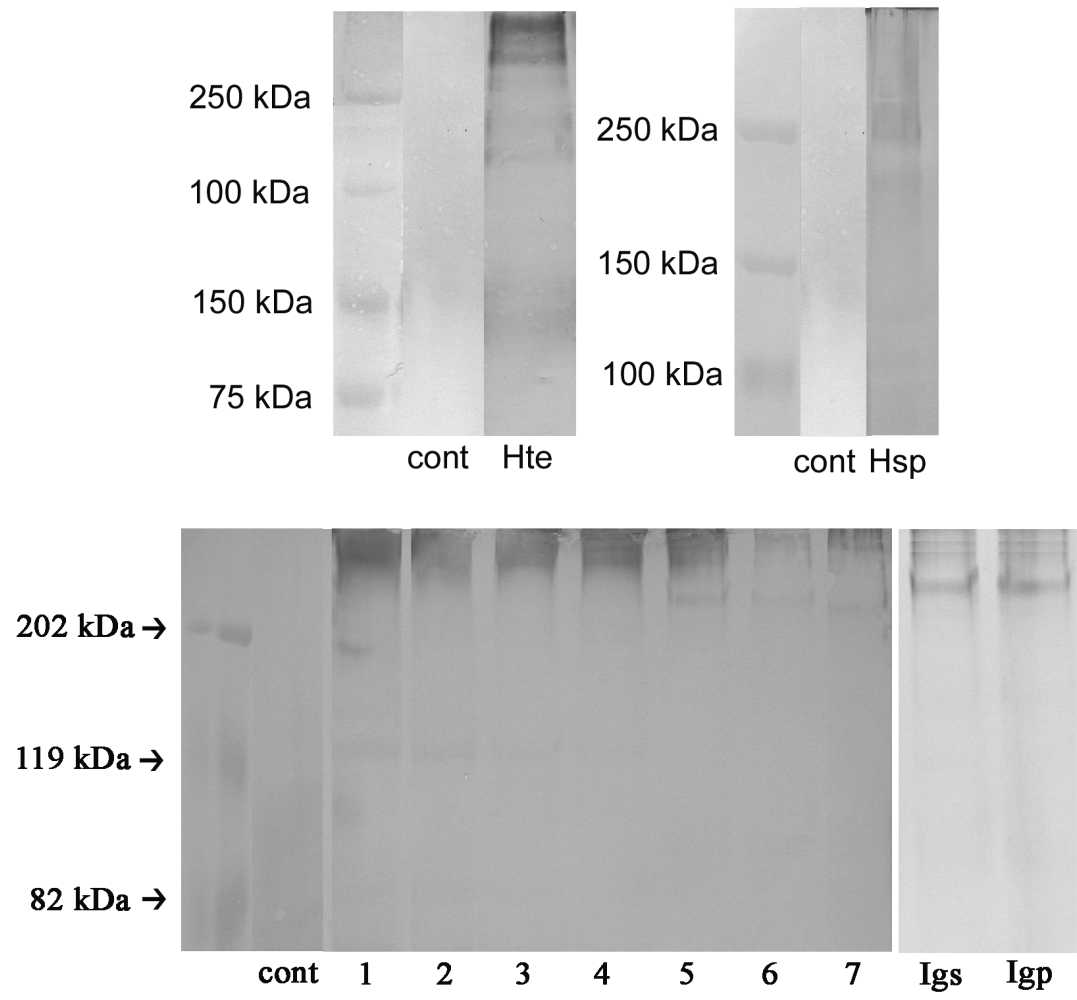


Figure 4


250 kDa
100 kDa
150 kDa
75 kDa
cont
Hte
250 kDa
150 kDa
100 kDa
cont
Hsp
202 kDa →
119 kDa →
82 kDa →
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5
6
7
Igs
Igp

Figure 5

CHARACTERIZATION OF A NEW MOUSE EPIDIDYMAL ANTIGEN RECOGNIZED BY THE MONOCLONAL ANTIBODY TRA 54

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Running Tittle: A new mouse epididymal antigen recognized by mAb TRA 54

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The epididymis participates in the maturation of spermatozoa necessary for fertilization, but little is known about the epididymal molecules involved in the corresponding spermatozoa modifications. We have previously described a novel pattern of expression for an antigen in testicular germ cells and epididymal epithelial cells that reacts with the monoclonal antibody (mAb) TRA 54. Immunohistochemical analyses suggested that this epididymal antigen was released into the epididymal lumen and subsequently bound to luminal spermatozoa in an androgen-dependent manner. In the present report, we describe the purification and structural characterization of the mouse epididymal antigen recognized by mAb TRA 54. Sequencing of tryptic fragments from the purified protein by a combination of MALDI-TOF-MS and LC-nano-ESI/MS/MS mass spectrometry followed by sequence alignments showed that the protein corresponded to an unnamed protein (BAC 34360; ~65 kDa, pI 5.49) initially identified in the mouse genome. The protein is originated from a cDNA related to the mouse albumin-1 gene. Functional experiments indicated that

this antigen is important for penetration of the zona pellucida during the acrosomal reaction. The epididymal antigen recognized by mAb TRA 54 plays an important role in spermatogenesis and in the epididymal maturation necessary to prepare the spermatozoa for fertilization.

The functions of the epididymis include the absorption of seminiferous fluid and the concentration, transport and storage of spermatozoa (1,2). Mammalian spermatozoa acquire their capacity to fertilize oocytes through multiple and sequential interactions with proteins present in the luminal fluid of the epididymis (3-5). The functional and antigenic modification of spermatozoa in the epididymal environment, a process known as epididymal maturation (2,6,7), changes the profile of antigen expression, partly through the addition of new molecules to the surface of these gametes (8-13). Several enzymes are secreted into the luminal fluid by different epididymal regions and contribute to the surface modifications that culminate in spermatozoa maturation (2-5).

Although spermatozoa alterations during maturation in the epididymis are essential for successful fertilization, relatively little is known about the role of the epididymis in this process and about the modifications involved (14). The use of monoclonal antibodies (mAb) to study antigens expressed by cells of the male reproductive organs has contributed to our understanding of the role of these proteins in spermatozoa maturation and in the formation of functional male gametes (13,15-17). The mAb TRA (Testicular Germ Cells Immunized to rat - monoclonal antibody) 54, raised by immunizing rats with mouse testicular germ cells, was initially identified by its ability to recognize an antigen expressed in spermatids of the seminiferous tubules of C57 BL/6 older than 24 days (18,19). A detailed analysis of the intracellular biosynthetic pathway of this antigen in testicular germ cells showed that the translation of the mAb TRA 54 antigen started in the Golgi system of early spermatids and that the antigen was incorporated by the acrosomal system of these cells during spermiogenesis (19).

Recently, we investigated the expression pattern of the mAb TRA 54 antigen in epithelial cells of the mouse epididymal caput and observed that this protein was expressed independently of that found in testicular germ cells (20). This finding confirmed previous observations that the TRA 54 antigen was expressed by early spermatids but not by spermatozoa in the seminiferous tubules (18). Interestingly, we detected the TRA 54 antigen in spermatozoa present in the epididymal lumen, which suggested that molecules containing the epitope recognized by mAb

TRA 54 could be released by epithelial cells of the epididymal caput and subsequently bind to the surface of spermatozoa moving down this duct (20).

The antigen recognized by mAb TRA 54 in the epididymis and luminal spermatozoa is a high molecular mass (260 kDa) protein (20). Preliminary experiments in our laboratory have suggested that this antigen may be important during the acrosomal reaction for penetration in the zona pellucida, a glycoprotein layer surrounding the oocyte (unpublished observations). However, no data concerning the molecular identity of this protein have been reported so far.

In this work, we used a combination of column chromatography and mass spectrometry (MS) to isolate and sequence the antigen recognized by mAb TRA 54 in mouse epididymal epithelial cells. Structural analysis of the epididymal antigen indicated that this protein belongs to the prealbumin epididymal-specific (PES) class of proteins, the members of which can bind to the spermatozoa membrane during the passage of these gametes through the epididymal lumen.

Experimental Procedures

Animals - Male C57 BL/6 mice were obtained from a breeding colony maintained by the Multidisciplinary Center for Biological Investigation (CEMIB) at UNICAMP and was housed at 25^{o}C on a 12 h light/dark cycle, with free access to food and water. The animal experiments described here were approved by the institutional Committee for Ethics in Animal Experimentation (CEEA/IB/UNICAMP, protocol number 590-1) and were done within the guidelines of the Brazilian College for Animal Experimentation (COBEA).

Preparation of monoclonal antibody - The monoclonal antibody TRA 54 was obtained as described elsewhere (18).

Preparation of tissue samples - Adult (60-day-old) C57 BL/6 mice were sacrificed by cervical dislocation and the epididymal caput was collected and homogenized in 1 ml of 10 mM Tris-HCl, pH 7.4, containing 10 mM EDTA (Mallinckrodt, Paris, France), 100 mM NaF, 10 mM $Na_4P_2O_7$, 10 mM Na_3VO_4, 2 mM PMSF and 0.1 mg of aprotinin/ml (Sigma-Aldrich, St. Louis, MO). Fresh spermatozoa were allowed to disperse in the same buffer after cutting the epididymal cauda. The tissue suspensions were subsequently centrifuged for 15 min at 10,000 *g* at 4^{o}C followed by two additional centrifugations of the supernatant from the previous step (25 min each at 25,000 *g* and 4^{o}C). The protein concentration of the supernatants was determined using a Bradford protein

assay kit (Bio-Rad, Richmond, CA). Aliquots of the samples were separated by SDS-PAGE[1], and immunoblotting was done to confirm the distribution pattern of the immunoreactive bands before chromatography.

SDS-PAGE and immunoblotting - Aliquots of each sample (100 µg per lane) were diluted in sample buffer and applied to 7.5% polyacrylamide gels prior to SDS-PAGE, according to Laemmli (21). The proteins were subsequently transferred electrophoretically to polyvinylidenedifluoride (PVDF) membranes (Millipore, Bedford, MA) for immunoblotting with mAb TRA 54. The efficiency of electrotransfer was assessed by staining the gels with Comassie blue to detect untransferred protein bands. The PVDF membranes were blocked with 5% low-fat dry milk for 1 h and then washed with 0.05% Tween 20-Tris-buffer-saline (TBS-T). After incubation with mAb TRA 54 (diluted 1:2500 in TBS-T) overnight at 4ºC followed by washing in TBS-T, the membranes were finally incubated with goat anti-rat IgG conjugated with peroxidase (Dako A/S, Glostrup, Denmark) for 1 h at room temperature. Immunoreactive bands were detected by incubating the membranes with 0.03% hydrogen peroxide and 0.05% diaminobenzidine in 50 mM Tris-HCl, pH 7.2.

Chromatographic assays – The mAb TRA 54-reactive antigen was purified from supernatants of epididymal homogenates by a combination of affinity and ion exchange chromatography, as described below.

Lectin affinity chromatography - Epididymal supernatant (120 mg of protein) obtained as described above was loaded onto a column (HR 5/20) packed with the lectin concanavalin A (Con A) (Amersham Biosciences, Piscataway, NJ) that specifically binds □-D-glucopyranosyl□ -D-mannopyranosyl or sterically similar residues. The column was initially equilibrated with 0.1 M sodium acetate, pH 6.0, containing 1 M NaCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$, and unbound proteins were washed out with this same buffer. Bound proteins were eluted with a linear gradient (0-0.5 M) of glucose in this sodium acetate-salt buffer at a flow rate of 1 ml/min. The protein elution profile was monitored by measuring the absorbance at 280 nm using an ÄKTApurifier10 chromatographic system (Amersham Biosciences).

One milliliter fractions were collected and screened for reactivity towards mAb TRA 54 by dot blotting and/or immunoblotting. For dot blotting, 3 µl of each fraction was spotted onto a nitrocellulose membrane (BioRad) previously activated with 0.1 M sodium carbonate/bicarbonate buffer, pH 9.5. The spots were allowed to dry at room temperature and the membrane was

processed for immunodetection using mAb TRA 54 essentially as described for immunoblotting. Immunoreactive fractions were pooled and concentrated by centrifugation (4,000 *g*, 40 min, 4°C) in Amicon 5 kDa membranes (Millipore Bedford, MA) before anionic ion exchange chromatography.

Anionic ion exchange chromatography – The immunoreactive pool obtained in the previous step was applied to a HiTrap Q Sepharose™ High Performance column (5 ml; Amersham Biosciences) previously equilibrated with 50 mM Tris-HCl, pH 7.4. After washing the column to remove unbound material, bound proteins were eluted with a linear gradient of NaCl (0 – 1 M) in this same buffer. Dot blotting was used to detect the fractions that reacted with mAb TRA 54 and these were combined to form three pools (A, B and C) based on their elution profile. The samples were concentrated by centrifugation (4,000 *g* at 4°C for 40 min) in Amicon 5 kDa membranes (Millipore, Bedford, MA, USA) and analyzed by SDS-PAGE and immunoblotting with mAb TRA 54 to detect immunoreactive bands. Protein bands in the polyacrylamide gels were detected by staining with Commassie blue.

Gel In situ digestion - The mAb TRA 54-reactive band detected in the three pools was co-localized in gels stained with Commassie blue and excised with a sterile scalpel after destaining. The excised piece of gel was transferred to a 1.5 ml Eppendorf tube and cut into small pieces that were then suspended in 0.2 M ammonium bicarbonate containing 45 mM DTT followed by incubation at 30° C for 30 min. This liquid was subsequently removed after brief centrifugation and the gel pieces were incubated with undiluted acetonitrile for 10 min and then dried under vacuum (speed vacuum, Biotron, Modul 3180C, Puchon, Korea). The sample was rehydrated in 10 µl of 45 mM ammonium bicarbonate containing 0.5 µg of bovine trypsin (Sigma Aldrich) followed by incubation overnight at 37°C. The peptides generated by this digestion were extracted from the gel fragments by two incubations (at least 40 min each) with 100 µl of 0.1% trifluoroacetic acid (TFA) in 60% acetonitrile. Each incubation was followed by centrifugation to obtain a peptide-containing supernatant that was removed and dried by rotary evaporation (22).

Protein identification by mass spectrometry (*MS)* - Mass spectra were acquired using a matrix-assisted laser desorption ionization mass spectrometer fitted with a time-of-flight (TOF) analyzer (MALDI-TOF-MS), and a bidimensional capillary liquid chromatography (CapLC system, Waters, Manchester, UK) coupled to a tandem quadrupole orthogonal-TOF hybrid mass spectrometer

with a CID (collision induced dissociation) hexapole (QTOF, Waters-Micromass, Manchester, UK) with a nano Z-spray source, both operated in positive ion mode.

Peptide mass fingerprinting (PMF) by MS - The samples obtained by tryptic digestion were prepared for analysis using the dried droplet method. Initially, the samples were acidified by adding 1 µl of 0.1% TFA in water followed by incubation at room temperature for a few minutes to allow reduction of the droplet volume by evaporation in the MALDI microplate. The matrix solution of 0.1% (w/v) α-cyano-4-hydroxycinnamic acid was prepared in a solution of 0.1% TFA with acetonitrile (1:1, v/v), and 1 µl of this solution was added to the microplate. The sample was allowed to dry at room temperature. All of the measurements were done on a MALDI-TOF-MS instrument in the positive ion and reflectron modes. Subsequent data evaluation and peptide identification were done using the Mascot Wizard software package (Matrix Science, London, UK) (23).

Reverse phase capillary liquid chromatography/electrospray ionization mass spectrometry (ESI-MS) - 2D-LC-nanoESI-MS was done using the QTOF instrument and a CapLC ternary pump system connected through a stream-select valve module to the nano Z-spray source. The sample was injected into the system through the CapLC autosampler using the “microliter pickup algorithm” injection mode. Solvent A consisted of 5% acetonitrile in 0.1% formic acid, solvent B consisted of 95% acetonitrile in 0.1% formic acid, and solvent C consisted of 0.1% formic acid in H_2O. The protein digest was pre-concentrated and desalted on a Waters Opti-Pak C_{18} trap column and connected to pump C through the stream-select valve module. The pre-concentration/desalting step was done at 5 µl/min over 20 min using pump C. After switching to pumps A and B, a gradient was applied to the precolumn cartridge and was then used to elute the sample from the capillary column (NanoEase 75 µm i.d. x 15 cm) packed with C_{18} resin (Waters, Manchester, UK). The column was equilibrated with 5% of solvent B and a linear gradient of this solvent (from 5% to 70%) was applied over 60 min at a flow rate of 200 nl/min using a pre-column split with the pump delivering at 2 ml/min. NanoESI-MS analysis was done in-line with capillary chromatographic separation of the alkylated protein tryptic digests over the m/z range of 300-2000 at a scan speed of 1 s/scan. Data-dependent acquisition (DDA) was done in-line with capillary chromatographic separation only for the alkylated protein tryptic digest. An initial nanoESI survey mass spectrum was acquired over the m/z range of 300-2000 each second, with switching criteria for MS to MS/MS that included ion intensity and charge state.

NanoESI-MS/MS data were acquired over the m/z range of 50-2000 each second for up to three co-eluting peptides but only for doubly and triply charged ions. Switch back from nanoESI-MS/MS to nanoESI-MS mode was allowed after 10 s. The collision energy used varied automatically according to the mass and charge state ratio of the eluting peptides.

Peptide sequencing by LC-nanoESI-MS/MS - The ionization conditions used included a capillary voltage of 3.5 kV and cone and RF1 lens voltages of 30 V and 100 V, respectively. The source temperature was 70°C and the cone gas was N_2 at a flow of 80 l/h; the nanoflow nebulizer gas was warmed. Argon was used to dissociate the ions in the hexapole collision cell. External calibration with phosphoric acid 0.1% (v/v) was done over the entire 50-2000 m/z range. All mass spectra were acquired with the TOF analyzer in "V-mode" (TOF kV = 7.2) and the MCP voltage set at 2100 V.

De novo sequencing of tryptic peptides - Alkylated tryptic peptides separated by reverse-phase capillary chromatography were lyophilized and resuspended in 20% acetonitrile in 0.1% formic acid prior to injection into the mass spectrometer source at a flow rate of 100 nl/min. The resulting product ion mass spectra were acquired using the TOF analyzer and deconvoluted using the MaxEnt3 algorithm. "Single-charged" MS/MS spectrum was processed manually using the PepSeq application included in the MassLynx software package. The nanoESI-MS/MS data were processed using the softwares ProteinLynx Global Server v 2.0.5 for peak-list (pkl)-generated output files and MASCOT MS/MS Ion Search module (http://www.matrixscience.co.uk).

Computer-assisted alignment of the tryptic peptide sequences - The tryptic peptide sequences were aligned automatically by MASCOT (http://www.matrixscience.co.uk) using the MOWSE scoring scheme. The peptide fingerprint analysis tool of MASCOT incorporates a probability-based implementation of the MOWSE algorithm that accurately models the proteolytic behavior and specificity of trypsin. After scoring the data generated by the output files in MASCOT, all of the peptides were used to search the databank for matching proteins followed by alignment with the protein that showed the best MOWSE-based probability scores (24).

Nucleotide Sequence Similarity Searching - Basic Local Alignment Search Tool (BLAST) was used to compares the UPP related cDNA sequence against the mouse mRNA sequence

database (GenBank mRNA) on National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov). Comparisons were also generated using the Geneious 2.0.10 software (http://www.geneious.com).

Signal Peptide Prediction - The program SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the exportation signal peptide sequence of both UPP and mouse albumin-1.

Molecular modeling and phylogenetic analysis - The amino acid sequences of the tryptic peptides determined by MS were used to construct models of an Unnamed protein product – BAC34360 (25-31), using as starting geometry coordinates those of the crystal structure of human serum albumin obtained at 2.50Å resolution Protein Data Bank (PDB) ID 1O9X (32). A comparison of the 2D structures of various conserved domains of albumin using the 3D-jury server (http://bioinfo.pl/Meta) (33) showed that human serum albumin shared the greatest identity with the protein BAC34360. Optimal alignment of the albumin sequences was used to predict the structure with ESyPred3D and neural networks (http://www.fundp.ac.be) (34). The 3D structure was built using the modeling package Modeller 7v7 (35). Alignments for the best model template were obtained by combining, weighting and screening the results of several multiple alignments and were confirmed using the PDB-Blast server (http://www.rcsb.org/pdb/). The resulting structure file was examined and evaluated using the software RIBBONS (36) and the Swiss-PDBViewer v.3.51 (http://us.expasy.org). The relationship of BAC34360 to other members of the albumin superfamily of proteins was assessed by constructing a phylogenetic tree based on the conserved domains of these proteins and was generated by a neighbor-joining algorithm (37) using the programs Clustal X and Phylip (38,39).

Prediction of hydrophobic and antigenic domains – The hydrophobicity (40) and antigenicity (41) profiles of BAC34360 were determined using the Protean module of Lasergene software (DNASTAR, Madison, WI, USA).

Immunolocalization and fate of the TRA antigen during the acrosomal reaction

Sperm collection, capacitation and induction of the acrosomal reaction - Spermatozoa were collected from adult male mice by cutting the vasa deferentia with a scalpel and the dense mass of spermatozoa was then squeezed out and dispersed in 200 µl of M16 culture medium

supplemented with 4 mg of BSA/ml in an in vitro fertilization (IVF) dish. For capacitation, the spermatozoa were incubated for 2 h at 37°C in a 5% CO_2 atmosphere and then pelleted by centrifugation (400 *g*, 10 min) in PBS (Dulbecco´s phosphate-buffered salt solution; Gibco). To induce the acrosomal reaction, capacitated sperm were resuspended in 30 nM calcium ionophore A23187 for 20 min (42) and the suspension then washed three times in PBS by centrifugation (400 *g*, 10 min). For immunohistochemical analysis, 5 µl samples of the capacitated and acrosome-reacted sperm were allowed to dry on glass slides and fixed with ice-cold methanol for 30 min prior to immunostaining.

Immunostaining of sperm with mAb TRA 54 - Immunostaining was done as previously described (18,20) with slight modifications. Non-specific binding sites on slides prepared as described above were blocked with 20% normal goat serum. The sperm (capacitated or acrosome-reacted) on the slides were subsequently incubated overnight with mAb TRA 54 ascitic fluid (diluted 1:4000 in PBS/1% BSA) followed by incubation with a biotinylated anti-rat secondary antibody and detection with a Strept ABC kit (both from DAKO A/S, Glostrup, Denmark). The reaction was developed by incubating the slides with hydrogen peroxide and diaminobenzidine. Control sections were treated similarly but without mAb TRA 54. To assess the status of the acrosome, slides containing capacitated or acrosome-reacted sperm were labeled with FITC–conjugated peanut agglutinin lectin (FITC-PNA) (43,44). Spermatozoa that showed no labeling were considered to have undergone a complete acrosomal reaction (45). The resulting preparations were examined by fluorescence or phase-contrast microscopy using a Nikon microscope (Nikon, Tokyo, Japan).

Immunogold labeling with mAb TRA 54 - Capacitated sperm or sperm that had undergone an acrosomal reaction were washed three times in PBS by centrifugation and fixed in 1 ml of 4% paraformaldheyde, 0.2% glutaraldheyde in 0.1 M sodium cacodylate buffer, pH 7.4, at 4°C for 1 h. The samples were washed three times in 0.05 M Tris-HCl, pH 7.4 and dehydrated in a graded series of N-N-dimethylformamide and embedded in LR-White resin at -20°C for 48 h to ensure complete polymerization. Ultrathin sections (100 nm) were collected on nickel grids and then washed for 5 min in 0.05 M Tris-HCl, pH 7.4, and preincubated for 30 min with 0.05 M Tris-HCl, pH 7.4, containing 1% BSA. The grids were incubated overnight at 4°C with ascitic fluid containing mAb TRA 54 (diluted 1:3000), followed by washing with 0.05 M Tris-HCl, pH 7.4, and incubation for 1 h with a goat anti-rat secondary antibody conjugated to gold particles (15 nm

diameter) (Dako A/S, Glostrup, Denmark). The sections were stained in uranyl acetate. Gold-labeling control reactions were done by omitting the incubation with the ascitic fluid containing mAb TRA 54.

RESULTS

Detection of mAb TRA 54-reactive molecules in spermatozoa and epididymal caput homogenates - Western blots of extracts of freshly isolated epididymis revealed bands of ~200 kDa to 260 kDa that reacted with mAb TRA 54. No immunoreactive bands were seen in control experiments in which the mAb was omitted (Fig. 1).

Purification of the epididymal antigen - Figure 2*A* shows the elution profile of an epididymal homogenate after affinity chromatography. Although virtually no material bound to the column, this chromatographic step yielded two fractions of unbound material that reacted with mAb TRA 54 in dot blots (Fig. 2*B*). Anionic ion exchange chromatography of these combined fractions resulted in three immunoreactive regions: fractions 35-38 (pool A), 39-43 (pool B) and 44-50 (pool C) (Fig. 3*A*), based on dot blots (Fig. 3*B*). SDS-PAGE and immunoblotting of pool A showed a 65 kDa band that reacted strongly with mAb TRA 54 (Fig. 4); pools B and C showed only a weak reaction and were not used for band excision. The protein band corresponding to the immunoreactive band seen in pool A was excised from Commassie blue-stained gels for subsequent digestion with trypsin.

Digestion with trypsin, peptide sequencing by MS, and protein identification - Tryptic peptides of the purified protein were detected by MALDI-TOF-MS (Fig. 5) and sequenced by LC-nanoESI-MS/MS (Fig. 6) as described in the Methods. The accuracy of detection was 50 ppm for MALDI-TOF-MS and 15 ppm for nanoESI-MS/MS. Incubation of the purified protein with trypsin yielded 17 peptides for PMF and 15 for *denovo* sequencing from MS/MS spectra. The peptide sequences were subsequently used to search the National Center for Biotechnology Information (NCBInr) database for similar sequences using the software Mascot PMF and MS/MS Ion Search module with the MOWSE algorithm (http://www.matrixscience.co.uk) (46). All comparisons indicated that the tryptic peptides of the molecule recognized by mAb TRA 54 is related to a protein identified as “Unnamed protein product (UPP) - *Mus musculus*” (protein identification number BAC 34360), with a nominal molecular mass of 64,961 Da and pI of 5.49.

The sequence for UPP was deposited by Carninci and Hayashizaki (25) during the preparation of an *M. musculus* cDNA library for the Mouse Genome Encyclopedia Project of the Genome Exploration Research Group at the Riken Genomic Sciences Center and Genome Science Laboratory (27).

The mouse epididymal protein identified here was concluded to be UPP based on the protein score of the MOWSE algorithm, defined as -10*Log(P), where P is the probability that the observed match is a random event (46). In this analysis, protein scores higher than 62 are significant ($p<0.05$) for MALDI-TOF-MS. For MS/MS spectra, the ion score is also -10*Log(P), although in this case P is the probability that the observed match is a random event. Individual ion scores >33 indicate identity or extensive homology ($p<0.05$). For UPP, the scores were 85 and 699 for PMF and MS/MS spectra, respectively. UPP is 576 amino acids long and has three large domains homologous to the albuminoid protein superfamily.

Nucleotide Sequence Similarity Searching – UPP (BAC 34360) was generated from the mouse (*Mus musculus*) cDNA sequence identified by the accession number AK 050644.1 The search in NCBI Blast mouse database indicated that the UPP aminoacids sequence has 99,8% of sequence identity to the mouse albumin-1 mRNA (Fig. 7), that is located on the chromosome 5 of mouse genome (Location: Chr5 E2) (Fig. 8a). While albumin-1 full length gene has 15 exons, the AK 050644.1 cDNA has 14 exons because the first exon is missing (Fig. 8b).

Signal Peptide Prediction – Albumin-1 protein has an initial peptide signal whose cleavage site is located between the aminoacides on the positions 18 and 19. No peptide signal sequence was predicted to the AK 050644.1 cDNA sequence (data not showed).

Phylogenetic analysis – The phylogenetic analysis based on the amino acid sequences of UPP and members of the albuminoid protein family showed that this protein and albumin are closely related (data not showed).

Protein modeling - The predicted three-dimensional structure of UPP shown in Fig. 9 revealed that the protein consisted essentially of α-helices, arranged in a “V” shape in face view. Comparison of the predicted model for UPP with that for human serum albumin (1O9X), which showed the highest homology with the former protein based on the 3D-jury-server, indicated a marked similarity in their three-dimensional structures, including the high content of α-helices.

Although the primary sequence of UPP was most related to that of albumin 1 from *Mus musculus*, comparison of the theoretical 3D structures showed that 1O9X provided the best match (Fig. 10).

Antigenic and hydrophobic domains - Figure 11 shows that UPP contains numerous predicted antigenic and hydrophobic domains distributed throughout the molecule. The presence of antigenic sites agrees with the immunoreactivity of this protein with mAb TRA 54.

Spermatozoa localization of the antigen recognized by mAb TRA 54 - As part of an initial characterization of the functional relevance of the antigen recognized by mAb TRA 54, we investigated the immunolocalization of this molecule in capacitated and acrosome-reacted sperm. Intact spermatozoa were labeled with FITC-PNA (Fig. 12*A*) and immunohistochemical staining with mAb TRA 54 was seen in the acrosomal region, with no positive staining in the flagellum or other region of the spermatozoa (Fig. 12*B*). Spermatozoa without fluorescent staining were considered to have undergone a complete acrosomal reaction (Fig. 12*C*) and no positive response to mAb TRA 54 antigen was seen in these cells (Fig. 12*D*).

Transmission electron microscopy of immunogold labeled-preparations showed a conspicuous positive reaction to mAb TRA 54, mainly in the plasma membrane surrounding the spermatozoa head and in the region corresponding to the acrosome (Fig. 12*E*). Acrosome-reacted spermatozoa showed no positive labeling with mAb TRA 54 (Fig. 12*F*).

DISCUSSION

Mammalian spermatozoa acquire their capacity to fertilize oocytes through multiple, sequential interactions with proteins present in the luminal fluid of the epididymis (1). Several enzymes secreted into this fluid by different epididymal regions contribute to the surface modifications that result in spermatozoa maturation (2-5). We recently described a new pattern for the epididymal expression of an antigen previously identified in mouse testicular spermatids by mAb TRA 54 (18,19), and suggested that this antigen is released from epididymal epithelial cells and binds to the surface of spermatozoa during its transit through this duct (20). Preliminary experiments indicated that this antigen is involved in the penetration of spermatozoa into oocytes during fertilization. Since the molecular identity of this antigen is currently unknown, in this study we purified, sequenced and identified the epididymal protein that reacts with mAb TRA 54. The

epididymis was used as the source of antigen since most of the molecules involved in sperm-egg fusion are produced and released by the columnar epithelium of the epididymis into the lumen, where they bind to the surface or acrosome of spermatozoa (3,47-56). Many of these molecules share other important characteristics with the epididymal TRA 54 antigen, including their expression in testicular germ cells (49,54,56,57), their androgen-dependent regulation (4,20) and their high molecular mass (5,20).

Using a combination of affinity (lectin) and anionic ion exchange chromatographies together with immunodetection (dot blotting and western blotting), we purified a 65 kDa protein that reacted with mAb TRA 54. Curiously, in crude homogenates of the epididymal caput, the major immunoreactive bands had a molecular mass of ~200-260 kDa compared to the 65 kDa for the purified protein. This discrepancy in the masses of the immunoreactive bands could reflect the formation of aggregates to yield high molecular mass complexes, such as occurs with several proteins in the epididymal lumen (5,58) or spermatozoan acrosome (45). Such complexes may dissociate during purification (59) and hence result in protein with lower molecular mass. Alternatively, under native conditions, the 65 kDa antigen may exist as a trimer (195 kDa) or tetramer (260 kDa).

The recognition of the epididymal antigen by mAb TRA 54 agreed with the various antigenic domains predicted for this protein. The testicular and epididymal antigens recognized by mAb TRA 54 have been classified as Golgi-derived molecules and share a common epitope that is added to these molecules by the trans-element of the Golgi complex (18-20). Previous immunoblot analysis showed that the molecules recognized by mAb TRA 54 in testis and epididymis are similar, but not the same (20). However, it is currently unclear whether the molecules expressed during spermatozoan differentiation in the seminiferous tubules and those expressed by epididymal epithelial cells are isoforms that vary in their carbohydrate content, or whether they are different molecules that have the same epitope recognized by mAb TRA 54, as suggested for other molecules (49,60).

Mass spectrometric analysis of the purified protein from epididymis and of the tryptic fragments by MALDI-TOF-MS and 2D-LC-nanoESI-MS/MS, respectively, yielded a molecular mass of 64,961 Da that agreed with the value of 65 kDa initially estimated by SDS-PAGE. Comparison of the peptide fingerprint mass spectra and sequences obtained in tandem nanoESI-MS/MS mass spectra by tryptic digestion with those deposited in the NCBInr database yielded a very high score between this epididymal protein and an *M. musculus* protein termed “Unnamed protein product” (UPP). Comparison of the amino acid sequence of the aligned tryptic peptides of

the epididymal antigen recognized by mAb TRA 54 with that of UPP indicated that these are most likely the same protein.

UPP was initially detected during the preparation of a BL6 *M. musculus* tissue cDNA library (25-27). Although no biological function had been attributed to UPP until now, we verify that it shares three structural domains with mouse albumin and is closely related to other proteins with albuminoid characteristics (i.e., alpha-fetoprotein). In fact, the UPP fragment was obtained from a mouse cDNA sequence derived of the mouse albumin-1 gene. The albumin-1 gene is a very long nucleotide sequence with 15 exons located on the mouse chromosome 5. These exons can be retained or targeted for removal in different combinations to create a diverse array of mRNAs (alternative RNA splicing) and further a great number of albumin-1 isoforms in different tissues and with differing or even opposite biological activities. The epididimal albumin-1 isoform recognized by the mAb TR 54 shows 14 exons and does not contain the signal peptide sequence, probably because the isolated protein was already in its secreted conformation.

Interestingly, one of the epididymal class of proteins was previously termed as prealbumin epididymal specific or PES (58-61). All of the molecules belonging to this class of proteins show structural domains with partial or complete homology with albumin domains (58,59). As occurs with the albumin-1 isoform recognized by the mAb TRA 54, the PES proteins are released in a testosterone-dependent manner and can bind to the membrane of spermatozoa during their passage through the epididymal lumen (58-61). Prealbumin epididymal specific isoforms were previously described to ovine (59), ram (62,63) and rat (61,64) models, and rat isoform was clearly demonstrated to participate in the spermatozoa binding to zona pellucida covering the oocite (64). These observations, together with the results reported here, strongly indicate that the epididymal antigen recognized by mAb TRA 54 is an albumin-1 isoform that belongs to PES class of proteins, clearly related to the epididymal maturation of spermatozoa prior to fertilization.

The role of the albuminoid domains during fertilization is still unknown. However, as occurs with the mouse epididymal albumin-1 isoform recognized by mAb TRA 54, other molecules with albumin domains are also internalized by cells and stored in different intracellular compartments (65). In agreement with this, the hydropathy plot of the antigen revealed a significant number of hydrophobic regions that could be involved in interaction with biological membranes and further internalization. In addition, several albuminoid proteins are reported to have hormone ligand/carrier transport functions, and possess or are involved in a variety of other

activities, including chemotaxis, cell-cell recognition, esterase activity, “heat shock protein” activity, oxygen free radical scavenging, copper-stimulated lipid peroxidation, and fatty acid, heavy metal and actin binding (65-67). The antigen recognized by mAb TRA 54 may exert functions similar to those described above, being involved in the transport of molecules across the spermatozoan membrane during epididymal maturation, in the movement of proteins during the acrosomal reaction, or in membrane remodeling during spermatozoa-oocyte membrane fusion (68).

To investigate the possible involvement of this protein in spermatozoan function, we examined the occurrence and distribution of this protein in capacitated and acrosome-reacted spermatozoa. The results of these functional studies indicated that the acrosomal antigen was not present in completely reacted spermatozoa, and probably dispersed gradually during acrosomal exocytosis. The location of the antigen in capacitated sperm and its behavior during the acrosomal reaction supported the hypothesis that this protein may be involved in the penetration of the oocyte zona pellucida during this reaction. As reported for other spermatozoan proteins, the antigen recognized by mAb TRA 54 may modify or co-activate intra-acrosomal enzymes that are released concomitantly during the acrosomal reaction. The transportation and coupling of these enzymes may involve the exposure to other intra-acrosomal proteins that become tightly bound to the spermatozoan surface and are required for the secondary binding of spermatozoa to the oocyte (45,69). *In vitro* assays to test a possible inhibitory effect of mAb TRA 54 on the initial recognition and binding of spermatozoa to the zona pellucida receptors, and in triggering the acrosomal reaction, are in progress. In addition, *in vitro* fertilization assays are being done to assess the possible role of this antigen in sperm-egg interactions, as previously described to the rat PES counterpart. In the future, epididymal albumin-1 mouse mutants will be important to clear the biological function of this protein concerning the mammalian fertilization.

In conclusion, we have identified the epididymal antigen recognized by mAb TRA 54 in homogenates of epididymal caput. This protein is an albumi-1 isoform and belongs to the PES class of proteins, the members of which are involved in the maturation and capacitation of spermatozoa prior to fertilization. Since infertility in mice and humans is strongly associated with a lack of epididymal antigens (70,71), and since the antigen characterized here is also expressed by human epididymis (unpublished observations), the purification of sufficient amounts of this protein should allow identification of the structural domains of this molecule that are involved in fertilization. The purified antigen could also be a useful starting point for the development of male contraceptives.

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FOOTNOTES

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1The abbreviations used are: EDTA, ethylenediaminetetraacetic acid; PES, prealbumin epididymal specific; PMSF, phenylmethanesulphonyl fluoride; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; DTT, dithiothreitol; TFA, trifluoroacetic acid; TOF, time-of-flight; LC, liquid chromatography; ESI, electrospray ionization; MS, mass spectrometry; RP-CapLC, reverse phase capillary liquid chromatography; UPP, unnamed protein product

FIGURE LEGENDS

Fig. 1. Western blot analysis with mAb TRA 54. Protein samples were electrophoresed and transferred to a PVDF membrane and immunoblotted with mAb TRA 54. The positions of the molecular mass markers are indicated on the left. Control immunoblot without TRA 54 showed no bands (cont). Immunoreactive bands of ~200 to 260 kDa were detected in epididymal (epi) extracts. Arrows indicate the upper and lower limits of the band complexes.

Fig. 2. Purification of the mouse epididymal protein detected with mAb TRA 54. A, Proteins (120 mg) from a homogenate of epididymal caput tissue were loaded onto a column (HR 5/20) packed with concanavalin A (Con-A) and preequilibrated with 0.1 M sodium acetate, pH 6.0, containing 1 M NaCl, 1 mM MgCl2 and 1 mM CaCl2. After washing the column with this same buffer to remove unbound material, bound proteins were eluted with a linear gradient (0-0.5 M) of glucose in sodium acetate-salt buffer at a flow rate of 1 ml/min, and the elution profile was monitored at 280 nm. Left Y axis indicates the absorbance (nm); right Y axis indicates the theoretical elution gradient. One milliliter fractions were collected and screened for reactivity towards mAb TRA 54 by dot blotting. B, For dot blotting, 3 µl of each fraction was spotted onto a nitrocellulose membrane previously activated with 0.1 M sodium carbonate/bicarbonate buffer, pH 9.5. The spots were dried at room temperature and the membrane was processed for immunodetection using mAb TRA 54 essentially as described for immunoblotting. Immunoreactivity with mAb TRA 54 was detected in fractions 1 and 2, and these fractions were pooled and concentrated by centrifugation in Amicon 5 kDa membranes before anionic ion exchange chromatography.

Fig. 3. Purification of the mouse epididymal protein detected with mAb TRA 54. A, The immunoreactive pool obtained in the previous step (described in Fig. 2) was applied to a 5 ml HiTrap Q-SepharoseTM High Performance column equilibrated with 50 mM Tris-HCl, pH 7.4. After washing the column to remove unbound material, bound proteins were eluted with a linear gradient of NaCl (0-1 M) in Tris-HCl buffer. One milliliter fractions were collected and screened for reactivity towards mAb TRA 54 by dot blotting. B, The fractions that reacted with mAb TRA 54 in dot blotting were combined to form three pools (35-38 - pool A; 39-43 - pool B; 44-50 - pool C) based on their elution profile. Left Y axis indicates the absorbance (nm); right Y axis indicates the theoretical elution gradient.

Fig. 4. A, SDS-PAGE and B, Western blot with mAb TRA 54 of the protein purified from mouse epididymis (epi). The immunoreactive pools obtained by ion-exchange chromatography on Q-Sepharose were concentrated prior to SDS-PAGE and immunoblotting. Aliquots (100 μg) of pool A (fA) were electrophoresed in 7.5% polyacrylamide gels and transferred to a PVDF membrane for immunoblotting with mAb TRA 54. The PVDF membranes were blocked with 5% low-fat dry milk for 1 h and then washed with Tris-buffered saline containing 0.05% Tween 20-(TBS-T). After incubation with mAb TRA 54 overnight at 4°C followed by incubation with goat anti-rat IgG conjugated with peroxidase, immunoreactive bands were detected with 0.03% hydrogen peroxide and 0.05% diaminobenzidine. The positions of the molecular mass markers are indicated on the left of each half membrane. Control immunoblots (ctrl) without mAb TRA 54 showed no bands. A strongly immunoreactive band of ~65 kDa was detected in pool A (pA, arrow). The corresponding protein band in the polyacrylamide gel stained with Commassie blue (arrowhead) was excised and the protein digested with trypsin.

Fig. 5. MALDI-MS peptide fingerprint showing the major protonated peptides ($[M+H]^+$) originating from tryptic digestion of the 65 kDa protein detected by mAb TRA 54. The samples obtained by tryptic digestion were prepared for analysis using the dried droplet method. All of the measurements were done on a MALDI-MS instrument in the positive ion and reflectron modes. Subsequent data evaluation and peptide identification were done using the Mascot Wizard software package.

Fig. 6. Representative product ion mass spectrum for the mass-selected protonated peptide LVQEVTDFAK acquired using a high-resolution, orthogonal time-of-flight QTOF mass spectrometer after collision-induced dissociation with argon. The ionization conditions used included a capillary voltage of 3.5 kV and cone and RF1 lens voltages of 30 V and 100 V, respectively. External calibration with phosphoric acid 0.1% (v/v) was done over the range 50-2000 *m/z*. The mass spectrum was acquired with the TOF analyzer in “V-mode” (TOF kV = 7.2) and the MCP voltage set at 2100 V.

Fig. 7. Sequence similarity alignment generated in NCBI BLAST. Query corresponds to the UPP aminoacides sequence. The UPP (BAC 34360) has 99.8% of sequence identity with the *Mus musculus* albumin-1 mRNA. The alignment was generated using the Geneious 2.0.10 software.

Fig. 8. A: Position of the albumin-1 gene on the chromosome 5 of mouse genome. The albumin-1 gene (Alb1) is setted on the position 50. B: The albumin-1 gene (Alb1, second line) has 15 exons (vertical lines setted on the single horizontal line). The AK050644.1 cDNA sequence corresponding to UPP (first line) shares all the exons, excepting the first one, with the albumin-1 gene.

Fig. 9. Mirror images of the predicted three-dimensional structure of UPP (BAC34360) showing the high content of □̃-helices in the X (A,B), Y (C,D) and Z (E,F) axes. The model was constructed as described in the Methods.

Fig. 10. Comparison of the three-dimensional structure of the template protein (human serum albumin, PDB accession number 1O9X) and UPP (BAC34360). Human albumin showed the greatest three-dimensional alignment (>70%) with UPP. The N- and C-terminals are shown. The models were constructed as described in the Methods.

Fig. 11. A, Predicted Jameson-Wolf antigenic domains and B, Kyte-Dolittle hydropathy plot of UPP (BAC34360) determined using the Protean plug-in option of Lasergene software (DNASTAR).

Fig. 12. A-D, Localization of the TRA 54 antigen in spermatozoa based on immunoreactivity with mAb TRA 54. The integrity of the spermatozoan acrosome was evaluated by the presence (A) or absence (C) of a FITC-PNA fluorescent area in the spermatozoan head. The antigen was detected within the acrosome in capacitated and intact spermatozoa (B), but was not seen after the acrosomal-reaction (D). The flagella of these spermatozoa were not abeled (B). E-F, Transmission electron microscopy (TEM) of spermatozoon. Preparations immunolabeled with mAb TRA 54. E, Images revealed a positive reaction in the plasma membrane that covered the sperm caput and in the acrosome (Ac) of capacitated spermatozoa. F, No antigen was detected in acrosome-reacted spermatozoa. Panels (A) and (C) are fluorescent images, (B) and (D) are phase-contrast images, and (E) and (F) are TEM images. Note that the images in panels (A) and (B), and in panels (C) and (D) are from the same preparations but with different illumination. Scale bars: a-d: 3.5 μm; e: 0.2 μm; f: 0.35 μm.

FIGURE 1

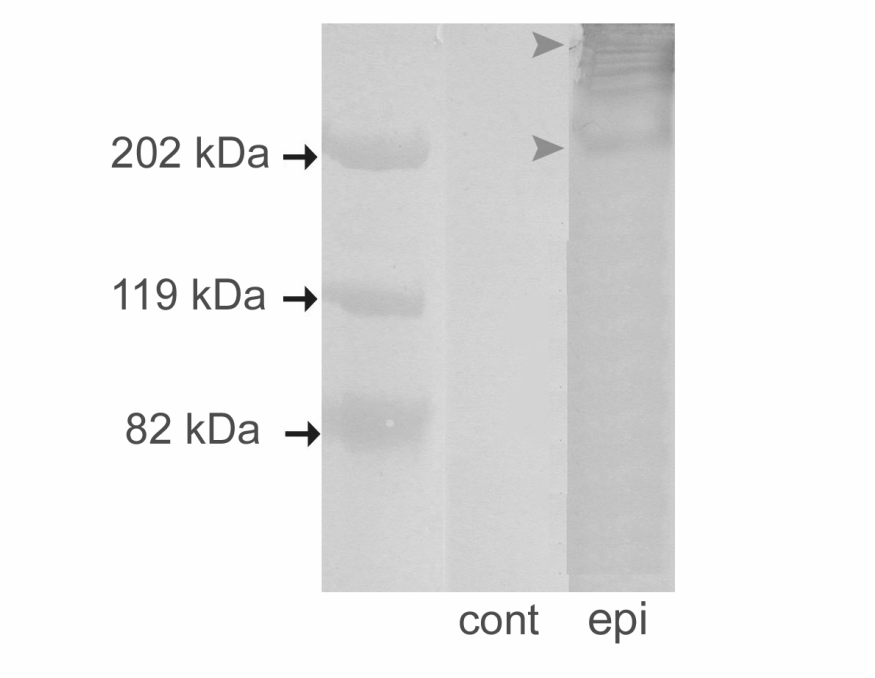

FIGURE 2

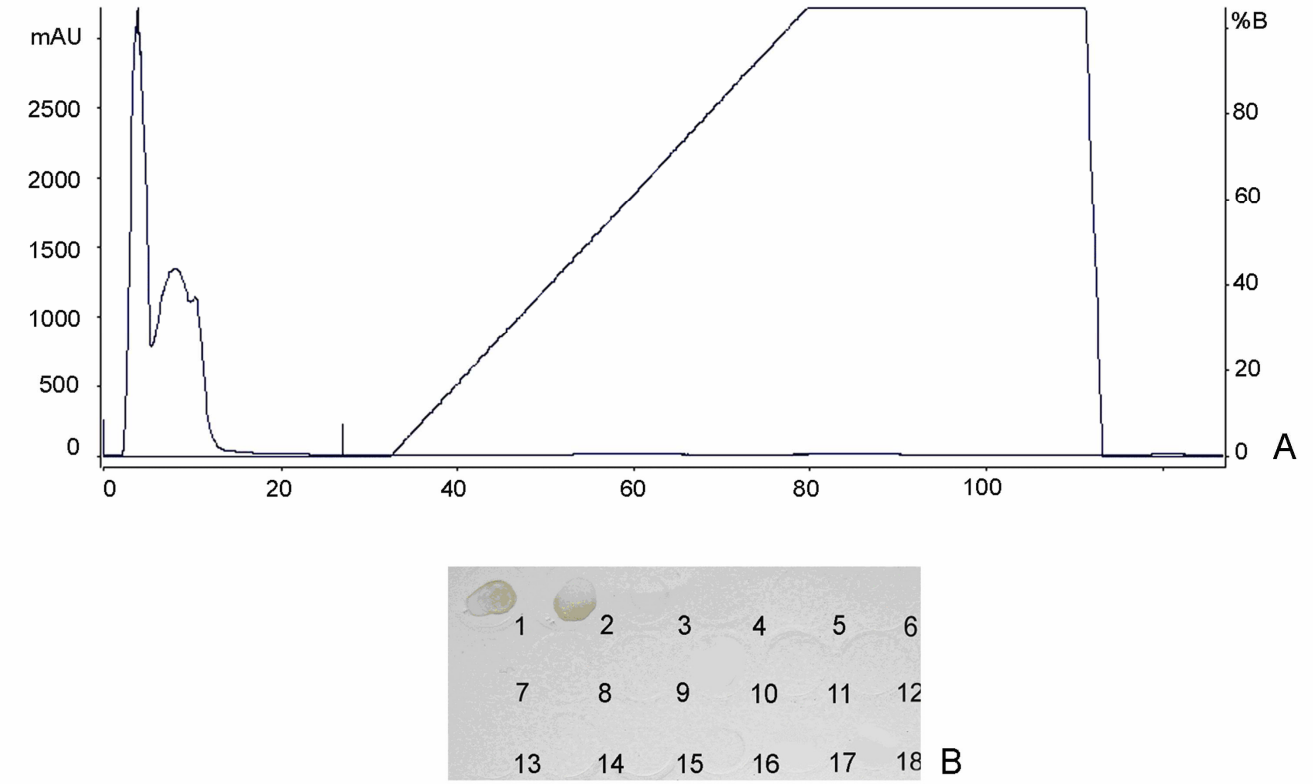
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FIGURE 3

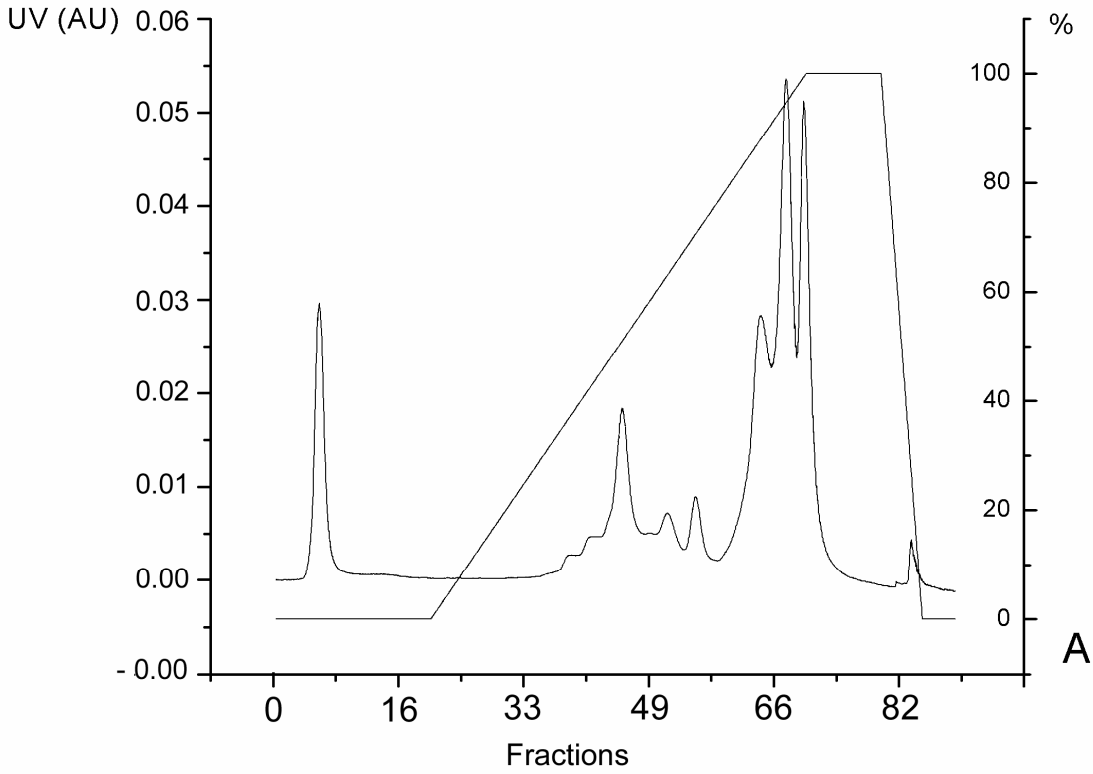
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FIGURE 4

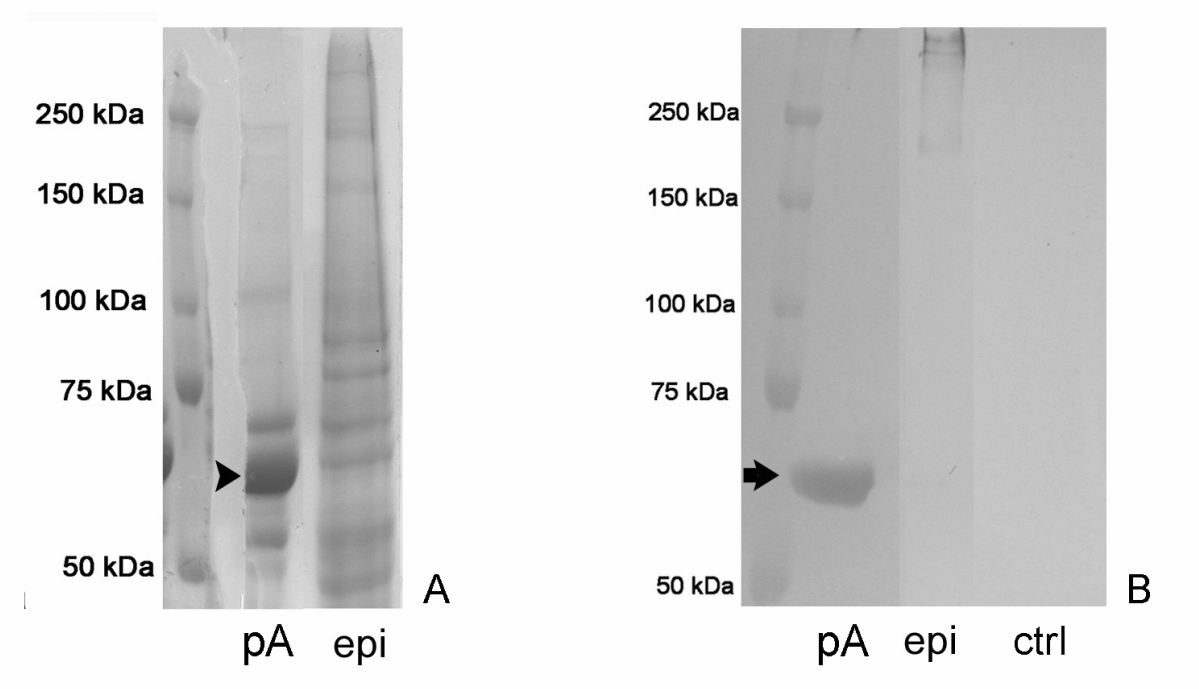
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FIGURE 5

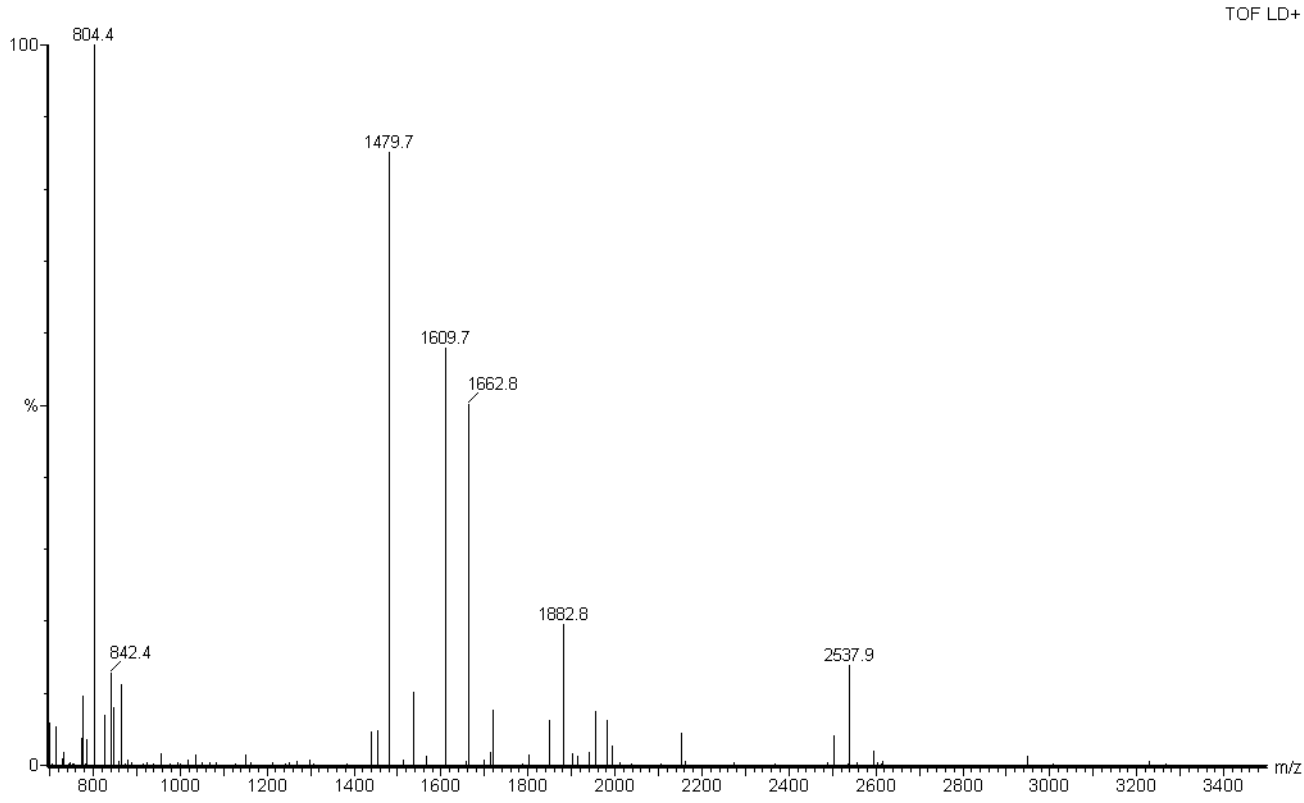
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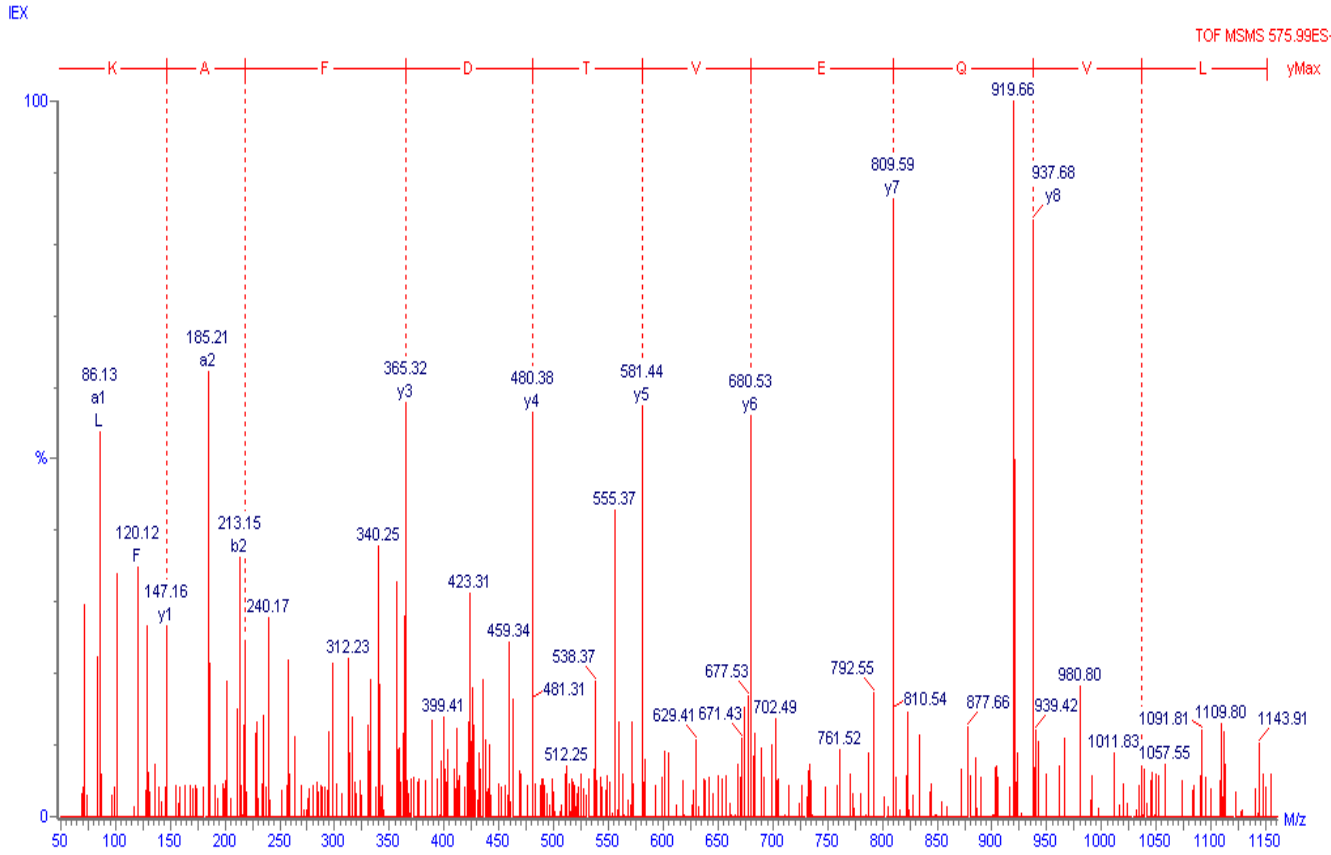
FIGURE 6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b2
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312.23
340.25
365.32
y3
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423.31
459.34
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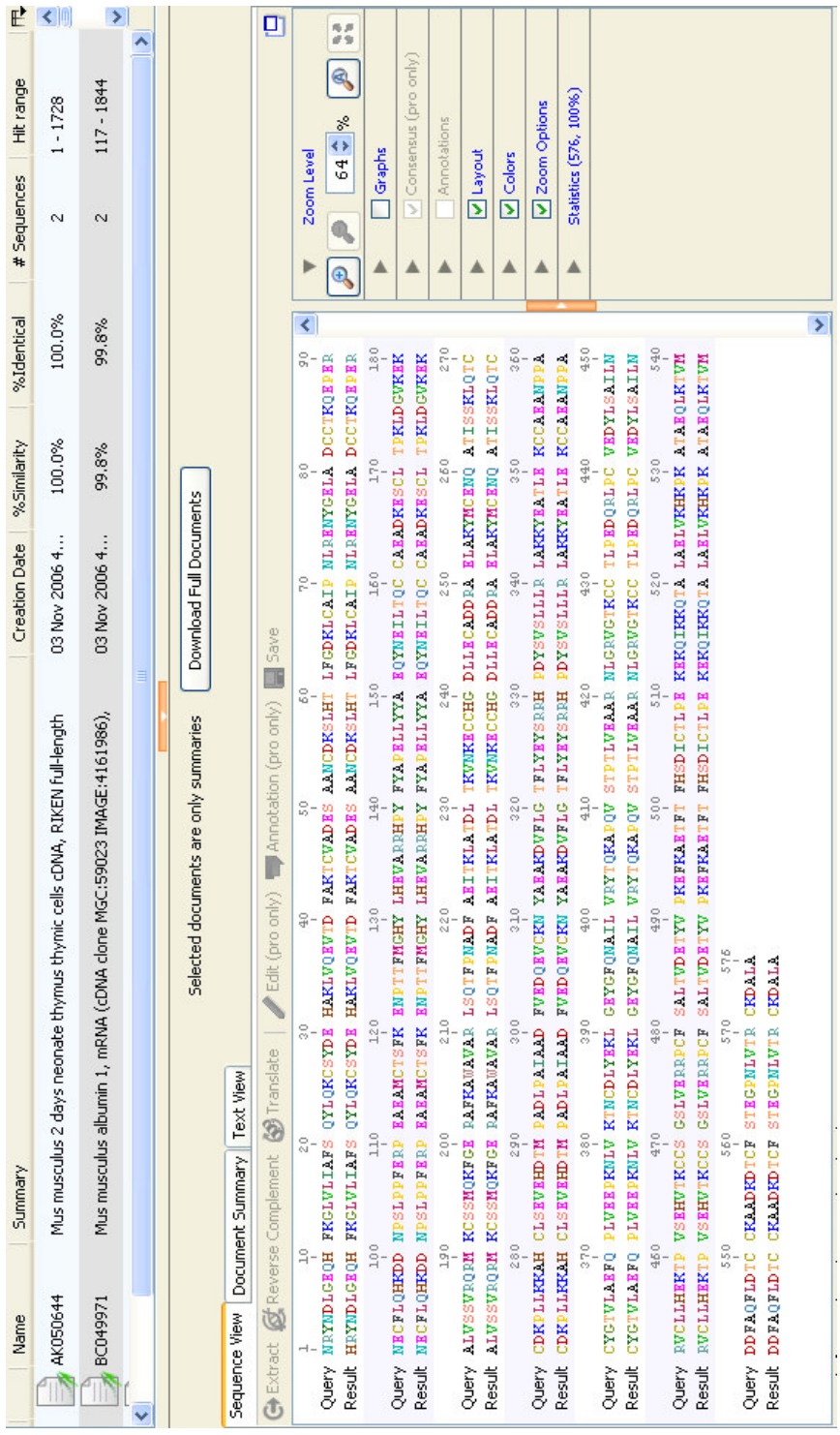
512.25
538.37
555.37
581.44
y5
629.41
671.43
677.53
680.53
y6
702.49
761.52
792.55
809.59
y7
810.54
877.66
919.66
937.68
y8
939.42
980.80
1011.83
1057.55
1091.81
1109.80
1143.91
M/z
50 100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000 1050 1100 1150

FIGURE 7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BC049971
Mus musculus albumin 1, mRNA (cDNA clone MGC:59023 IMAGE:4161986),
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Selected documents are only summaries
Download Full Documents
Sequence View
Document Summary
Text View
Extract
Reverse Complement
Translate
Edit (pro only)
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Colors
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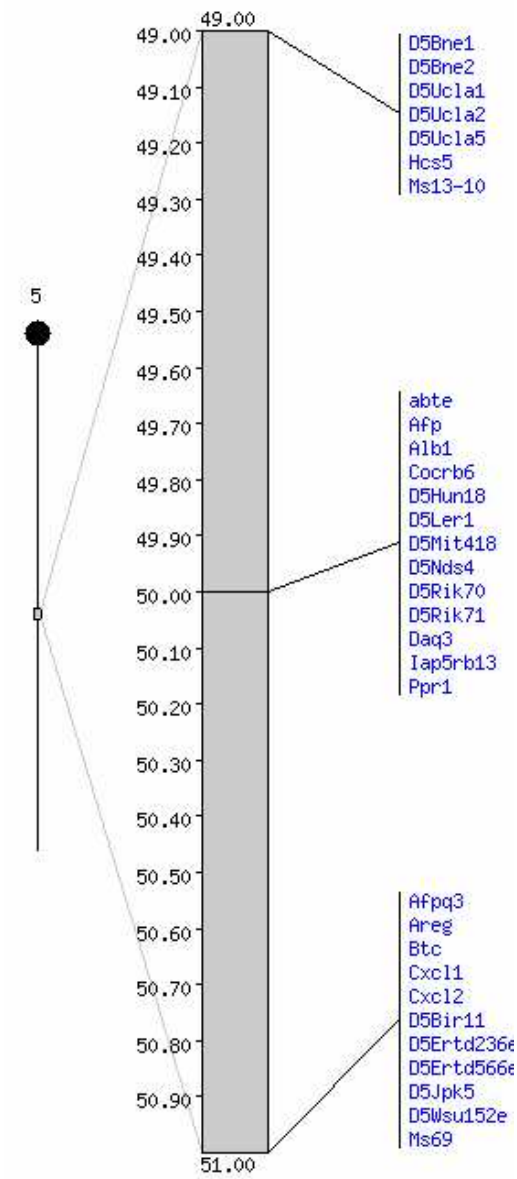
FIGURE 8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49.30
49.40
49.50
49.60
49.70
49.80
49.90
50.00
50.10
50.20
50.30
50.40
50.50
50.60
50.70
50.80
50.90
51.00
D5Bne1
D5Bne2
D5Ucla1
D5Ucla2
D5Ucla5
Hcs5
Ms13-10
abte
Afp
Alb1
Cocrb6
D5Hun18
D5Ler1
D5Mit418
D5Nds4
D5Rik70
D5Rik71
Daq3
Iap5rb13
Ppr1
Afpq3
Areg
Btc
Cxcl1
Cxcl2
D5Bir11
D5Ertd236e
D5Ertd566e
D5Jpk5
D5Wsu152e
Ms69

A


chr5:
91530000 91535000 91540000 91545000 91550000 91555000 91560000 91565000
Your Sequence from Blat Search
Blat Sequence
UCSC Known Genes Based on UniProt, RefSeq, and GenBank mRNA
Alb1
Afp
RefSeq Genes
Alb1
Afp
Non-Mouse RefSeq Genes
Other RefSeq

B

FIGURE 9

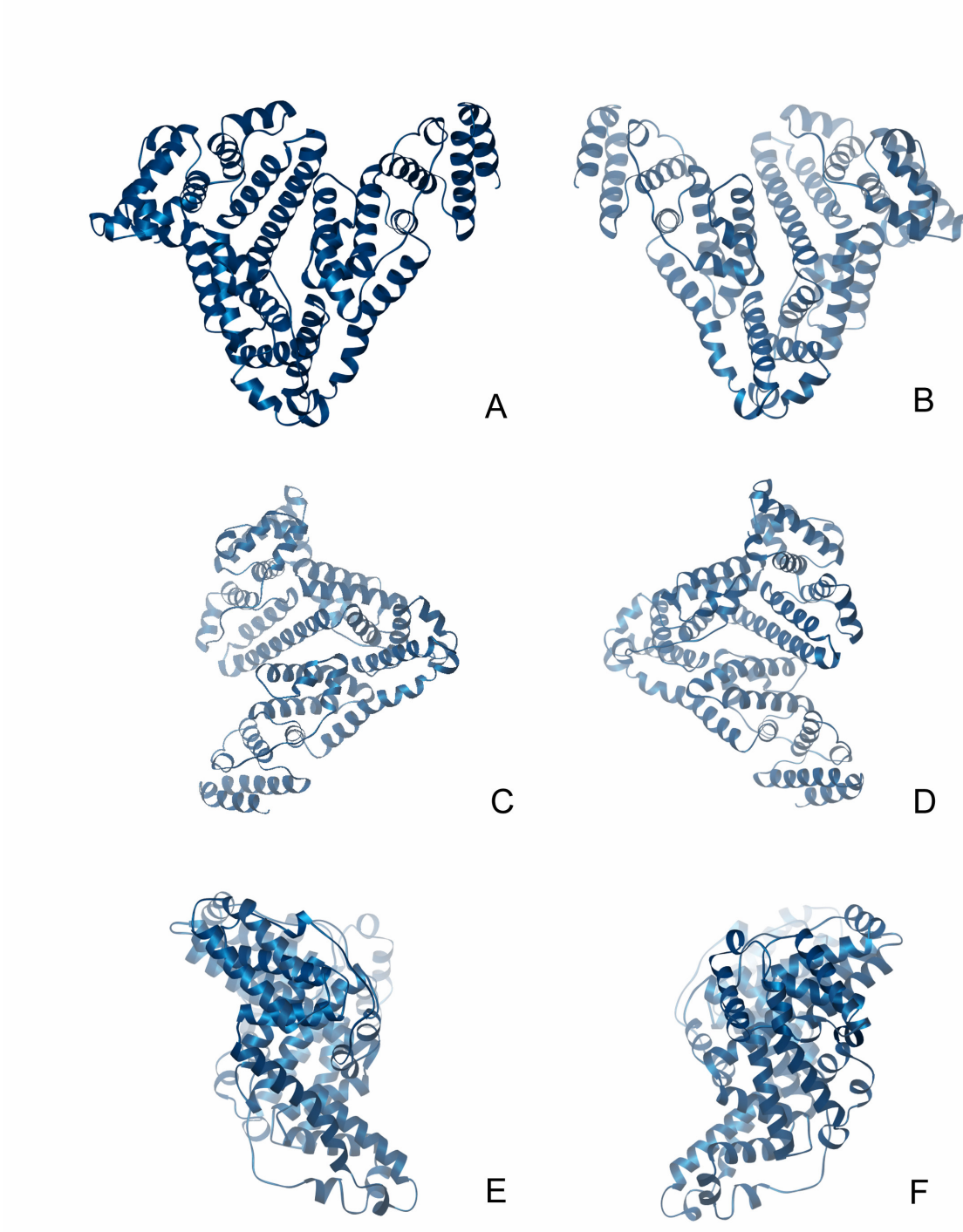


FIGURE 10

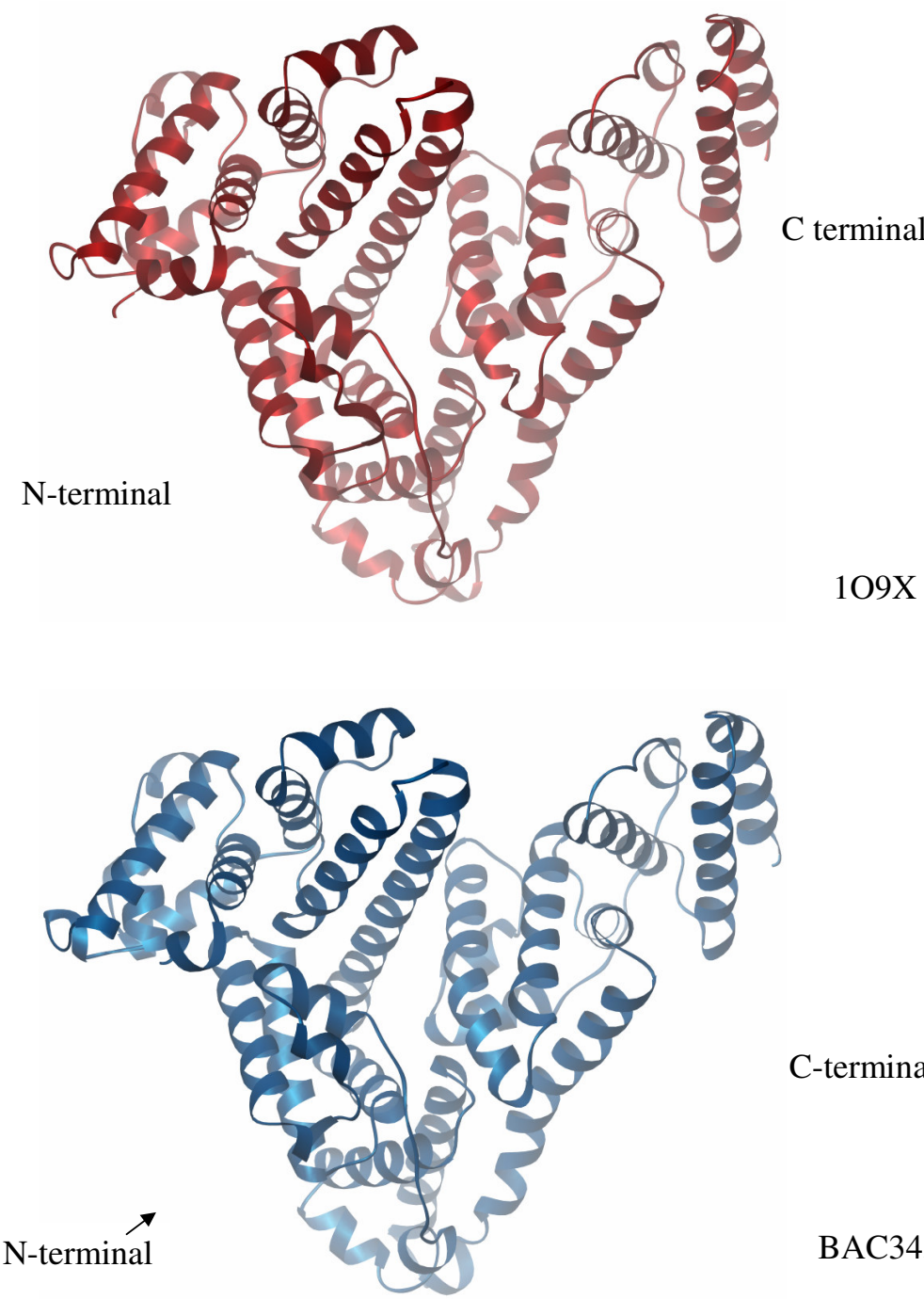


FIGURE 11

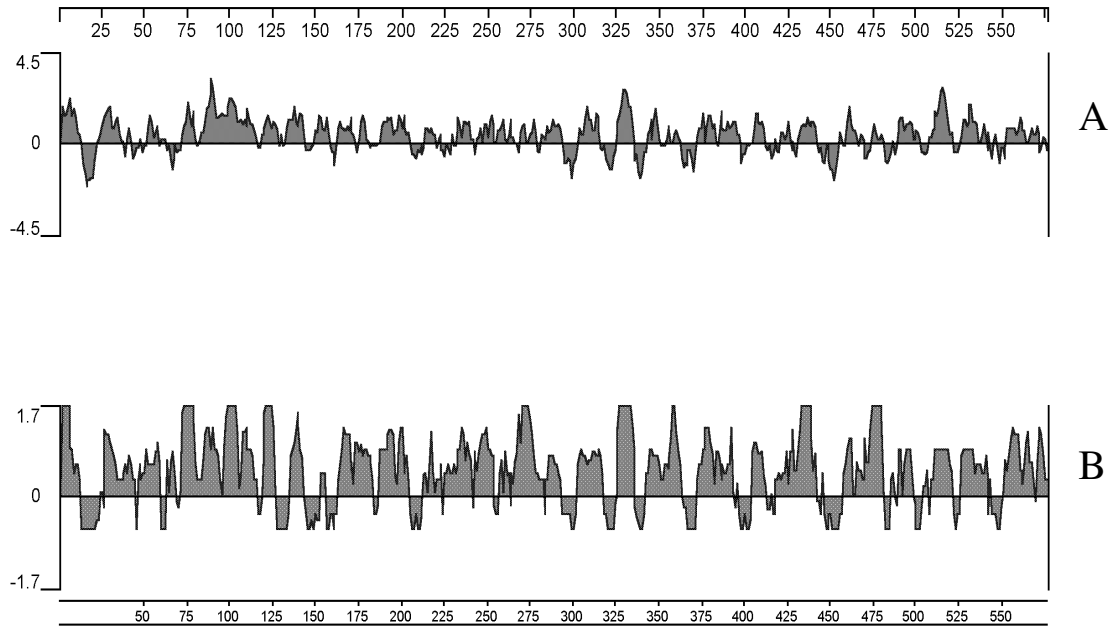
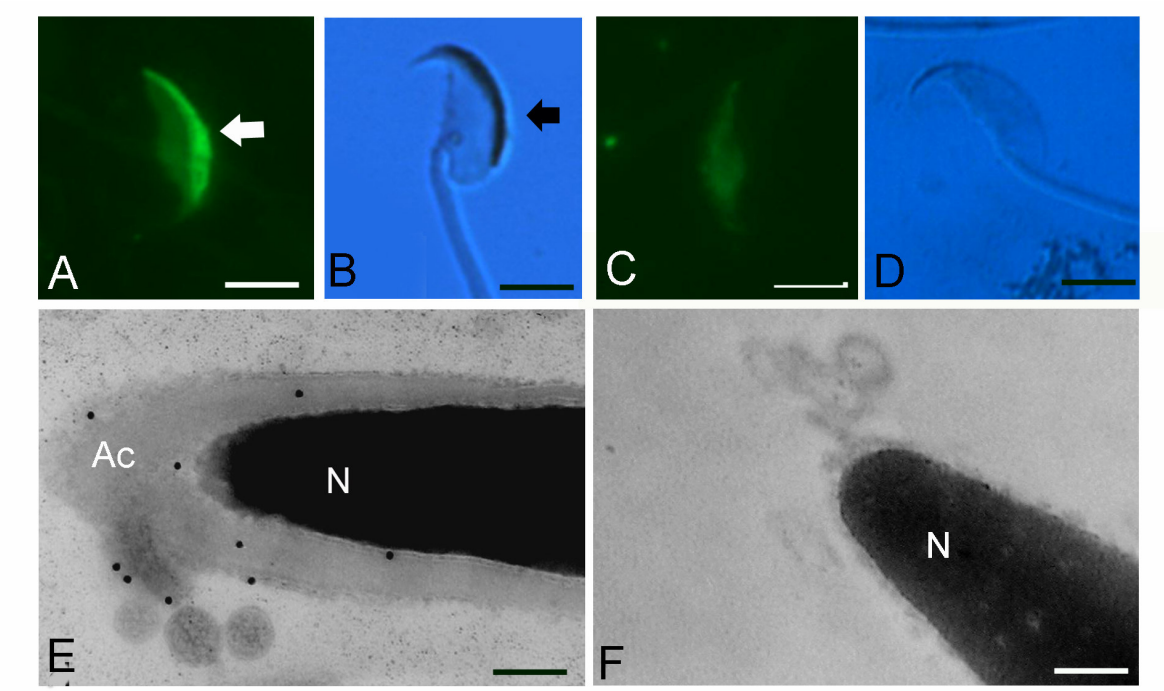

25 50 75 100 125 150 175 200 225 250 275 300 325 350 375 400 425 450 475 500 525 550
4.5
0
-4.5
A
1.7
0
-1.7
B
50 75 100 125 150 175 200 225 250 275 300 325 350 375 400 425 450 475 500 525 550

FIGURE 12



The mouse epididymal TRA 54 antigen: a novel molecule adhered to luminal spermatozoa and required to oocyte-zona pellucida penetration before fertilization.

Short Tittle: TRA 54 antigen is required to mouse fertilization.

Summary

The epididymis is involved in the biochemical maturation of spermatozoa. The monoclonal antibody (mAb) TRA 54 recognizes an antigen released by the epididymal cells and endocyted by the luminal spermatozoa to the acrosomal vesicle. Previous results demonstrate that the antigen is lost from the acrosome during the acrosome reaction. The aim of this study was to investigate the effect of the antigen inhibition by the mAb TRA 54 on the fertilization process was assessed by *In vitro* fertilization using both zona pellucida-intact and -free oocytes. The antibody significantly inhibited fertilization of the zona pellucida intact oocytes. These findings support the hypothesis that the antigen would be involved in the acquisition of fusion competence of the mouse spermatozoa.

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ABSTRACT

The epididymis is known to be involved in the biochemical maturation of sperms. The monoclonal antibody (mAb) TRA 54 recognizes an antigen released by the epididymal epithelial cells and further adhered to the luminal spermatozoa. Previous results obtained by immunostaining with the mAb TRA 54 on fresh, capacited and induced acrosome-reacted (ionophorum-treated) spermatozoa demonstrated that the antigen is released from spermatozoa acrosome after the acrosome reaction. To assess the effect of the TRA 54 antigen on mouse fertilization, capacited spermatozoa inseminated cumulus-invested oocytes and acrosome-reacted spermatozoa inseminated zona pellucida-free eggs in medium containing the mAb TRA 54. The antigen was demonstrated to be lost by the spermatozoa during the acrosome reaction, and the antibody significantly inhibited fertilization of zona-intact oocytes. These findings suggest that the antigen would be a final destined intra-acrosomal protein involved in the sperm-oocyte interaction at zona pellucida level, thus involved in the acquisition of the mouse spermatozoa fusion competence.

INTRODUCTION

Spermatozoa are very specialized cells, dedicated to fertilization of the oocyte (Vernet et al, 2001). For fertilization to occur, the ability of spermatozoa to reach and recognize the egg is a critical event of sperm-egg adeshion and fusion. Mammalian spermatozoa leaving the testis are unable to fertilize eggs (Myles and Primakoff, 1984; Lédare et al, 1999) until they undergo some physiological processes as epididymal maturation, capacitation in female reproductive tract and the acrosome reaction (Myles and Primakoff, 1984; Dacheuxs, 1987, Inoue et al, 2005). The epididymal maturation is a used collective term to assign the set of alteration underwent by the epididymal luminal spermatozoa to make them competent for fertilization. All the essential modification of several antigens profiles expressed by the spermatozoa are provided by a

specific epidydimal secretory activity (Jones et al, 1985; Toshimori et al, 1988; Vreeburg et al, 1992; Tulsiani et al, 1993; Eddy and O'Brien, 1994; Kirchhoff and Hale, 1996; Kirchhoff et al, 1998; Kaunisto et al, 1999, Mathur et al 2000).

The sequential events that culminate in the oocyte fertilization by the spermatozoa have been carefully investigated and reviewed (Cuasnicu et al, 2001; Inoue et al, 2005; Primakoff and Myles, 2002); however, the identification of the essential molecules involved in the adhesion and fusion of the spermatozoa and oocyte is still incomplete (Miller et al, 2000). The newly described epididymal antigen recognized by the monoclonal antibody (mAb) TRA 54 (mouse testicular germ cells immunized to rat) seems to be one of the many complementary proteins involved in the spermatozoa preparation leading to the fertilization process (Pereira et al, 1998; Arrotéia et al, 2004). In previous study, we have reported that this molecule is produced in an androgen-dependent manner by the principal cells of the epididymal caput. This molecule is further secreted into the lumen, where is adhered to the membrane overlaying the spermatozoa acrosomal region (Arrotéia et al, 2004), followed by the antigen incorporation inside of the spermatozoa acrosomal vesicle (submitted results).

It is well known that spermatozoa surface proteins are required to the capacited spermatozoa binding to the oocyte zona pellucida (the extra cellular matrix of the oocytes) to triggering the acrosome reaction and that acrosomal antigens are involved as in the zona pellucida enzymatic penetration as in the spermatozoa membrane reorganization needed for the membrane oocyte receptors recognition (Boué et al, 1994; 1995; Légaré et al, 1999; Saxena et al, 1999; Syntin and Cornwall, 1999; Cohen et al, 2000, 2001; Chu et al, 2000; Liu et al, 2000; Gaudreault et al., 2001; Pauls et al, 2003). We have recently demonstrated that the antigen recognized by the mAb TRA 54 is released from spermatozoa acrosome during the exocytic acrosome reaction. In view of this, the aim of the present work was to investigate a possible role

of the studied molecule in one or more of the specific steps involved in the sperm-egg interaction. Acrosome labeling and in vitro fertilization (IVF) assays were used to this propose.

MATERIAL AND METHODS

Animals

C57BL/6 mice (30-60 days old) were commercially obtained and housed under standard conditions of temperature and light as described in Guide for Care and Use of Laboratory Animals. The animals were killed by cervical dislocation.

Reagents and Antibodies

All the chemicals and culture medium (M16 and M2) used in this study were obtained from the Sigma-Aldrich Company (St Louis, MO, USA) unless otherwise stated. The monoclonal antibody was produced as described by Pereira et al, 1998. The protein concentration of the solutions was estimated using a Bradford protein assay kit (Bio-Rad, Richmond, CA, USA).

Spermatozoa Collection, Capacitation and Induction of Acrosomal Reaction

Vas deferens of male adult mice was cutted using a sterile blade. A dense spermatozoa mass was squeezed out from the vas deferens and the spermatozoa were allowed to disperse in 200μL of culture medium M16 supplemented with 4mg/ml of Bovine Serum Albumin (BSA) in plastic culture dish (Corning). For spermatozoa capacitation, cells obtained as described above were incubated for 2h in a culture stove at 37°C in a 5% CO_2 atmosphere. Capacited sperms were pelleted by centrifugation in PBS (Dulbeccos's Phosphate Buffer Salt Solution – Gibco) for 400 X g (10 min). To induce the acrosome reaction, capacited spermatozoa were ressuspended in 30 nM (IVF assays) or 1μM (acrosome reaction induction assays) calcium ionophorum A23187 and incubated for 20 minutes in a culture stove at 37°C in a 5% CO_2 atmosphere (Larson and

Miller, 1999). After the incubation, the spermatozoa suspensions were washed three times in PBS by centrifugation (400 X g, 10 minutes). For immunohistochemical analysis, 5μL samples of the capacited and acrosome-reacted spermatozoa were allowed to dry on glass slides and fixed with iced methanol for 30 minutes previously the immunostaining protocol.

Effect of the mAb TRA 54 on the Acrosome Reaction Induction

Spermatozoa from vas deferens allowed to disperse in culture medium M16 supplemented with 4mg/ml BSA were settled in four samples (I, II, III, and IV). Spermatozoa in sample I were incubated in M16-BSA supplemented medium for 2h in culture stove at 37°C in a 5% CO_2 atmosphere. Previously to the incubation in the culture stove, 1% mAb TRA 54 was added to sample II, 1μM calcium ionophorum A23187 was added to sample III, and both mAb TRA 54 and ionophorum were sequentially added to the sample IV. After the incubation periods, all samples were washed three times in PBS, allowed to dry on the glass slides and fixed with iced methanol as described above. To assess the spermatozoa acrosomal status, prepared slides were labeled with the FITC-conjugated *Peanut agglutinin* lectin (FITC-PNA) (Lee and Ahuja, 1987; Tao et al, 1993). Percentages of spermatozoa with intact acrosome or acrosome-reacted in each sample were determined after fluorescence microscopy analysis (filter with 494-blue excitation, 520 emission, 510-514 barrier). Fluorescence-negative spermatozoa were considered to be completely acrosome-reacted (Saxena et al, 1999). At least 400 spermatozoa were counted in each evaluation. The data were statistically analyzed using an one-factor analysis of variance (ANOVA) followed by Tukey means comparison. Probability values less than 5% ($p<0,05$) were considered significant.

Oocytes Collection and Preparation for In vitro fertilization Experiments

To assess the effect of the TRA 54 antigen inhibition on fertilization, female mice (4 wk old) were super-ovulated by consecutive intraperitoneal injections of eCG 5 IU (Syntex S.A., São Paulo, Brazil) and 48h later with hCG 5 IU (Calier, Barcelona, Spanish). Ovulated oocytes masses were collected by rupturing of oviductal ampullae 20 h after the hCG administration. The oocytes were immediately transferred to culture medium M2 pre-equilibrated at 37°C in a 5% CO_2 atmosphere. The cumulus complexes of the oocytes were removed by brief treatment with 0,01% hyaluronidase in M2 (Saxena and Toshimori, 2004). The zona pellucida-free oocytes were obtained after briefly incubating the cumulus-free oocytes with low pH (2,5) Tyrode's solution (Manandhar and Toshimori, 2001).

In vitro fertilization Experiments

The zona pellucida-intact oocytes were co-incubated with capacited spermatozoa and the zona pellucida-free oocytes were co-incubated with acrosome-reacted spermatozoa in M16-BSA supplemented (control I), under mineral oil. Part of the IVF dishes contained 10% rabbit normal serum (control II) or 10% mAb TRA 54 (test assay). The final spermatozoa concentration was adjusted to 2 X 10^6 spermatozoa/ml in a 60µL drop of medium. After 5 h of co-incubation in the culture stove, the oocytes and adhered spermatozoa were transferred to a fresh drop of medium. Fertilization was assessed using an inverted microscope by parameters as the presence of two pronucleus at 8h or more, or two cells after 24 h of the gametes co-incubation. Each assay was conduced 6 to 12 times according the purpose. The data were statistically analyzed using an one-factor analysis of variance (ANOVA) followed by Tukey means comparison. Probability values less than 5% ($p<0,05$) were considered significant.

RESULTS

Effect of the mAb TRA 54 on the Induction of the Acrosome Reaction

Spermatozoa in sample I showed basal rates of acrosomal reaction (approximately 36,7%) that was not altered by the addition of the mAb TRA 54 to the medium ($p<0,001$, Table 1). The mobility of the spermatozoa was not altered after the antibody addition. Addition of calcium ionophorum to the spermatozoa sample (III) significantly increased the rates of the acrosomal reaction to approximately 73.3% ($p<0,001$, Table 1). Previous mAb TRA 54 addition to spermatozoa does not alter the acrosome reaction inducted by ionophorum (sample IV, 76.2%, $p<0.001$, Table 1). Thus, acrosomal reaction rates were not significantly different if the mAb TRA 54 was absent (sample III) or present (sample IV) before the ionophorum induction.

In vitro fertilization

When zona pellucida-intact oocytes were used, fertilization rate was 86.4% in control I group and 84.1% in the presence of the rabbit normal serum (control II) but only 30.4% when the mAb TRA 54 was added ($p<0.001$, Table 2). A fertilized oocyte and an unfertilized oocyte are respectively represented in Fig. 1a and 1b. Most of the oocytes fertilized in the presence of the mAb TRA 54 showed a time delay in the cleavage process (Fig. 1c).

Acrosome reacted spermatozoa induced with calcium ionophorum were able to bind zona pellucida-free oocytes (Fig. 1d). Spermatozoa in the three conditions (control I, II and test group) were equally able to fertilize zona pellucida free-oocytes since the fertilization rates were respectively 87.2%, 82.0% and 85,7% ($p=0.789$,Table 3). A fertilized zona pellucida-free oocyte is represented in the Fig. 1e.

DISCUSSION

Spermatozoa in testicular fluid have weak forward motility and can not interact with unfertilized eggs. During the transit throughout the epididymis, the spermatozoa undergo further maturation to acquire the ability to fertilize eggs (Dacheux et al, 2005). Enzymes, proteins and glicoproteins secreted by different regions of the epididymis are destined to alter the spermatozoa surface fashion in order to capability them to the fertilization process (Hamil et al, Robaire et al, 2000; Sullivan et al, 2005). Numerous studies have been done to identify these kind of molecules (Myles and Primakoff, 1984; Boué et al., 1995; Légaré et al., 1999; Syntin and Cornwall, 1999; Cohen et al., 2000, 2001; Chu et al., 2000; Liu et al., 2000; Gaudreault et al, 2001; Nonaka et al., 2003; Pauls et al, 2003), however no consensus has emerged on the precise roles of the many of the specific molecules that seem to be essential during the sperm-oocyte interaction (Saxena et al 1999). In order to contribute with the understanding of this complex biological process, we have recently identified a novel molecule that is synthesed and released by principal cells of the epididymal caput to be further incorporated by luminal spermatozoa (Arrotéia et al., 2004). The main goal of this study was to investigate if this antigen, recognized by the mAb TRA 54, could be involved in one or more of the sequential events occurred during the fertilization process.

After the gametes approach each other, the spermatozoa is supposed to be able to penetrate the structures surrounding the oocyte in order to reach and untimely fuse with the oocyte plasma membrane (Lin et al, 1994). Whereas the surface molecules provided by epididymis enable spermatozoa to bind the zona pellucida receptors (Flasch and Gadella, 2000), another set of molecules is involved in the disintegrin-integrin anchoring of the two gametes membranes (Flasch and Gadella, 2000, Naz et al 1992; Saxena and Toshimori, 2004; Busso et al 2005). Previous results (submitted article) obtained from the immunostaining and immunogold experiments clearly demonstrate that the epididymal antigen recognized by the mAb TRA 54 is

incorporated to inside of the acrosome in fresh and capacited spermatozoa, as reported for other epididymal-released antigens (Myles and Primakoff, 1984; Nonaka et al., 2003). Although the molecular mechanisms underlying this antigen incorporation are still unknown, these circumstantial evidences strongly indicates that acrosome constitutes the final destine of the epididymal-released antigen recognized by the monoclonal TRA 54.

Previous evidences showed that the mAb TRA 54 antigen is completely released and dispersed during the acrosome reaction probably to participate in the zona pellucida penetration for further fertilization (submitted results). In according with this hypothesis, the inner acrosomal antigen should not be involved in the initial recognition / binding to the zona pellucida receptors nether in the acrosome reaction triggering - events provided by proteins in the spermatozoa surface (Saxena et al 1999). Our present results are in according with this hypothesis since that the acrosome-reaction triggering was not altered during *in vitro* assays using the mAb TRA 54 as the antigen inhibitor (Table 1). In addition, *in vitro* fertilization experiments demonstrated that the mAb TRA 54 added to the fertilization medium significantly inhibited the fertilization rate of zona pellucida-intact oocytes. Furthermore, most of the embryos generated in the mAb TRA 54 presence showed a delayed cleavage process. Interestingly, there were no decreases in the fertilization rates when the antigen inhibition with the mAb TRA 54 was performed in zona pellucida-free IVF assays, indicating that the antigen has no function at the oocyte membrane level. Taken together, these findings clearly suggest that the studied antigen is involved in the optimization of zona pellucida penetration by an acrosome-reacted spermatozoon. As reported to other spermatozoa proteins, the role of the antigen recognized by the mAb TRA 54 in the spermatozoa passage through the zona pellucida could involve the modification and co-activation of others intra-acrosomal enzymes concomitantly released, the carry and coupling of a different enzymes or the exposition of other intra-acrosomal proteins that will become tightly

bound to the spermatozoa surface to allow the spermatozoa secondary bind to the oocyte (BROOKS, 1988; Primakoff, 1994; SAXENA et al., 1999).

In summary, evidence provided in the present work indicates that the epididymal-released antigen recognized by the mAb TRA 54 would be a final destined intra-acrosomal protein involved in the acquisition of fusion competence of mouse spermatozoa. Although the exact mechanisms are unclear, this antigen seems to be required to the acrosomal matrix dispersion and was demonstrated to be certainly involved in the optimization of zona pellucida penetration by the spermatozoa. Further investigations concerning the molecular structure of the antigen will help to reveal the exact molecular mechanism of the antigen action and will clarify the relevance of this molecule for the gamete interaction.

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Table 1. Effect of the mAb TRA 54 on the ionophorum-induced acrosome reaction. A=control sperm; B= mAb TRA 54 added to sperm; C= calcium ionophorum added to sperm; D= mAb TRA 54 added to sperm before the addition of the calcium ionophore. Media were analyzed by ANOVA followed by Tukey media comparison. Values not connected by the same letter are different. Values are represented in percentage +/- SEM.

	A (control)	B (mAb TRA 54)	C (calcium ionophorum)	D (mAb TRA 54 + calcium ionophorum)
F=119.33 P<0.0001	36.75 +/- 2.1 (b)	38.12 +/- 3.0 (b)	73.37 +/- 1.5 (a)	72.62 +/- 1.8 (a)

Table 2. Effect of the mAb TRA 54 on the zona pellucida-intact oocytes fertilization. Media were analyzed by ANOVA followed by Tukey media comparison. Values not connected by the same letter are different. Values are represented in percentage +/- SEM.

		Control I	Control II	TRA 54
Fertilization (%)	F=25.10 P<0.0001	86.44 +/- 12.4 (a)	84.16666 +/- 13.4 (a)	30.4583 +/-9.2 (b)

Table 3. Effect of the mAb TRA 54 on the zona pellucida-free oocytes fertilization. Media were analyzed by ANOVA followed by Tukey media comparison. Values not connected by the same letter are different. Values are represented in percentage +/- SEM.

		Control I	Control II	TRA 54
Fertilization (%)	F=0.24 P=0.785	87.2 +/- 11.5 (a)	82.0 +/- 14.8 (a)	85.76 +/-10.2 (b)

Figure Legend

Figure 1. In vitro fertilization results. In vitro fertilization assays using zona pellucida-intact oocytes showed normal two-cell embryos after 24h in control groups (a), but the presence of the mAb TRA 54 inhibited (b) or delayed (c) the fertilization. Induced acrosome-reacted spermatozoa were able to bind (d) and fertilize (e) zona pellucida-free oocytes in presence or absence of the mAb TRA 54. Scale bars - a: 20μm; b: 18μm; c: 17μm; d-e: 20μm.

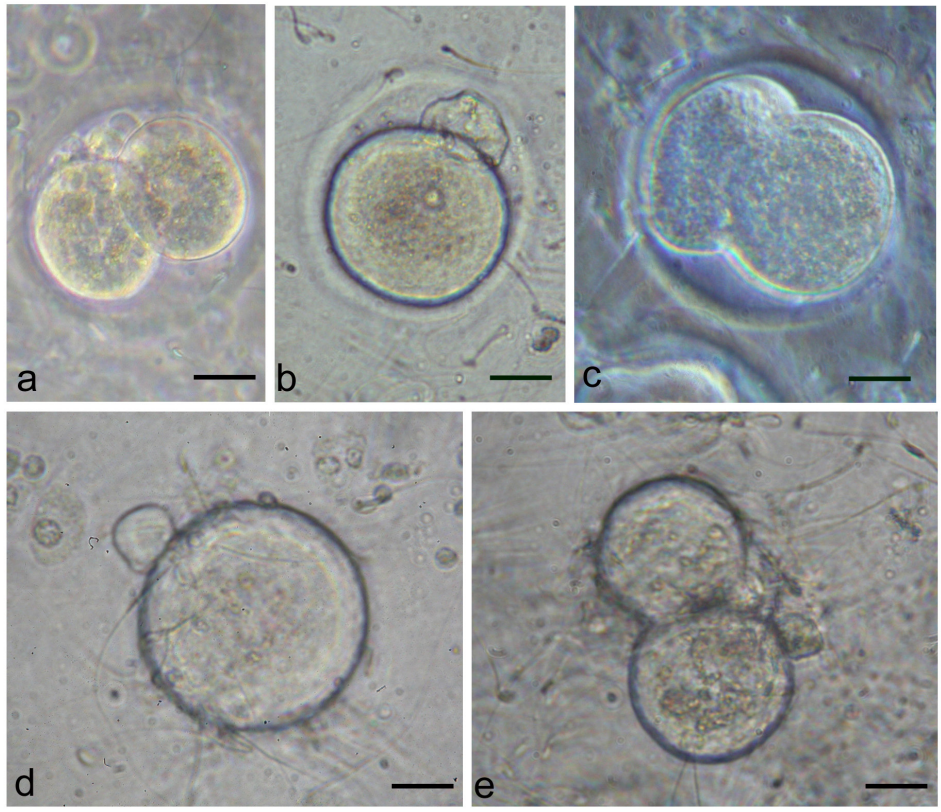

Figure 1

THE MAMMALIAN EPIDIDYMIS: A MORPHO-PHYSIOLOGICAL OVERVIEW

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Running Tittle: Morphophysiology of mammalian epididymis

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ABSTRACT

Mammalian epididymis is a several meters-long tubular organ anatomically connected to the testis. Far from being only a uniform channel that transports, concentrates and storages spermatozoa, epididymis is known to produce a very specific microenvironment involved in the spermatozoa maturation for acquisition of fertilization ability. Enzymes, proteins and glycoproteins, most of them secreted by different regions of the epididymis under androgenic control, are meant to prepare spermatozoa to recognize and fuse with oocyte. This physiological role makes epididymis really essential for male fertility capability. In fact, some cases of male infertility, diagnosed as idiopathic, are nowadays attributed to epididymal pathophysiologies directly affecting spermatozoa maturation process. The purpose of this review is to introduce epididymis in several aspects, as well as to strengthen the understanding of this organ as fundamental in male reproductive physiology and fertility.

Key words: epididymis – spermatozoa maturation – epididymal protein secretion – fertility – aging - androgens

1. Introduction

Epididymis is a tubular organ anatomically connected to the testis, specifically located between the vas efferens and the vas deferens. This excurrent duct is present in all vertebrates with the exception of cartilaginous fishes and it is particularly developed in species with internal fertilization (Jones, 1998). This organ shelters a several meters-long contorted duct and its length is estimated to be 5–7 m in men, and as long up to 80 m in large mammals as bulls and stallions (Sullivan, 2004). Different segments are possibly identified in epididymis, such as an initial segment, caput, corpus and cauda, and each region is formed by a lumen and a polarized epithelium composed mostly of principal and basal cells (Lasserre et al, 2001; Dacheux et al 2005).

Several descriptive anatomical and histological studies of the testicular excurrent duct system from various species appeared at the beginning of the twentieth century. The authors of this studies hypothesed that epididymis played an active role in the maturation of the spermatozoa (Benoit, 1926; Young 1929). Relatively little research was done on the excurrent duct system during the ensuing three decades, until Orgebin-Crist demonstrates, in 1967, that spermatozoa, in fact, acquire the ability to swim and fertilize eggs by passing through this organ (Orgebin-Crist, 1967).

Nowadays, with the advent of more sophisticated techniques, there is a resurgence of interest in epididymis. The epididymal duct is recognized to be not only a uniform channel that transports, concentrates and storages spermatozoa. It is well known that spermatozoa leaving the testis are immovable, immature and unable to fertilize an oocyte (Yanagimachi 1994, Hamil et al 2000, Flesch and Gadella 2000), and that under androgen control, the epididymal epithelium secretes proteins within the intraluminal compartment creating a very complex environment around the spermatozoa (Hermo et al 2004; Sullivan, 2004). This luminal compartment stores spermatozoa until ejaculation and acts in very specific place and moment, in terms of temperature, oxygen tension, pH and energy substrate available, essential for sperm preparation to fertilization (Dacheux et al 2005). All this epididymal specificity provides morphological, biochemical, physiological and functional changes on the spermatozoa structures in order to convert them in fertilization-competent cells, in a process termed *epididymal maturation* (Toshimori, 2003; Gatti et al 2004; Flesch and Gadella, 2000).

Since 1966, over 12.000 research articles have been published on epididymis. These articles concern epididymis in several aspects, and all of them agree that epididymis is crucial for the spermatozoa preparation preceding fertilization. It is well known that proteins and small molecules secreted by the epididymal epithelia into the lumen will interact with the transiting spermatozoa and directly or indirectly affect the spermatozoa surface, working as a signaling molecule for other epididymal proteins activity (Dacheux et al 1987; Lye and Hinton, 2004; Gatti

et al 2004). A significant number of these molecules will be taken up by the spermatozoa (Lye and Hinton, 2004), and several acrosome molecules previously formed during spermiogenesis, that are involved in the acrosome reaction or in the sperm-zona pellucida (ZP) interaction and sperm-egg fusion, will be gradually rearranged and compartmentalized in a stage-specific manner during sperm maturation (França et al, 2005).

As far as it can be determined until now, the general organization of the human epididymis, the regional character of its epithelium and luminal environment, and the maturation changes that spermatozoa undergo are very similar in both animals and man (Bedford 1994; Kirchhoff, 1999; Légaré *et al*, 1999; Marty et al, 2003). The understanding of the epididymal structure, its biochemical properties and function is currently deep but there are yet huge gaps in all aspects. The complexity of the cellular properties, the spatial and temporal organization of protein syntheses and secretion and the dynamic interactions between the epithelial cells and the contents of the luminal compartment has represented challenges. There are strong reasons to believe that a better understanding of this organ will allow the development of therapeutic agents to treat certain types of infertility and, in another hand, to develop male contraceptive agents.

This review intends to approach a complete overview in several aspects related to the mammalian epididymal structure and function.

2. Intra-uterus and post-natal development of the epididymal duct

Most of the crucial events related to the normal function of the testis and to the male reproductive tract take place during the fetal and early postnatal period (França et al 2005).

In mammals, sex is determined by the presence or absence of a single gene on the Y chromosome, Sry, that is required to initiate male-specific pathways and repress female-specific pathways (Tilmann and Capel, 2002). On the onset of sex differentiation, the reproductive primordia look indistinguishable in genetic male and female embryos and the gonads (testicles

or ovaries), the genital ducts (Wolffian or mesonephric and Mullerian or paramesonephric) and the urogenital sinus of both sexes emerge from the morphologically undifferentiated primordia (Larios and Mendoza, 2001). Therefore, it can be assumed that genes involved in the establishment of these primordia are the same for both sexes. During development, the first morphologic sex difference appears in gonads of male embryos, followed by genital ducts; at last and subsequently, sex differences appear in the urogenital sinus (Larios and Mendoza, 2001).

The epididymal duct is known to be derived from the Wolffian or mesonephric duct, which are longitudinally arisen in the intermediate mesoderm in order to give rise to nephrogenic structures and to the male genital duct (Fig. 1). In humans, differentiation of the genital ducts (such as male epididymis or female oviduct) occurs through the seventh week subsequently to the previous gonadal differentiation. This development is under control of genetic products derived mainly from the sexual chromosomes (Vohra and Morgentaler, 1997; França et al 2005).

Testicular Sertoli cells, triggered by a mechanism not well known which is mediated by the Y chromosome, differentiate, produce and secrete the anti-Müllerian substance, a non-steroid hormone involved in the involution of the paramesonephric (Müllerian) duct. Subsequently, under the control of the placental gonadotrophin - human chorionic gonadotrophin (hCG) - the Leydig cells differentiate and begin producing androgens, that positively regulates the ipsilateral mesonephric duct in a paracrine way. These hormonal combination induces the mesodermic cells of the cranial portion of mesonephric ducts to develop, proliferate and evolute to form the epididymis in late gestation (Fig. 2A-B). Anatomically, cords arising from the rete testis form the efferent duct and fuse with the mesonephric tubules adjacent to the testis becoming the epididymal duct (Vohra and Morgentaler 1997; Marty et al 2003). The arrival of germ cells into the spermatogenic cords in fetal testis is believed to contribute to epididymal differentiation (Pryor et al 2000). As

develpment proceeds, the tubule becomes longer and the degree of convolution increases from the cranial to distal pole of the epididymis. Prenatal exposures to compounds that reduce testosterone synthesis/androgenic signal interfere with epididymal development, frequently resulting in the absence of entire sections of the organ (Vohra and Morgentaler 1997; Marty et al 2003).

The post-natal development of the epididymis has been studied mainly in rats but there are evidences that male postnatal reproductive system development has similar patterns in humans and other mammals as dogs and mice (Marty et al 2003). In general, post-natal development of mammalian epididymis was demonstrated to follow a biphasic pattern. Although a progressive development occurs from the fetal period to infants (first phase), this development is transient and regresses during the early infancy, when few changes in epididymal histology can be verified (Robaire et al 2000). This postnatal pattern of epididymal development requires hormonal signals from the hypothalamic-pituitary (HP) axis, a subsequent testicular response, and feedback from the testis to the HP axis to modulate gonadotrophin release. Initially during the neonatal period, serum gonadotrophin rates are high in male, but decline rapidly within a few days (Döhler and Wuttke, 1974).

A definitive development starts in late childhood, and is completed by puberty (second phase), when the highest rate of cell division and epididymal expansion occur. Establishment of the regionalized protein secretion takes place progressively during post-natal development and before puberty (Dacheux et al 2005). Epididymal regionalization seems to be related to the different stages of testicular maturation, the steroidogenic activity of Leydig cells and the androgen-dependence of the epididymis itself (De Miguel et al 1998). Although not well developed at birth, the blood-epididymis barrier is completely formed before the first spermatozoa reach the epididymal lumen (Agarwal and Hoffer, 1989).

3. Hormonal regulation of the developing and developed epididymis

In mammals, intra-uterus development of the epididymis and post-natal differentiation of the epididymal epithelium are dependent upon androgens from the testicular Leydig cells and upon constituents of the luminal fluid produced by the testis (Rodríguez et al 2001). Besides in early development, the adulthood epididymis remains dependent on androgens and/or testicular factors derived from the lumen for the maintenance of the epithelial functions. Both epididymal lumen and epithelia contains androgen receptors and are able to bind testosterone and also dihydrotestosterone (DHT) (Jones and Connel, 1982; Nitta et al, 1993; McMahon et al, 1995; Sonea et al, 1997; Syntin et al, 1999; Robaire et al, 2000; Hess et al 200; Pastor-Soler et al, 2002).

Dihydrotestosterone (DHT) is a metabolite derived from testosterone by acting of the 5α-reductase enzyme and can be produced locally in epididymis since the principal cells contain 5α-reductase, mainly in the initial segment of the duct (Dacheux et al, 2005; França et al, 2005). In fact, the predominant androgen in epididymal fluid is DHT, and it is believed that this molecule is the main androgen responsible for maintaining epididymal structure and function. Both testosterone and DHT affect epididymal structure and physiology more significantly by a paracrine than by a serum via (Robaire et al, 2000).

Both regionalized differentiation of the epididymis and variation in the luminal fluid composition take place under the control of androgens (Toshimori 2003). Thus, it is known that production and secretion of at least half of the epididymal proteins, including those further incorporated by transiting spermatozoa, are under androgenic control, which may act positively or negatively, in different levels of sensitivity (Tézon et al, 1985; Boué et al, 1995; Ellerman et al, 1998; Robaire et al, 2000, Dachuex et al 2005). It is believed that many promoters and transcriptional factors produced in the testis are able to target gene expression with region-specificity for epididymis (Robaire et al, 2000; Dacheux et al, 2005).

Orchidectomy models are currently employed to investigate the effects of testicular androgens on the epididymis. It has been demonstrated that there is a dramatic time-dependent loss weight in the epididymis after androgen withdrawal. This epididymal weight loss is in part due to the loss of spermatozoa and fluid of the lumen and in part due to changes in the epithelium, particularly cell death by apoptosis (Robaire et al, 2000, Hess et al 2001). Removal of circulating androgens by bilateral orchidectomy promotes the epithelium, and resemble the precursor epithelium usually only seen in prepubertal juveniles (Arrotéia et al 2004; Avram et al 2004). After orchidectomy, apoptosis first appears in the epithelium of the initial segment and subsequently in more distal segments, reaching mainly the principal cells. The remaining also undergo a reduction in length and wide (Hess et al 2001). In general, the effects of the orchidectomy on the epididymis are reversible by testosterone administration (Hess et al 2001; Arrotéia et al 2004). However, evidences raised by androgen replacement indicated that apoptotic cell death in epididymis can be adverted by androgens, but the initial segment is dependent on luminal components coming from the testis besides androgens alone (Fan and Robaire 1998, Avram et al 2004). The exact nature of these required components has not been systematically examined.

Additionally to the testosterone and other androgenic derived metabolites, estrogens are now reported to target epididymal epithelial cells (Nitta et al 1993, Hess et al 2001). The cytochrome P450-aromatase is the terminal enzyme responsible for the irreversible transformation of androgens into estrogens and it is present in the endoplasmic reticulum membrane of the cells. The aromatase gene expression and its translation into a fully active protein have been shown to occur in the testicular germ cells and more recently in germ cells as well as in the epithelial cells of the epididymis in mammals (Carreau 2003; França et al 2005). Current evidences indicate that estrogens can reach relatively high concentrations in the male reproductive system and that estrogen receptor-like protein exists in epididymal tissues and an estrogen binding activity was shown in epididymis of several mammals (reviewed by França et al

2005). It has been recently demonstrated that the luminal fluid reabsorption that occurs in the efferent ductules and in the initial segment of the epididymis is regulated by estrogens. It is also been attributed to the estrogen present in epididymis the regulation of the fluid transport through this duct, being the estrogen responsible for increasing the concentration of the sperm as they enter the caput of the epididymis (França et al, 2005). However, other results have demonstrated that estrogen influence seems more likely to be important during development of the epididymis rather than in adult function, when it seems to exert a minor role. Actually, most of the mechanism of estrogen actions on the epididymis remains to be determined (Hess et al 2001).

Other substances for which either receptors or the substance itself have been found in the epididymis include prolactin, retinoic acid, and vitamin D, whose active metabolite is synthesized primarily in the cauda epididymis (Robaire et al 2000). In a complementary way, another regulator of epididymal function is vitamin E, which plays important role in maintaining viability and the functional and structural appearances of epididymal duct epithelial cells (França et al 2005).

4. Morphological features

Mammalian epididymis is an elongated coiled duct suspended within the mesorchium and firmly or loosely bound to the testicular tunica albuginea. The epididymal tubular lumen is continuous with the lumina of vasa efferentia in the testis and ends in the vas deferens (Verma 2001) (Fig. 2B).

It is well known that epididymis is more than a uniform channel that transports and stores spermatozoa. Ultrastructural studies revealed occurrence of two separate and functionally distinct compartments, epithelial layer and lumen, which are sealed off by occluding tight junctions joining the adluminal ends of epithelial cells. The presence of tigh junctions between the adjacent epididymal epithelial cells forms the blood-epididymal barrier that prevents blood

cells to contact luminal spermatozoa and ensures protection to the male gametes from the leucocytes non-self recognition and immunological attack until the ejaculation (Verma 2001; Dacheux et al 2005). The epididymal tubular epithelium is surrounded by smooth musculature along its whole length, helping the sperm transport (Verma 2001). In addition, boar and monkey epididymis are known to present numerous arterioles and capillaries that penetrate directly into the subepithelial region from the underlying connective tissue, resulting in a large number of vessels that are close related to the epithelial layer (Dacheux et al 2005).

Gross aspect of the epididymis allows the identification of different segments comprising proximal caput, corpus and distal cauda (Flickinger et al 1978; Goyal, 1985; Cummins et al 1986; Calvo et al 1997; Yeüng et al 1994; Lasserre et al 2001; Verma 2001; Toshimori 2003). The most proximal region of the caput segment, termed initial segment, is also well distinguished in many species. Besides this gross anatomical division, it is well demonstrated that epididymis exhibits region-specific and cell-specific regions within the epithelium of a given segment (Rodríguez et al 2001). According to this high regionalization of the epithelium and luminal protein secretion, the three main epididymal segments can be subdivided into several regions (França et al 2005). In general, five different physiological regions are distinguished: (i) proximal caput or initial segment, (ii) middle caput, (iii) distal caput and proximal corpus, (iv) distal corpus and (v) cauda (Dacheux et al 2005) (Fig. 3A).

Histological characteristics allow the easy identification of the anterior and posterior extremities of the mammalian epididymis. The thickness of the epididymal epithelium varies from thickest in the proximal caput to thinnest in caudal region, and conversely the luminal diameter and thickness of the peritubular smooth muscle increases from proximal to distal regions (Flickinger et al 1978; Goyal, 1985; Cummins et al 1986; Calvo et al 1997; Yeüng et al, 1994; Lasserre et al, 2001; Verma 2001; Toshimori 2003) (Fig. 3A-C). Few sperms are found in the initial segment, because the reduced luminal size, and large mass of sperm aggregates are located in the cauda (Avram et al 2004, Arrotéia et al 2004, 2005) (Fig. 3B-C). In all of these

segments, the epididymal duct is lined by an epithelium composed by principal and basal cells, while other cells such as apical, narrow, clear and halo cells (intraepithelial lymphocytes) are also present in this duct in a segment-specific manner (Fig. 3D-F).

The most abundant and extensively studied cell type found in the epididymal epithelium is the principal cell, which constitutes 65-80 % of the total epithelial cell from caput to cauda segment. These columnar cells present prominent stereocilia extended into the lumen (Fig. 4A). Ultrastructurally, the supranuclear region of this cell type contains abundant presence of large stacks of Golgi sacules, mitochondria, multivesicular bodies and apical dilated membranous elements, while infranuclear region is densely packed with rough endoplasmic reticulum and (Robaire et al 2000, Dacheux et al 2005) (Fig. 4B-C).

Basal cells are the second most abundant cell type found in the epididymal epithelium, covering 10-20 % of the total cells. They are triangular and flat cells resident in the base of the epithelium (Fig. 3D-E) and contact the basement membrane forming an extensive cellular sheet surrounding the epididymal epithelium (Leung et al 2004). The goblet-shaped apical cells comprised about 10 % of the total epithelial population in the proximal initial segment but only around 1 % in the distal zone of epididymis (Adamali and Hermo, 1996). They are well characterized by many mitochondria in the apical cytoplasm, few microvilli at the luminal border and nucleus located in the upper half of their cytoplasm (Adamali and Hermo, 1996; Goyal 1985). The slender elongated narrow cells increase from 3 % in the proximal initial segment to 6 % in the intermediate zone. These cells presented numerous C-shaped vesicles and mitochondria and a small flattened nucleus located in the upper half of their cytoplasm. The structural features of both apical and narrow cells suggest these cells are involved in intracellular transport, in the degradation of specific proteins and carbohydrates within their lysosomes and in protecting spermatozoa from a changing environment of harmful electrophiles (Goyal 1985; Adamali and Hermo, 1996). In addition, localization of carbonic anhydrase II exclusively within narrow cells suggests that these cells may modify the pH of the lumen resulting in the

quiescence of sperm motility in the proximal end of the epididymis. Clear and halo cells together constitute just fewer than 5% of the cells in the epithelium. Clear cells are equally distributed through caput, corpus and cauda segments and halo cells, recognized by their dark-stained nucleus surrounding by a pale-staining cytoplasm. They are also present in all levels of the epididymal epithelium. Halo cells originate from the immune system in a combination of B and T lymphocytes as well as monocytes (Flickinger et al 1997, Dacheux et al 2005). Together, all the described cell types exert several functions that are necessary for epididymal function as protein secretion and absorption (principal cells); endocytosis (clear and apical cells); secretory activities responsible for acidification of the luminal fluid (clear cells and narrow cells); immune defense, phagocytosis (halo cells) and production of antioxidants (basal cells) (Yeüng et al 1994; Robaire et al 2000; França et al, 2005). Nowadays, basal cells are reported to absorb substances and possibly provide mechanical function of lending stability to the epithelium by virtue of possessing tonofilaments (Verma 2001). In rat, recent evidences indicate that electrolytes and water transport in principal cell may be regulated in a paracrine way by basal cells through local formation of prostaglandins (Leung et al 2004; Cheung et al 2005). It is important to understand that each cell type may express different proteins within the distinct epididymal regions, indicating that they can perform different functions according the location (Robaire et al 2000).

In most of the species studied, the anterior part of the epididymis has been shown to be the most active region in protein secretion (Robaire et al 2000). Some specific genes or proteins are expressed only in the initial segment of the epididymis, and caput and corpus regions of the epididymis synthesize about 80% of the components of the fluid. Interestingly, one single protein can represent more than 50% of the secretion of one unique epididymal zone (Gatti et al, 2004). It is interesting to note that very few proteins are secreted specifically in the cauda segment. The reasons for this are not fully understood, however it is speculated that this occurs either because there is no need for new secretions, as the sperm maturation process is completed, or because

proteins secreted in more proximal segments are accumulated in the fluid and remain present around the sperm within the epididymal cauda (Robaire et al 2000). This typical pattern for the epididymal protein expression was described for several molecules including an epididymal antigen studied in our laboratory (Arrotéia et al 2004), recognized by the monoclonal antibody TRA 54 (Fig. 5A-C). All these evidences are enough to demonstrate the high degree of regionalization in the epididymis activity. This epididymal regionalization, attributed to different patterns of gene expression, is critical to the formation and maintenance of the different functions of by the epididymal duct (Kirchhoff et al, 1998; Rodríguez et al 2001; Suzuki et al, 2004).

Although a number of mechanisms are involved in the regulation of this complex pattern of gene expression, it is known that sets of genes are expressed within each segment and each gene is regulated by different groups of enhancers and repressors. Each region may well express the same gene, but its level of expression in combination with the expression of other genes, defines specifically a given segment of the epididymis (Suzuki et al 2004). In fact, three levels of complexity of gene expression in the epididymal epithelium become evident: (a) a cell type specific gene expression, (b) a pattern of gene expression for a given cell type within a specific region, and (c) the different subcellular localizations of a given marker for a cell type in different regions of the organ (Robaire et al 2000). Nowadays, the understanding of the clear mechanism by which the regionalized gene expression occurs, the reason and the way the gene expression is maintained, have yet not been completely elucidated (Suzuki et al, 2004). Gene silencing agents, such as RNAi, used to manipulate gene expression may prove useful for the analysis of the epididymal genes involved in the maturation process (Lye and Hinton, 2004). The identification of epididymal regulatory proteins may lead to the development of “designer” contraceptives drugs or used as a drug to target therapeutic genes (Sipila et al 2004, Suzuki et al 2004).

4.1 Epithelial activities

Most of the epididymal secreted proteins are attributed to secretory activity of the epithelial cells of the anterior region of the duct. In boars, for example, the protein secretion in caput, corpus and cauda represents 83, 16 and 1% of the overall secretion of epididymis, respectively (Dacheux et al 2005).

The cellular type involved in the epididymal secretion and reabsorption, thus directly involved in the control of luminal proteins concentration, is the principal cell, that is distributed in the whole extension of the epididymis. They show as a well-developed endocytic apparatus, including coated pits, pinocytic vesicles, coated vesicles, endosomes and lysosomes, as well as organelles involved in the classical pathway of protein secretion referred to “merocrine secretion”, including abundant cisternae of rough endoplasmic reticulum (ER), sacules of Golgi apparatus and Golgi-derived apical secretory vesicles (Dacheux et al, 2005).

In addition to the traditional “merocrine secretion”, an unusual protein secretion has now been described in principal cells of several mammals including rodents and humans (Yanagimachi et al 1985; Frenette and Sullivan, 2001; Nickel et al 2003). In this new described category of secretion, referred as “apocrine secretion”, protein synthesis take place in the cytoplasm in an ER and Golgi-independent manner. The release of these proteins occurs at small protrusions at the apical surface of the cells (apical blebs) followed by their fragmentation. This mechanism results in the release of the small membranous vesicles named "epididymosomes" into the epididymal fluid. Although its relevance is still not fully understood, it is known that the "epididymosomes" interact with sperm surface during the epididymal transit. Many different proteins are associated with epididymosomes and some of them are selectively transferred to specific membranous domains of luminal spermatozoa. As the proteins released by “apocrine secretion” do not have a signal peptide, they are often GPI anchored. However, the exact mechanism of protein transfer from epididymosomes to spermatozoa remains to be determined (Nickel, 2003). An aldose reductase known for its 20alpha-hydroxysteroid

dehydrogenase activity (P25b) and a specific cytokine (macrophage migration inhibitory factor or MIF) have been identified in association with epididymosomes and may be important in adding functionality to the male gametes while they pass through the epididymis (Saez et al, 2003, Frenette et al 2003).

4.2 Protein in the luminal compartment

Most of the secreted proteins that have been identified appear to be conserved among species and are present in many mammalian species studied (Robaire et al 2000). Up to now, the amount of the proteins secreted by the epididymis is underestimated because the methods of protein detection are still limited. These methods are not able neither to detect weakly expressed proteins nor clearly separate large (bigger than 250-300 kDa) and basic (pH > 8.4) compounds, commonly found in the epididymal lumen (Dacheux et al 2005).

The medium surrounding sperm is completely and periodically renewed (Gatti et al 2004). Although many luminal proteins are not yet characterized, several hundreds of proteins, identified by mass spectrometry or evaluated by advanced technical methods in proteomic approach, have been specifically characterized in the fluid from different regions of epididymis (Fouchecort et al 2000). Luminal concentration of each secreted protein has been described as region and protein-specific. The major proteins found in the epididymal fluid are lactoferrin, clusterin, cholesterol transfer protein, glutathione-peroxidase, albuminoid proteins, prostaglandin, prostaglandin D2 synthase, CRISP 1 protein, hexosaminidase, procathepsin D, among others (Fouchecort et al 2000; Suzuki et al, 2004). Once most of the proteins on the surface of maturing spermatozoa show glycan chains or carbohydrates moieties, a number of glycosidases and glycosyltransferases are found free in the epididymal fluid or are associated with sperm plasma membrane in order to perform specific glycan-modifying (Tulsiani et al 1998; Toshimori 2003).

Although the proteins present in the epididymal fluid are mainly resultant from the secretory and reabsorptive activity of the principal epithelial cells (Vernet et al, 2004; Dacheux et al, 2005), rete-testis compounds that enter through the efferent duct in the anterior region of the epididymis also contribute to form this fluid. However, almost all of these proteins are rapidly absorbed or degraded in the first segment of the epididymis. Proteolysis of the pre-existing proteins and even metabolic activity of the spermatozoa also contribute to the protein profile of the epididymal fluid (Dacheux et al 2005).

The main characteristic of proteins secreted by epididymal epithelia into the lumen is the high polymorphism in molecular mass and pI. Many proteins are identified in several different isoforms, mainly attributed to post translational modifications or to different degrees of glycosylation or partial proteolysis (Dachuex et al 2005). Other evidences suggest that some proteins could be released as a pro-form and become activated after processing in the fluid (Gatti et al 2004).

Several steps of the spermatozoa maturation involving changes in the protein profile of the plasma membrane are mediated by external enzymes and secretions derived from the epithelium lining of the epididymis (Toshimori, 2003). Some of the mechanisms involved in epididymal maturation of the spermatozoa are discussed below.

5. Adulthood biological function

5.1. Epididymal maturation of spermatozoa

Mammalian testicular spermatozoa present in the seminiferous tubules of testis are either immotile or display very restricted vibratory motions; they are described as non-viable with no fertilizing capacity haploid cells (Verma 2001; Gatti et al 2004).

The final stages of spermatozoa differentiation occur outside the gonad, in the epididymal tubule, where spermatozoa undergo substantial remodeling (Dacheux et al, 2005). As the spermatozoa progress from the caput down to the caudal region of the epididymis, they attain

progressive motility and fertilizing ability (Orgebin-Crist,1967; Verma 2001;Toshimori, 2003; Vernet et al, 2004). Enzymes, proteins and glycoproteins secreted to the fluid by different regions of the epididymis are destined to interact sequentially with spermatozoa surface in order to alter their molecular fashion and to enable them to the fertilization process (Hamil et al 2000, Robaire et al, 2000; Gatti et al 2004; Dacheux et al, 2005; Sullivan et al, 2005) (Fig. 5D). This process is termed epididymal maturation and results in the spermatozoa ability to move, recognize and bind the zona pellucida (ZP) and fuse to the oocyte plasma membrane (Dacheux et al 2003).

Numerous studies have been done in order to identify the specific epididymal molecules that seem to be essential during the sperm-oocyte interaction (Myles and Primakoff, 1984; Boué et al. 1995; Légaré et al. 1999; Syntin and Cornwall, 1999; Cohen et al. 2000, 2001; Chu et al. 2000; Hamil et al 2000; Liu et al. 2000; Gaudreault et al 2001; Nonaka et al. 2003; Pauls et al 2003). However, no consensus has emerged on the precise roles of most of these molecules (Saxena et al 1999). Although the real molecular mechanisms that induce sperm maturation had not been definitively identified to date, it is well established that the regionalized epididymal fluid microenvironment promotes numerous spermatozoa changes that go through the entire length of the epididymal tubule (Dacheux et al, 2005).

Principal and basal cells of the polarized epithelium are the main cell types responsible for creating both progressive and continuous changes in the fluid composition that acts as a very complex environment around the spermatozoa (Hermo et al, 2004). Active secretion and reabsorption made by epithelial cells and the presence of significant restrictions in the exchange between luminal compartment and blood plasma are involved in the maintenance of the specific characteristics of the epididymal fluid. Some components of this fluid are present in an unusual concentration, and some of them are not found in other body fluids. In general, epididymal secreted proteins exhibit transport and binding functions or enzymatic activity and contribute to

the fertilization ability of the spermatozoa by facilitating the exchange of proteins or lipids between spermatozoa and the surrounding fluid (Dacheux et al 2005).

For the fertilization to occur in the female tract, capacitated spermatozoa initially interact, in a specie-specific manner, with the zona pellucida (ZP), an extracellular coat that surrounds all mammalian eggs. This process, called *primary binding*, is mediated by ZP glycoconjugates that recognize sperm receptors located on the surface of the male gamete. Bound spermatozoa undergo the acrosome reaction, and initiate penetration of the ZP. Sperm penetration involves both digestion of the ZP and vigorous sperm motion, while keeping the sperm associated to the matrix, via sperm receptors; this interaction has been named *secondary binding* (Flesch and Gadella 2000). Numerous candidates have been postulated as sperm receptors for primary and secondary binding. For instance, Human P43H, an epididymal sperm protein secreted predominantly by the principal cells in the proximal-distal section of the corpus epididymis (Boué et al 1994, 1995; Boué and Sullivan, 1996) and N-acetylglucosaminidase (NAG), a epididymal glycosidase responsible for the hydrolysis of non reducing terminal N-acetylglucosamine (GlcNAc) residues from ß-glycosidic boundaries in numerous glycoconjugates (Miranda et al 2000), are two extensive studied examples of the molecules known to be involved in sperm interaction with the ZP. The monoclonal antibodies named mAb 4A8 and mAb TRA 54 were produced by independent laboratories in order to allow fertilization studies since these antibodies are able to inhibit the zona penetration, although their recognition epitopes are still not fully characterized (Batova et al 1998, Arrotéia, submitted results).

Acrosome reacted spermatozoa that have completed ZP penetration reach the perivitelline space, bind and fuse to the egg plasma membrane, releasing the genetic material and initiating zygote development. Protein complexes with both binding and fusion functions, would be present in both the egg and the spermatozoa and would interact with the counterpart on the surface of the other gamete (Töpfer-Petersen, 1999). Sperm binding to the oolema has been proposed to involve interaction of sperm receptors located in the equatorial segment and

postacrosomal region with carbohydrates from oligosaccharides located on the egg plasma membrane (Gabriele et al, 1998; Flesch and Gadella 2000).

Several proteins of epididymal origin have been proposed to participate in the sperm - egg membrane fusion process. In general, fusion proteins may have sequences of hydrophobic residues and mostly of alpha-helical structure, which are considered as fusion peptides (Pécheur et al 1999). The protein named “fertilin” is one of the best studied sperm adhesion molecules, but the real mechanism is yet unknown (Myles et al 1994). Rat and mouse epididymal protein DE and its well studied human epididymal secretory protein counterpart, named ARP (Acidic Epididymal Glycoprotein Related Protein or AEG Related Protein), have been characterized as a putative sperm receptor in the egg fusion process (Cuasnicú et al 1984a,b; Rochwerger et al, 1992; Rochwerger and Cuasnicú 1992; Hayashi et al 1996, Kratzschmar et al 1996, Ellerman et al 1998). Among others, ARP and DE belong to the CRISP family members that share a highly conserved cluster of cysteines near to the carboxy-*terminus* of protein. Using recombinant ARP expressed in bacteria, human oocytes showed in their surface the presence of complementary sites to ARP (Cohen et al 2000). These evidences strongly support a role of ARP/DE in sperm-egg fusion process. Sperm Oocyte Binding 2 protein (SOB2) and a protein referred as FLB1, both human epididymal proteins, were reported to participate in the sperm-egg fusion process, since binding and penetration of human spermatozoa into the zona-free eggs is inhibited in the presence of their specific antibodies (Boué et al 1992, 1995). Finally, the involvement of integrins in the interaction of spermatozoa with the oocyte plasma membrane has been supported by several findings (Bronson and Fusi 1990, Fusi et al 1992, Schaller et al 1993, Miranda and Tézon 1992), although He et al (2003), using integrins null eggs, reported that none of the integrins known and present on the mouse egg are really essential for sperm-egg binding and fusion.

The sperm acrosome is a highly specialized organelle overlying the anterior part of the sperm nucleus and containing a number of hydrolytic enzymes that are believed to be required

for fertilization. During epididymal maturation, several intra-acrosomal proteins firstly expressed in the testis continue to undergo biochemical and/or immunocitochemical modifications. Such maturation-dependent modifications may be attributed to glycosylation/deglycosylation and/or proteolytic processing (Yoshinaga and Toshimori, 2003). For example, acrosomal human SP-10 protein decreases from 45 kDa to 18-25 kDa as a result of proteolytic process when spermatozoa pass into the caput epididymis. Other molecules, as acrin-3 of the mouse spermatozoa, increase its antigenicity but its molecular size is not changed. Acrin 1 e acrin 2 move from one acrosomal domain to another one, resulting in a compartmentalization of these molecules during epididymal maturation. Thus, intra-acrosomal proteins initially generated in testis can change their location and their size simultaneously during epididymal maturation (Saxena et al 1999 ; Yoshinaga and Toshimori, 2003).

Besides the changes in acrosomal proteins, extern proteins may extensively be affected. Most of the sperm surface proteins brought from the testis are removed or further processed by proteolysis in epididymis. This process induces either a change in domain distribution of these proteins (within a domain or across domains) or a release of the cleaved proteins into the epididymal lumen (exo-proteolysis) or into inside spermatozoa (endo-proteolysis) (Toshimori 2003; Dacheux et al, 2005). Besides, the set of these alterations exposes new functional peptides domains able to interact with the surrounding molecules that may trigger the sperm-egg interactions (Toshimori, 2003). For instance, fertilin-β, a protein initially covering the entire testicular sperm head, is degraded by two successive cleavages during caput transit and is further restricted to the post-acrosomal domain of the spermatozoa. Other proteins transformed in the same way are hyaluronidase pH20, protein CE9 and alpha-D-mannosidase (Gatti et al 2004).

Some of the new proteins identified on spermatozoa during the transit in epididymis are transitory, but most of the low weight components remain in the spermatozoa after leaving the epididymis (Dacheux et al, 2005). Some of these proteins are loosely bound and probably

prevent direct interactions with the sperm surface by masking sites that will be activated during the fertilization. In another hand, several external epididymal proteins are closely bound or may be integrated into spermatozoa membrane due hydrophobic properties (as GPI-anchored proteins) or proteolysis of their carboxy-terminal region (Kirchhoff, 1996; Gatti et al, 2004). In addition, a number or proteins can be transferred from the epididymal epithelium to the spermatozoa by a very specific and not fully elucidated mechanism by epididymosomes.

5.2. Epididymis and the protection of the mature spermatozoa

In human, the spermatozoa takes approximately 10 days to reach the cauda epididymides (Vernet et al, 2004; Toshimori 2003), but this time varies from five approximately 16 days in several mammalian species already investigated (França et al 2005). Once fully mature, spermatozoa may be stored in the terminal region of the cauda epididymis for days or weeks depending on the species, until ejaculation occurs (Yanagimachi, 1994). In fact, ejaculated spermatozoa can survive for 12–24 h, but the male gametes within the cauda epididymis remain viable for a 10-day period or more (Sullivan, 2004). The mechanisms of sperm survival in the distal epididymidis remain poorly explained. It is known that spermatozoa are at risk during their epididymal transit and storage, since the predominance of polyunsaturated fatty acids (PUFA) in their plasma membrane renders spermatozoa highly susceptible to lipid peroxidation due to attacks from reactive oxygen species (ROS). Developing enzymatic and non-enzymatic strategies to protect the spermatozoa during this extremely vulnerable period is another role attributed to the secretory activity of the epididymal epithelium (Vernet et al 2004). Some of the proteins released by the epididymal epithelium into the lumen seem to be involved in protecting the spermatozoa from both oxidation reaction and /or bacterial attack (Robaire et al 2000; Gatti et al, 2004).

A system of regulated storage of spermatozoa in the distal region of the organ has been developed in mammals, ensuring that the stored cells are quiescent and unreactive (Bedford

and Yanagimachi, 1991). The exact mechanism of this physiological phenomenon, known as *sperm quiescence*, is not clearly understood. One hypothesis to explain this remaining of the quiescent state resides in the drastic change in ionic composition of the medium in the cauda epididymis (Verma 2001).

Finally, there are a number of epididymal sperm-coating proteins that exert their effects on the male gamete not in the male tract, but in the female one. These proteins are produced by epididymis and bind luminal sperm but become functional in the female oviduct. Some of these molecules are considered to be decapacitating factors and might associate to the spermatozoa surface and prevent premature sperm activation.

6. Epididymis and Infertility

Approximately 12% of couples will encounter fertility problems during their reproductive life. Whereas diagnostic tools and therapies to treat female infertility are relatively well developed, male infertility often remains unexplained. It is generally accepted that male factors contribute to approximately 40% of infertility cases and many of the causes of male infertility are considered idiopathic (Sullivan, 2004).

Spermogram is an exam that can easily evaluate the semen quality according to previously defined parameters edited by the World Health Organization (WHO) (WHO, 1999). The normal spermogram values were based on multi-centered population studies on fertile men. However, several men presenting normal spermogram are diagnosed as idiopathic infertile. In these cases, these men can present post-testicular defects resulting in ejaculation of spermatozoa with normal morphology but sub-optimal fertilizing ability (Sullivan, 2004). Particularly, epididymis could be involved in a number of pathophysiologies affecting sperm maturation in some of these cases of male infertility (Boué and Sullivan, 1994; Sullivan, 2004).

As discussed in this review, the specific microenvironment of the epididydimal luminal compartment are believed to be essential for sperm maturation and the intraluminal fluid of the

epididymis differs markedly in composition from blood and varies within different regions of the epididymis. A disruption in any of the features of the epididymal microenvironment (temperature, pH, protein composition and concentration) may lead to sperm fertility and conservation disorders (Dacheux et el, 2005).

It is known that the osmotic pressure of the epididymal fluid in cauda is higher than blood and uterus microenvironment and that the ability to regulate semen volume is developed during epididymal transit. As volume regulation is essential for spermatozoa survival, their fertilizing capacity is clearly dependent on osmolyte secretion by epididymis (Cooper and Yeung, 2003). Interest on the sperm volume regulation has arisen from the suggestion that a dysfunction of the volume regulation of the semen was the cause of infertility in certain transgenic mice. In these mice, spermatozoa are sharply angulated at the midpiece. This is interpreted as evidence of swelling since it can be mimicked by immersion of normal sperm in hypotonic medium. The mechanisms regulating sperm volume were barely examined, but evidences suggest that epididymis could influence the ability of sperm to regulate their volume (Cooper and Yeung, 2003).

The proteins secreted along the epididymis under androgenic control vary from one segment to the other and modify the maturing male gamete in a sequential manner (Sullivan, 2004). Numerous attempts have been made in order to correlate the fertility potential of the spermatozoa to the proteins present in the seminal plasma including epididymal proteins. The presence or absence of given antigens on the spermatozoa has often been correlated to a sperm property such as recognition and binding ZP or oocyte membrane or to the spermatozoa movement (Dacheux et el, 2005). The utility of molecular markers of the epididymal function to identify male idiopathic infertility is under investigation. Few years ago, the protein P34H was related to these cases of male idiopathic infertility. P34H, a 34 KDa human epididymal sperm protein synthesized and secreted predominantly by the principal cells, is physiologically undetectable on spermatozoa in the caput epididymidis and progressively accumulates on the

sperm surface covering the acrosomal cap from the corpus to the distal cauda epididymidis. This protein remains in capacitated spermatozoa, but apparently is lost during the acrosome reaction (Boué et al 1994, 1996), suggesting that P34H is a human epididymal protein involved in sperm interaction with the ZP. Western blot analysis showed that P34H is associated with the spermatozoa of semen samples obtained from fertile donors, but it is undetectable in approximately 40% of the semen samples obtained from men presenting idiopathic infertility. Spermatozoa with undetectable levels of P34H were related to be unable to bind to the ZP through *in vitro* assays. Thus, in these men, infertility could be attributed to the inefficient epididymal maturation of male gametes that are unable to fertilize. Human P34H protein and other epididymal molecules could be considered as markers for the epididymal function in sperm maturation and can be used as a diagnostic tool to identify cases of infertility in men, which cannot be identified by classical semen analysis (Boué and Sullivan, 1996).

A number of transgenic mice exhibit a phenotype of defects in fertility due to indirect effects on epididymis. Recently, a variety of transgenic mice were generated to display male infertility by epididymal epithelium disruption. The use of these transgenic mice may allow us to solve the mode of action of epididymal specific genes and may suggest new targets for the treatment of some forms of male infertility (Lye and Hinton, 2004).

7. Influence of temperature in epididymal development, physiology and fertility

Testis temperature was found to be 1–5℃ lower than body temperature according to the mammal species (Harrison and Weiner 1949; Ivell and Hartung, 2003). Heat and certain substances, such as gases, steroids and peptide hormones, can pass from venous blood, interstitial fluid and lymph to the arterial blood; this process is called *local counter-current transfer* and was also found to occur also from the testis to epididymis and from ovary to ovary (Einer-Jensen and Hunter, 2005).

Both caput and part of the corpus epididymis are supplied by the testicular artery, which presents a reduced temperature. Testis will communicate locally with the ipsilateral caput and corpus epididymidis. This way, temperature of these segments is, therefore, lower than body temperature, which may decrease the damage to spermatozoa during storage (Einer-Jensen and Hunter, 2005). In fact, the coolest area in the scrotum is that where the cauda epididymis is located (Toshimori 2003).

It is known that a small increase in the temperature of the testis does not destroy the germinal epithelium; however, it reduces testis weight and sperm production and brings a greater incidence of morphologically abnormal spermatids and spermatozoa. Testicular Sertoli cells also appear to be temperature-dependent, although the mechanisms involved are not clear (Ivell and Hartung, 2003). Selective imposition of abdominal temperature on the epididymis solely does not suppress sperm maturation there, and bilaterally cryptepididymal males remain fertile for long periods. However, deep body temperature alterations changes the ionic and protein composition of cauda fluid yielding effects on the cauda epithelium, eliminating the special ability of the cauda to store and prolong the life of spermatozoa by promoting a rapid epididymal transport (Bedford 1991). The damage caused by the epididymal heating is reflected in a reduced diameter, length and shape of the epithelial cells of the duct (Fig. 6A-B) (Arrotéia et al 2005). One clinical consequence of epididymal temperature increase and cryptepididymal condition change is a much smaller and atypically declined number of spermatozoa in the ejaculate (Bedford 1991).

The epididymal maturation and storage of sperm depends, to a large extent, on specific proteins secreted from the epididymal epithelium (Toshimori 2003). In a series of studies on the epididymides of cryptorchid animals and on primary epithelial cell cultures, it was shown that some of these epididymal gene products are exquisitely sensitive to small changes in temperature (Pera et al, 1996; Kirchhoff et al 2000). Although there is no information whether epididymal function (and hence sperm maturation) is irreversibly compromised in adult humans

who were treated for cryptorchidism as children, experimental results indicate that gross and histological alterations caused by cryptorchidism can be restored by orchidopexy (Ivell and Hartung, 2003; Arrotéia et al 2005).

9. The aging process of epididymis

During aging, the male reproductive tract is characterized by testicular dysfunction resulting atrophy in the seminiferous epithelium and the Leydig cells. Although the aging process has been studied extensively for the testis and prostate, there is little information on how aging affects the epididymis (Robaire et al, 2000).

The epididymis is highly dependent on the presence of androgens, particularly 5α-reduced androgens, in order to work. Evidences arisen from a developed rat model showing long life and being relatively free of diseases demonstrated that while ability of the epididymis to produce 5α-reduced androgens is compromised with age, the ability of this tissue in respond to androgens is not (Wright et al, 1993).

Epididymal alterations due to aging affect epididymis in a segment- and cell-dependent manner: basal cells are primarily affected in the initial segment, clear cells are the most modified in the caput segment and principal cell are the most damaged in the corpus. In the proximal cauda segment, aging results in more dramatically changes in the appearance: clear cells are enlarged and filled with dense lysosomes and some principal cells contain large vacuoles. Dysregulated intracellular trafficking, decreased protein degradation and oxidative stress are hypothesis to explain the molecular mechanisms behind these changes (Robaire et al 2000).

Finally, blood-epididymis barrier may be compromised in structure and function with age, resulting in an increase of the number of monocytes/macrophages mainly in the initial segment. Several kinematics parameters of sperm motility in the cauda epididymis may also be decreased. High incidences of pre-implantation loss, lower fetal weight and higher post-natal death rates were observed in the offspring of young females mated with old male rats (Serre and

Robaire, 1998), but whether these effects are due to deficient epididymal maturation or due to a testicular dysfunction remains to be solved.

Conclusion

Mammalian epididymis promotes spermatozoa modifications that are necessary for spermatozoa to become fertilization-competent cells and to be stored safely in the male. All epididymal physiology is adapted to create sequential changes around the sperm during its progression through the organ, especially on membrane properties.

Understanding of epididymal physiology is not an easy task, since the complex pattern of gene regulation system, the protein secretion pattern and the hormonal dependence model are unique in the body. Since epididymal dysfunctions are related to cases of idiopathic male infertility, focusing on major epididymal proteins with spermatozoa final fate urges. However, there is a lot of work to be done in order to comprehend this phenomenon, and it is not clear if all the efforts will be enough to fully understand how spermatozoa become fertile. In a clinical point of view, increasing of our knowledge on spermatozoa maturation should provide specific markers in order to develop new efficient criteria in predicting and improving male fertility. Implementation of genomics and proteomics will help, in a near future, in the characterization of some already identified proteins, as well as in the description of novel epididymal components, anticipating great advances in the elucidation of the sperm maturation process.

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Figure Captions

Fig. 1. Embryologic origin of epididymis. Epididymal duct is derived from the mesonephric duct, an anatomical structure longitudinally arisen in the intermediate mesoderm (IM) of the mammal embryo. Mesonephric duct and primordial gonade are closely located.

Fig. 2. Intra-uterus development of epididymis. Testicular production and secretion of anti-Müllerian substance and testosterone positively regulate the ipsilateral mesonephric duct and induce the cranial portion of mesonephric ducts to develop, proliferate and evolute to contact testicular sexual cords (A). Cords arising from the rete testis form the efferent duct and fuse with the mesonephric tubules adjacent to the testis becoming the epididymal duct by late gestation (B). As gestation proceeds, the tubule becomes longer and the degree of convolution increases from the cranial to distal pole of the epididymis.

Fig. 3. Regionalization of epididymis. A: according to the high regionalization of the epithelium and luminal protein secretion, five different physiological regions are distinguished: (I), proximal caput or initial segment; (II), middle caput; (III), distal caput and proximal corpus; (IV), distal corpus; (V), cauda. B: initial segment (IS) and proximal caput (caput) of the epididymis. Asterisk (*): connective tissue septum. C: cauda of the epididymis. Asterisk (*): aggregated of spermatozoa. Arrow: peritubular smooth muscle. The thickness of the epididymal epithelium varies from thickest in the proximal caput to thinnest in caudal region, and conversely the luminal diameter and thickness of the peritubular smooth muscle increases from proximal to distal regions. D: proximal caput. E: middle caput. F: cauda. In all of these segments, the epididymal duct is lined by an epithelium composed of principal (arrow) and basal (arrowhead) cells. Apical (double arrowhead), clear (double arrow), halo (thin arrow) and narrow (large arrow) cells are also distributed in the different segments. Scale bar: A: 1100 μm; B: 63 μm; C: 120 μm; D-F: 32 μm.

Fig. 4. Transmission electron microscopy of principal cells in epididymal caput of mice. A: apical region of the cell. st: stereocilia; vr: vesicular region with membranous elements; mb:

multivesicular bodies; mt: mitochondria area; arrow: tight junction region in lateral membrane. B: supranuclear area of cytoplasm showing abundant flattened and distended portions of the Golgi sacules (gs). N: nucleus. C: infranuclear area of cytoplasm showing Golgi sacules and abundant rough endoplasmic reticulum (rer) cisternae. Scale bar: A: 1,0 μm; B: 0,5 μm; C: 0,4 μm.

Fig. 5. Regionalized expression of the epididymal antigen recognized by the monoclonal antibody TRA 54 in mice. A: whole epididymis; B: epididymal caput; C: epididymal cauda; D: epididymal spermatozoa; detail: vas deferens spermatozoa. The molecule is synthesed by principal cells of the epididymal caput (arrow), released to the lumen and accumulated in the cauda (asterisk), where is incorporated by the acrosomal region of the spermatozoa (C). The antigen is maintened after spermatozoa leave epididymis. Scale bar: A: 1000 μm; B-C: 55 μm; D: 10 μm; detail: 4 μm.

Fig. 6. Photomicrographs of epididymal caput. A: control adult epididymis; B: epididymis from cryptorchidic mice. The damage caused by the epididymal heating includes a reduced diameter, length and shape of the epithelial cells. Sperms are absent due to production failure by cryptorchidical testis. Scale bar: 40μm.

Figure 1

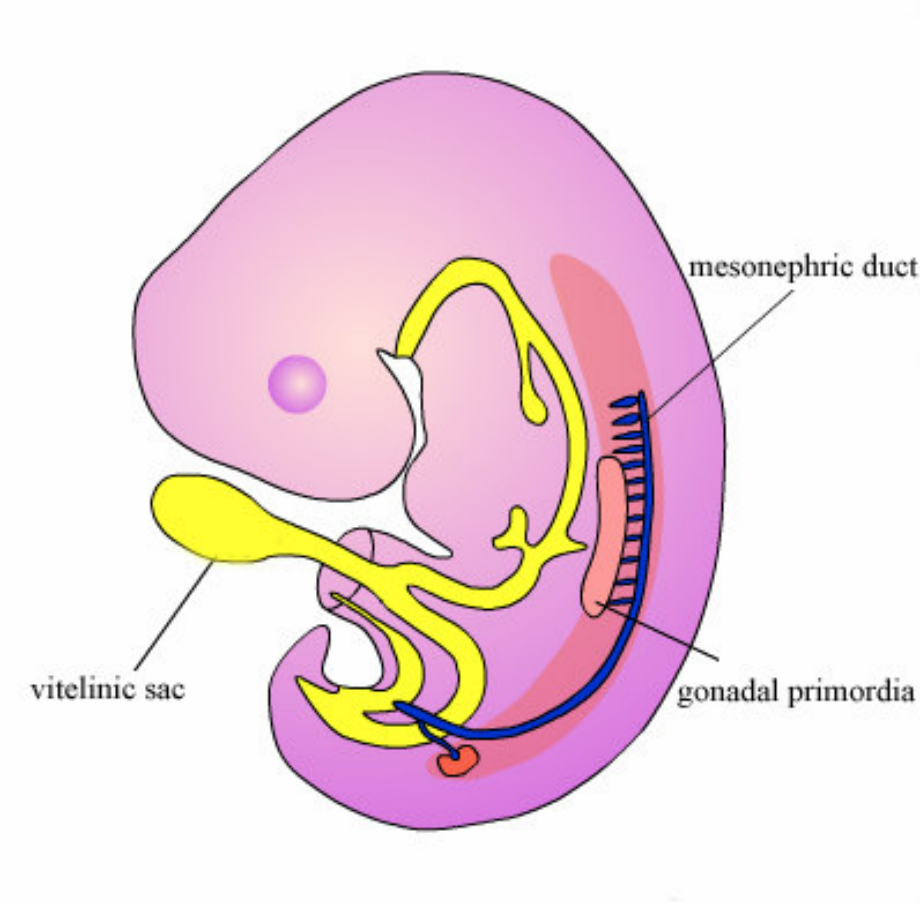


Figure 2

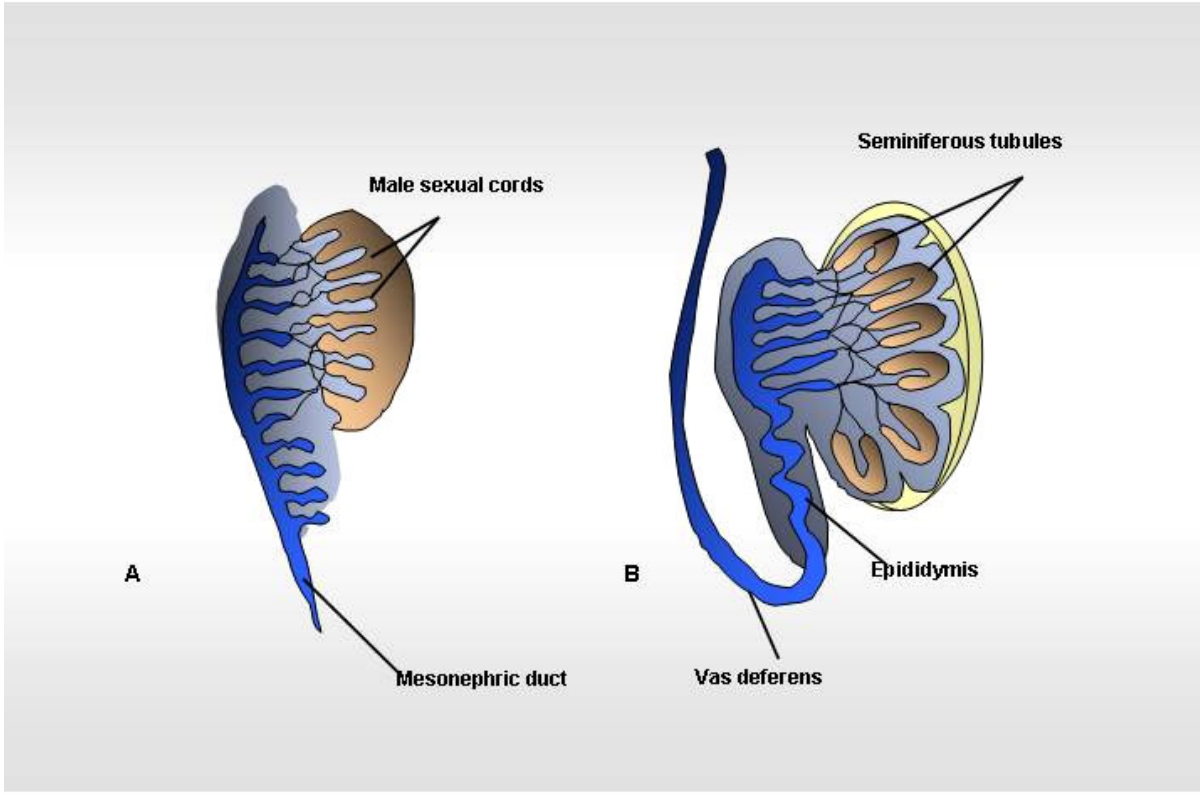

Male sexual cords
A
Mesonephric duct
Seminiferous tubules
B
Epididymis
Vas deferens

Figure 3

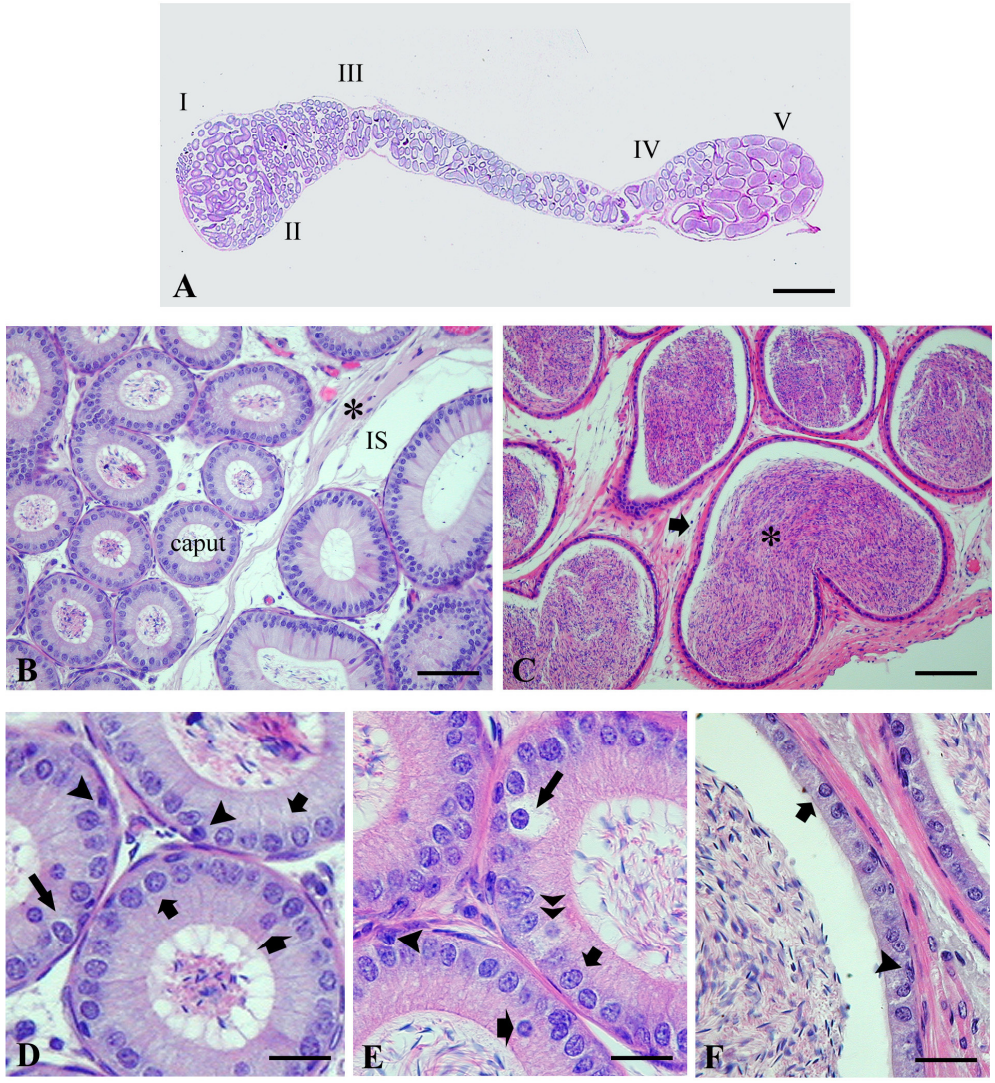


Figure 4

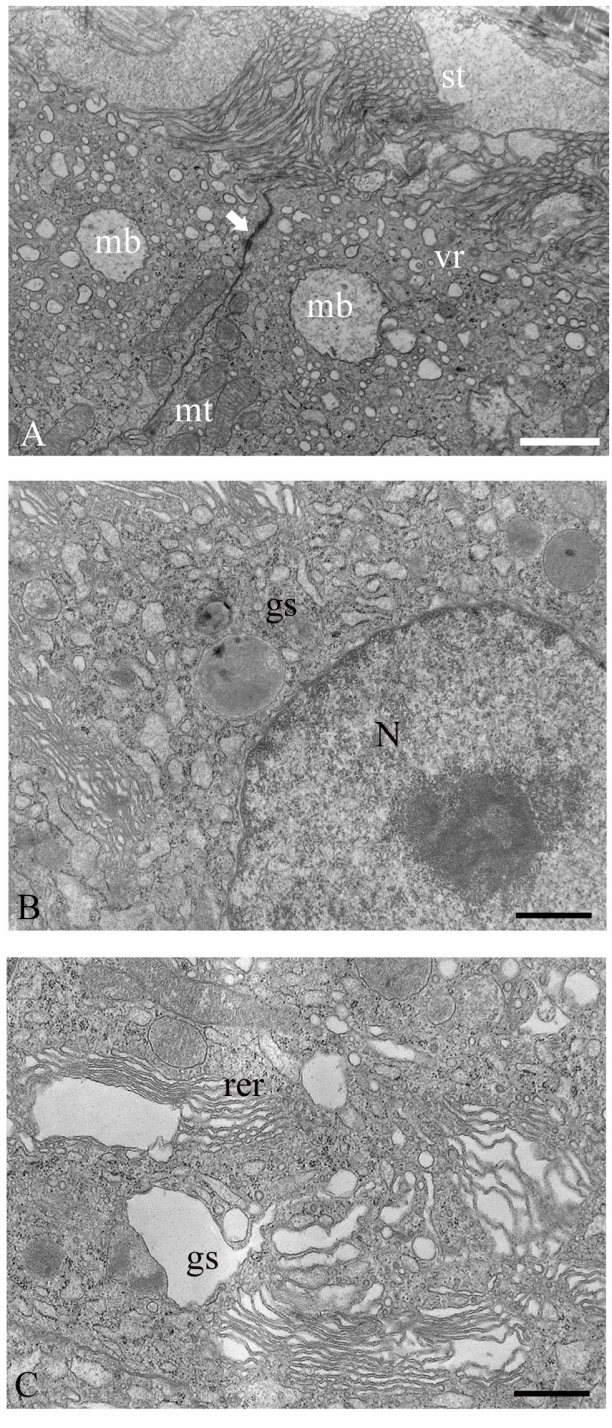


Figure 5

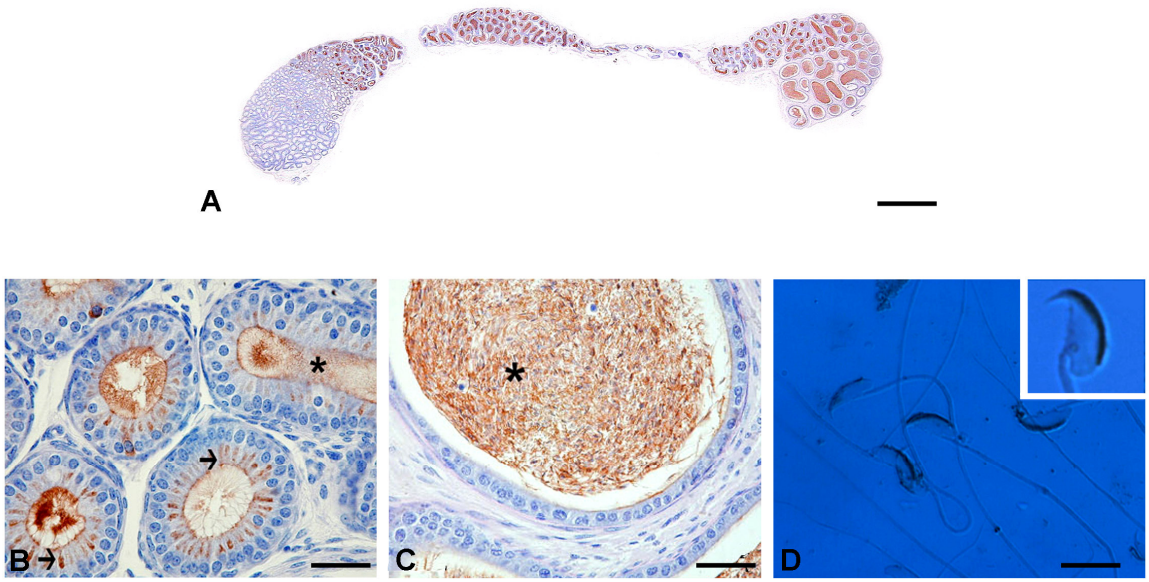
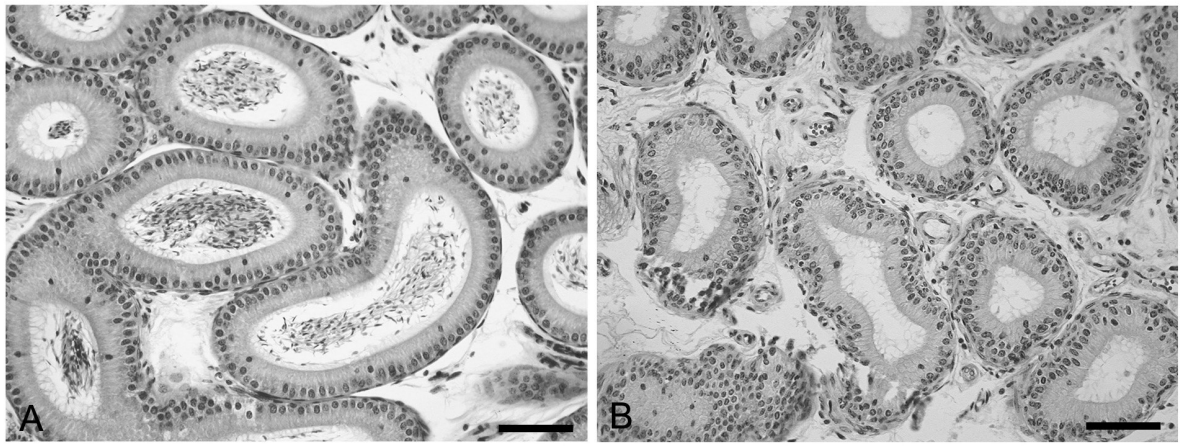


Figure 6



Capítulo VII

Considerações e Conclusões Finais

Este trabalho permite as seguintes conclusões:

(1) A molécula antigênica reconhecida pelo Amc TRA 54, reconhecidamente expressa inicialmente em uma população específica de células germinativas em desenvolvimento (espermátides), é também expressa por uma população limitada de células da região da cabeça do epidídimo (células principais do epitélio epididimário);

(2) O antígeno expresso nas células principais do epitélio epididmário é sintetizado e liberado à luz do epidídimo pela via clássica de secreção celular envolvendo retículo endoplasmático rugoso, complexo de Golgi e vesículas apicais de secreção.

(3) Após liberado no lumen epididimário, o antígeno é inicialmente adsorvido sobre a superfície do espermatozóide, polarizando-se sobre a região da membrana que recobre a região acrossomal. Como destino, o antígeno em questão é gradativamente endocitado e transferido para o interior da vesícula acrossômica durante o processo de maturação epididmária;

(4) O antígeno pode ser reconhecido pelo Amc TRA 54 dentro do acrossomo de espermatozóides mesmo após o período de capacitação, ocorrido, *in vivo*, dentro da tuba uterina do organismo feminino, demonstrando sua manutenção e possível importância em momentos prévios à fertilização;

(5) O antígeno em estudo é também expresso por células germinativas e epididimárias de tecidos humanos. Esta expressão obedece aos padrões descritos para o antígeno expresso em camundongos, sugerindo uma conservação inter-específica da expressão desta molécula no trato reprodutor masculino. O antígeno liberado pelo epidídimo humano é aderido à membrana de superfície da região pós-equatorial do espermatozóide, na qual permanece após findado o processo de capacitação, à semelhança do ocorrido para espermatozóides e murinos;

(6) A reação acrossômica (processo que resulta no rearranjo de membranas da cabeça do espermatozóide e que permite ao mesmo atravessar a zona pelúcida que envolve o oócito) altera o padrão de expressão do antígeno reconhecido pelo Amc TRA 54 em espermatozóides murinos e humanos. Em espermatozóides murinos, o antígeno em questão é gradual e totalmente liberado durante o evento exocítico da reação acrossômica. parecendo ser requerido para a dispersão da matriz acrosomal e para a otimização da passagem do espermatozóide pela zona pelúcida, visto que a inibição de sua atividade pelo anticorpo monoclonal TRA 54 impede ou retarda, de maneira significativa, a capacidade de fertilização dos espermatozóides. Por sua vez, a alteração observada para espermatozóides humanos durante a reação acrossômica consiste no deslocamento do antígeno presente na região pós-equatorial do gameta para uma posição mais posterior (peça média), provavelmente permitindo a exposição de proteínas de fecundação previamente camufladas e importantes para a interação com a membrana plasmática do oócito.

(7) O processo de isolamento e seqüenciamento do antígeno reconhecido pelo Amc TRA 54 nas células epiteliais do epidídimo de camundongos sugerem que o mesmo corresponde a à proteína albumina-1 de camundongo, cuja seqüência de aminoácidos estava previamente depositado em bancos de dados de proteína, e à qual nenhuma função reprodutiva havia sido atribuída até o momento. O antígeno em estudo pode ser incluído na superfamília das proteínas albuminóides, especificamente na família PES (*prealbumin epididymal-specific*), cujos membros, liberados pelo epidídimo de maneira andrógeno-dependente, apresentam em comum a propriedade de agregarem-se em macromoléculas, a presença de domínios moleculares homólogos à albumina sérica e, interessantemente, a propriedade de aderir à superfície do espermatozóide durante o trânsito epididimário e a propriedade de serem internalizados (devido à abundância e disposição dos sítios hidrofóbicos presentes).

Isoformas com características semelhantes foram previamente descritas para outros mamíferos.

Em resumo, as evidências relatadas neste trabalho sugerem que, embora sob diferentes mecanismos de ação e em diferentes momentos, o antígeno reconhecido pelo Amc TRA 54 está claramente envolvido na aquisição da competência fecundativa dos espermatozóides de mamíferos, adquiridas no epidídimo previamente ao momento da fertilização. Este trabalho ineditamente contribuiu com a identificação funcional, bioquímica e estrutural de uma proteína envolvida no processo de maturação epididimária do espermatozóide, contribuindo para a geração de um mapa, ainda incompleto, das diferentes moléculas essenciais às diversas fases da fertilização em mamíferos.

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Descrição detalhada de protocolos metodológicos

O presente anexo tem por intuito reproduzir detalhadamente os protocolos metodológicos utilizados para o desenvolvimento desta tese.

As etapas de cada protocolo estão descritas passo a passo. Ao final de cada protocolo, segue um compilado das receitas dos reagentes e soluções específicos de cada metodologia descrita.

Alguns protocolos compartilham etapas com mais um ou dois protocolos adicionais, os quais estão devidamente indicados no decorrer do texto.

1. Isolamento de espermatozóides de camundongos C57Bl6

1.1. Sacrificar camundongos machos (4 a 8 semanas) por deslocamento cervical e efetuar incisão abdominal ventral;

1.2. Identificar e remover os órgãos do sistema reprodutor;

1.3. Para obtenção de espermatozóides do epidídimo, colocar a cauda do órgão sobre uma gota (60 µl) de tampão fosfato salina (PBS 1x, Gibco) em placa de petri plástica (Cornning) e incubar a placa por 5 min a 37^{o}C para a dispersão dos gametas;

1.4. Para obtenção de espermatozóides do canal deferente, dissecar cuidadosamente o órgão removendo vasos sanguíneos externos e tecido adiposo. Depositar o órgão devidamente dissecado em posição esticada na placa de petri de modo que a extremidade distal esteja em contato com a gota de PBS. Com auxílio de espátulas de ponta fina, raspar delicadamente a parede do ducto, forçando a saída do agregado de espermatozóides. Transferir o agregado para outra gota de PBS e aguardar a dispersão por 5 min a 37^{o}C.

1.5. Transferir a gota de PBS contendo os espermatozóides para tubo eppendorff;

1.6. Proceder a lavagem do sêmen: acrescentar 600 µl de PBS ao eppendorff, centrifugar por 5 min a 450 X g e descartar o sobrenadante. Ressuspender o sedimento celular com 900 µl de PBS e repetir o procedimento de centrifugação e descarte do sobrenadante;

1.7. Ressuspender o sedimento de espermatozóides em fixador de Boiun, tampão PBS ou meio de cultura (M16, Sigma Aldrich), dependendo do procedimento a ser realizado em seguida.

<u>Soluções específicas</u>

- Bouin: misturar 75 ml de solução aquosa de ácido pícrico saturado e 20 ml de formaldeído (37%). Adicionar 5 ml de ácido acético glacial no momento da utilização.

- Solução aquosa de ácido pícrico saturado: dissolver 3g de ácido pícrico em 100ml de água destilada.

2. Capacitação e Indução da Reação Acrossômica – Espermatozóides murinos e humanos

2.1. Coletar os espermatozóides murinos como nos itens 1.1 a 1.6; espermatozóides humanos coletados após masturbação devem ser lavados como descrito no item 1.6;

2.2. Para espermatozóides humanos, acrescentar sobre o sedimento celular resultante do processo de lavagem por centrifugação 1 ml de meio de cultura HTF (Human Tubal Fluid, Gibco) enriquecido com 35 mg/ml de albumina bovina sérica (BSA, fração V, Sigma Aldrich). Para espermatozóides murinos, acrescentar 1 ml de meio M16 (Sigma Aldrich) enriquecido com 4 mg/ml de BSA (fração V, Sigma Aldrich);

2.3. Incubar as amostras em eppendorff inclinado a 45º por 20 h (espermatozóides humanos) ou por 1 h(espermatozóides murinos) em estufa de cultura a 37ºC, com atmosfera de 5% de CO_2;

2.4. Aspirar 700µl do meio pela porção superior do eppendorff (não tocar no sedimento celular). Esta fração conterá os espermatozóides capacitados e que apresentam melhor motilidade (técnica de *swim up*). Estes espermatozóides podem ser utilizados para ensaios de fertilização *in vitro*;

2.5. Para indução da reação acrossômica de espermatozóides humanos, acrescentar ao sedimento celular de espermatozóides capacitados 100 µl de meio HTF contendo fluido folicular humano na concentração final de a 20% e incubar a amostra por 20 min em estufa de cultura estufa de cultura a 37ºC, com atmosfera de 5% de CO_2. Para espermatozóides murinos, acrescentar ao sedimento celular 100 ul de meio de cultura M16 suplementado com BSA contendo 10µM de cálcio ionóforo (C7522, antigo A23187, Sigma Aldrich) por 30 min em estufa de cultura. O ionóforo de cálcio deve ser previamente preparado em solução estoque de 10 mM em dimetilsulfóxido (DMSO, D2650, Sigma Aldrich);

2.6. Realizar a lavagem do espermatozóide como no item 1.6.

3. Imunocitoquímica indireta com o Amc TRA 54 em espermatozóides e concomitante identificação do status acrossomal.

3.1. Para este procedimento, podem ser utilizados espermatozóides maturados (obtidos como nos itens 1.1 a 1.6), capacitados (obtidos como nos itens 2.1 a 2.4) ou "reagidos" (obtidos como nos itens 2.1 a 2.6);

3.2. Ressuspender o sedimento de espermatozóides em 50 µl de tampão Tris 0,02M contendo 0,05% de tween (TBS-T). Depositar 10 µl da amostras sobre lâmina histológica silanizada, em área redonda delimitada (raio de 1 cm). Aguardar a secagem e pós-fixar com um banho rápido (30 seg) de metanol absoluto a - 20°C;

3.3. Isolar cada corte histológico ou área contendo espermatozóides com caneta hidrofóbica (Pappen);

3.4. Permeabilizar as membranas dos espermatozóides incubando as lâminas com solução de Triton 100-X diluído à concentração de 1% em TBS-T por 30 min;

3.5. Lavar as lâminas com 3 banhos de 5 min de TBS-T;

3.6. Incubar as lâminas por 30 min com soro normal de coelho diluído a 20% no tampão TBS-T contendo 1% de albumina bovina sérica (BSA, B4287, Sigma Aldrich, St Louis, MO) para bloqueio dos sítios inespecíficos;

3.8. Lavar as lâminas com TBS-T (3 x 5 minutos) e incubar com o líquido ascítico contendo o Amc TRA 54, diluído na proporção de 1:3000 em TBS-T/BSA 1%. Esta incubação deve ser realizada *overnight* a 4° C ou por no mínimo 3h à temperatura ambiente.e incubar por 30 min com o anticorpo secundário IgG anti-rato biotinilado, 1:300 em TBS-T/BSA 1% (Dako A/S, Glostrup, Denmark), seguido de lavagem com TBS-T (3 x 5 minutos) e incubação por 45 min com o Kit Strept ABC (Dako A/S, Glostrup, Denmark). Alternativamente, pode-se substituir o anticorpo secundário IgG anti-rato biotinilado pelo conjugado com peroxidase (Dako A/S, Glostrup, Denmark), suprimindo-se então a etapa de incubação com o Kit ABC;

3.9. Revelar a reação através de solução de diaminobenzidina (DAB) e peróxido de hidrogênio (Sigma Aldrich, St Louis, MO) nas seguintes proporções: 4,5 ml de TBS sem tween, 500 µl de solução de DAB e 30 µl de peróxido de hidrogênio preparado a 30% em água destilada;

3.10. Identificar o surgimento de pontos acastanhados por observação ao microscópio de luz. Após 40 a 60 seg, parar a reação imergindo as lâminas em água corrente por 5 min. Se oportuno, desidratar as lâminas em série crescente de etanol, diafanizar com dois banhos

de xilos (5 min cada) e montar as lâminas com lamínulas e Bálsamo do Canadá. Deixar secar por 24 h para análise por microscopia;

3.11. Para concomitante marcação do acrossomo do espermatozóide, incubar as lâminas com solução de lectina *Pisum sativum* (PSA) conjugada com FITC (FITC-PSA, L0770, Sigma Aldrich) (espermatozóides humanos) ou solução de lectina *Peanut agglutinin* (PNA) conjugada com FITC (FITC-PNA, L7381, Sigma Aldrich) (espermatozóides murinos), diluídas a 40μg/ml em PBS 1x (Gibco), por 20 min a temperatura ambiente e ao abrigo da luz;

3.12. Lavar as lâminas com PBS 1x por (5 x 5 minutos), escorrer o excesso de tampão e montar com lamínulas com 5 μl de meio de montagem Vectashield. Armazenar no escuro a 4ºC até o momento da análise.

3.13. A localização do antígeno em estudo nos espermatozóides é avaliada por microscopia de luz com auxílio de filtros de interferência, e a determinação da presença ou ausência de acrosomo é determinada pelo padrão de fluorescência emitida pela região do acrossomo.

❖ Obs. Controle negativo da reação de imunocitoquímica: submeter algumas lâminas ao mesmo procedimento acima exceto à etapa de incubação com o Amc TRA 54, que deve ser substituída por uma incubação com o tampão de lavagem.

4. Procedimento de fertilização *in vitro*

4.1. Para indução controlada da ovulação de camundongos C57Bl6, aplicar intra-peritonealmente 5 UI de gonadotrofina corônica eqüina (eCG, Novormon 5000, Syntex S.A., São Paulo, Brasil) ou PMSG (G4527, Sigma Aldrich), preparados conforme recomendação do fabricante, a fêmeas de 25 a 30 dias de idade (usualmente volume de 200 µl por aplicação);

4.2. Transcorridas 48 h, aplicar intra- peritonealmente 5 UI de gonadotropina coriônica humana (hCG, Vetecor 5000, Calier, Barcelona, Espanha). Imediatamente após a injeção do segundo hormônio, colocar cada fêmea junto a um macho previamente vasectomizado para estimulação copulatória (usualmente volume de 200 µl por aplicação);

4.3. Transcorridas 14 h da aplicação do hCG, sacrificar as fêmeas por deslocamento cervical para coleta das tubas uterinas. Após incisão ventral pélvica, identificar e remover as tubas uterinas, imergindo-as imediatamente em 100 µl de meio de cultura M2 (Sigma Aldrich) suplementado com 4 mg/ml de BSA, em placa de cultura (Corning, Poliestireno Estéril);

4.4. Dissecar as ampolas das tubas uterinas em meio de cultura M2 suplementado, com auxílio de instrumental cirúrgico delicado e limpo, sob lupa (recomenda-se aumento de 6x);

4.5. Sempre com auxílio do estereomicroscópio (lupa), transferir o agrupamento de oócitos coletados para um poço de placa plástica estéril de 4 poços (Nunc) preenchido com 400 µl de meio M2 suplementado. Separar os oócitos uns dos outros por consecutivas aspirações com pipeta Pasteur de vidro afilada acoplada a um sistema de aspiração bucal;

4.6. Remover as células do *cumulus oophuros* (células foliculares) incubando os oócitos em 400 µl de solução de hialuronidase testicular (H3506, Sigma Aldrich) preparada a 0,1% em meio de cultura M2 durante no máximo 30 segundos. Esta solução deve ser colocada no segundo poço da placa de 4 poços. Em seguida, submeter o oócito a diversas passagens em meio M2 suplementado, que deve preencher os dois demais poços da placa de 4 poços (em média 10 aspirações por poço);

4.7. Para ensaios em que se necessite remover a zona pelúcida, transferir os oócitos para o primeiro poço de nova placa de 4 poços e incubar os oócitos em 400 µl de solução ácida de Tyrode por 10 a 20 seg, sob rápidas aspirações consecutivas, seguindo-se a lavagem em meio M2 suplementado. Transferir o mínimo possível de solução ácida para o meio de lavagem, sob risco de rompimento da membrana do oócito. A incubação e lavagem devem ser realizadas como descrito no item 4.6;

4.8. Transferir os oócitos para uma placa de cultura estéril (Cornning, 30 mm de diâmetro) contendo 3 ml de meio M2 suplementado. Incubar por 3 h em estufa de cultura a 37ºC, com atmosfera de 5% de CO_2; para maturação (liberação do primeiro corpúsculo polar);

4.9. Para ensaios de FIV utilizando oócitos com zona pelúcida, coletar e capacitar os espermatozóides de camundongos C57Bl6 conforme descrito nos itens 2.1 a 2.4. Para ensaios de FIV utilizando oócitos sem zona pelúcida, preparar os espermatozóides conforme itens 2.1 a 2.6, substituindo-se, porém, a concentração do ionóforo de cálcio para 30 nM. Ressuspender os espermatozóides (capacitados ou reagidos) em 3 alíquotas diferentes (100 µl) contendo de 1 x 10^6 espermatozóides/ml em meio M16 suplementado (utilizar a câmera de Makler para ajuste da concentração);

4.10. Acrescentar em uma das alíquotas (controle II) soro normal de coelho na concentração final de 10% e à outra alíquota o líquido ascítico contendo o Amc TRA 54 em concentração final de 400 µg/ml (1:10) de proteínas. Uma das alíquotas não deve receber acréscimos (controle I). Incubar em condições de cultura por no mínimo 1h;

4.11. Após as 3 h de incubação dos oócitos em condições de cultura, os mesmos devem ser classificados em maduros ou imaturos, respectivamente, pela presença ou ausência de primeiro corpúsculo polar. Somente oócitos maduros devem ser selecionados para o experimento de fecundação *in vitro*;

4.12. Preparar três placas de cultura estéril (Cornning, 30 mm de diâmetro) com 3 ml de óleo mineral. Em cada placa, adicionar gotas (40 a 60 µl / gota) das alíquotas de espermatozóides (uma ou mais placas para cada tratamento de espermatozóides – controle I, controle II e TRA 54). Cada placa de FIV deve receber no máximo 3 gotas. Transferir os oócitos maduros para as gotas de espermatozóides, utilizando para esta transferência o mínimo de meio possível. Cada gota deve receber no máximo 3 oócitos.

4.13. Co-incubar os gametas por um período de 5 horas. Após este período, transferir os oócitos para novas gotas de meio de cultura M16 suplementado.

4.14. Analisar a taxa de fertilização após 24 h da co-incubação por observação em microscópio invertido (NIKON). Para determinação de fertilização, devem ser considerados os seguintes critérios: presença de dois corpúsculos polares, presença de dois pró-núcleos ou detecção de pré-embrião de dois blastômeros.

<u>Soluções específicas:</u>

- Ácido de Tyrode: NaCl 137mM, $CaCl_2$ 1,3mM, $MgCl_2$ 0,5mM, Na_2HPO_4 0,3,M, glicose 0,1mg/ml, água destilada q.s.p.. Acertar o pH para 2,5 com solução de HCl 0,1M.

5. Imunohistoquímica indireta com o Amc TRA 54 em tecidos

5.1. Sacrificar camundongos machos (4 a 8 semanas) por deslocamento cervical e efetuar incisão abdominal ventral. Identificar e remover os órgãos do sistema reprodutor. Dissecar e remover vasos sanguíneos excesso de tecido adiposo per-gonadal. Imergir os órgãos de interesse (testículos e epidídimos) imediatamente em solução fixadora de Bouin, por 12 horas;

5.2. Processar as amostras de acordo com a técnica histológica de inclusão em parafina. Após microtomia (30 μM), desparafinizar os cortes com xilol (banho de 5 min) e hidratá-los com série decrescente de etanol;

5.3. Delimitar e isolar cada corte histológico com auxílio de caneta hidrofóbica (Pappen);

5.4. Incubar as lâminas por 5 min com tampão Tris 0,02M contendo 0,05% de tween (TBS-T);

5.5. Incubar as lâminas por 30 min com soro normal de coelho diluído a 20% no tampão TBS-T contendo 1% de albumina bovina sérica (BSA, B4287, Sigma Aldrich, St Louis, MO) para bloqueio dos sítios inespecíficos;

5.6. Lavar os cortes com TBS-T (3 x 5 minutos) e incubar com o líquido ascítico contendo o Amc TRA 54, diluído na proporção de 1:3000 em TBS-T/BSA 1%. Esta incubação deve ser realizada *overnight* a 4^{o} C ou por no mínimo 3h à temperatura ambiente.

5.7. Lavar os cortes com TBS-T (3 x 5 minutos) e incubar por 30 min com o anticorpo secundário IgG anti-rato biotinilado, 1:300 em TBS-T/BSA 1% (Dako A/S, Glostrup, Denmark). Após lavagem em TBS-T (3 x 5 minutos), incubar 45 min com o Kit Strept ABC (Dako A/S, Glostrup, Denmark). Alternativamente, pode-se substituir o anticorpo secundário IgG anti-rato biotinilado pelo conjugado com peroxidase (Dako A/S, Glostrup, Denmark), suprimindo-se então a etapa de incubação com o Kit ABC;

5.7. Revelar a reação através de solução de diaminobenzidina (DAB) e peróxido de hidrogênio (Sigma Aldrich, St Louis, MO) nas seguintes proporções: 4,5 ml de TBS sem tween, 500 □l de solução de DAB e 30 μl de peróxido de hidrogênio preparado a 30% em água destilada;

5.8. Identificar o surgimento de marcação acastanhada por observação ao microscópio de luz;

5.9. Parar a reação (40-60 seg após o início da reação) imergindo as lâminas com os cortes em água corrente por 5 min;

5.10. Contra-corar os cortes com 100 μl de hematoxilina de Harris por 30 segundos;

5.11. Desidratar os cortes em série crescente de etanol, diafanizar com 2 banhos de xilos (5 min cada) e montar as lâminas com lamínulas e bálsamo do canadá;

- Obs. Controle negativo da reação: submeter alguns cortes ao mesmo procedimento acima exceto à etapa de incubação com o Amc TRA 54, que deve ser substituída por uma incubação com o tampão de lavagem.

Soluções específicas:

- Bouin: misturar 75 ml de solução aquosa de ácido pícrico saturado e 20 ml de formaldeído (37%). Adicionar 5 ml de ácido acético glacial no momento da utilização.

- Solução aquosa de ácido pícrico saturado: dissolver 3g de ácido pícrico em 100ml de água destilada.

- Solução de DAB: dissolver 40 mg de DAB em pó (Sigma) em 10 ml de tampão tris-HCl 0,05M (pH 7,4). Armazenar a -20^{o}C, protegido da luz.

6. Preparação de amostras para microscopia eletrônica de transmissão

6.1. Para coleta de amostras de órgãos, anestesiar intra-peritonealmente camundongos adultos com uma mistura de cloridarato de xilazina (0,13g/Kg), ketamina (0,26 g/Kg) e água na proporção de 3:3:1, respectivamente (0,2 ml por camundongo);

6.2. Submeter os animais anestesiados à perfusão intra-cardíaca através do ventrículo esquerdo, durante 15 minutos, com 30 a 40 ml de solução fixadora (4% de paraformaldeído e 0,2% de glutaraldeído em tampão cacodilato de sódio 0,1M, pH 7,4, contendo 0,1M de sacarose);

6.3. Após a perfusão, realizar incisão ventral pélvica e coletar fragmentos (5 mm^3) da região da cabeça do epidídimo. Imergir os fragmentos coletados na mesma solução fixadora a 4ºC por um período de 2 h;

6.4. Para análise de amostras de espermatozóides, coletar e preparar os espermatozóides conforme descrito nos itens 1.1 a 1.6, ou 2.1 a 2.4 ou 2.1 a 2.6. Ressuspender os espermatozóides com a solução fixadora de perfusão (item 6.2) e incubar por 1 h a 4ºC. Após este período, centrifugar a amostra por 5 mim a 450 x g para formação do *pellet*;

6.5. As etapas seguintes são as mesmas tanto para os fragmentos de órgãos como para o sedimento de espermatozóides. Descartar a solução fixadora e acrescentar às amostras 1 ml de tampão Tris (0,05M, pH 7,4). Descartar após 5 min. Cuidado para não desmanchar o *pellet* de espermatozóides;

6.6. Desidratar as amostras a 4ºC em série crescente de N-N-dimetilformamida (20, 30, 45, 60, 70, 80, 95 e 3 vezes de 100%, 10 min cada incubação. No caso de amostras de espermatozóides, submeter a amostra à centrifugação (5 mim a 450 x g) se o *pellet* for desfeito durante a desidratação;

6.7. Incubar as amostras com misturas de N-N-dimetilformamida e resina Lowcryl K4M, nas proporções 1:2 (2 horas) e 2:1 (*overnight*), sob rotação a -20ºC;

6.8. Incubar as amostras com resina Lowcryl K4M por 12 horas, sob rotação a -20ºC. Incluir os fragmentos dos órgãos em cápsulas plásticas de fundo cônico ou chato contendo a resina pura. No caso de sedimento de espermatozóides, acrescentar a resina pura no próprio eppendorff, deixando o *pellet* ao fundo;

6.9. Manter as cápsulas a -20ºC, por 48 horas, sob luz ultravioleta, para a completa polimerização da resina.

6.10. Levar os blocos de resina Lowcryl K4M à microtomia. Obter inicialmente cortes semifinos (1µm de espessura) com navalha de vidro. Os cortes devem ser aderidos em lâminas histológicas com auxílio de calor (exposição à chapa de metal aquecida), cobertos com

solução de azul de toluidina a 0,5% (em borato de sódio a 5%), aquecidos por 1 min, lavados com água destilada e secos por novo aquecimento na chapa aquecida;

6.11. Após visualização das áreas de interesse, lapidar o blodo de resina para selecionar a face de corte. Obtér os cortes ultrafinos (70 nm) com navalha de diamante. Coletar os cortes em telas de níquel de 200 *mesh* e deixar secar sobre papel filtro. Proteger do pó e outras impurezas.

Soluções específicas

- Tampão cacodilato de sódio 0,1M: dissolver 2,14 g de cacodilato de sódio em 80 ml de água destilada. Utilizar gotas de solução de HCl 0,1 M para abaixar o pH para 7,4. Completar com água destilada para o volume final de 100 ml.

- Resina LowCryl K4M:

- Acrescentar a um béquer 2,7g do reagente “cross-linker A”;
- Inserir agitador magnético e agitar em velocidade baixa;
- Acrescentar 14,6g do reagente “monômero B”;
- Acrescentar 0,1g do podo “inibidor C”.
- Imediatamente colocar um béquer com os reagentes em câmara de vácuo e efetuar a agitação mecânica à vácuo;
- Após 30-40 min, armazenar o preparado em seringas (evitar bolhas), embrulhar as extremidades com parafilm, cobrir as seringas com papel alumíneo e armazenar a -20ºC.

7. Imuno-eletro-marcação com o Amc TRA 54 para microscopia eletrônica

7.1. Para este procedimento, filtrar a solução tampão em filtro de 0,22 μM de poro (Millipore). Centrifugar as soluções de anticorpos por 2 min a 2000 rpm para deposição de partículas. Manter as soluções em banho de gelo.

7.2. Lavar as telas de níquel contendo os cortes ultrafinos em tampão fosfato salina (PBS) a 0,02M (pH 7,4), passando cada tela por 10 gotas seqüenciais de tampão (5 seg por gota; volume da gota: 50 μl);

7.2. Incubar as telas com solução de PBS/BSA 1% por 30 min (uma tela por gota), para bloqueio de sítios inespecíficos;

7.3. Incubar as telas com solução do Amc TRA 54 (diluído na concentração de 1:3000 em PBS/BSA 1%) por aproximadamente 16 horas, em câmara úmida, à temperatura de 4ºC;

7.4. Lavar as telas com PBS (passagem por 10 gotas em seqüência) e incubar as mesmas com o anticorpo secundário anti IgG de rato conjugada com partículas de ouro de 5 ou 15 nm, (DAKO), diluído na proporção de 1:100 em PBS/BSA 1%, por 30 minutos;

7.5. Lavar as telas abundantemente em água deionizada (jato contínuo direcionado às bordas da tela);

7.6. Para contrastação dos cortes, incubar as telas com solução de uranila preparada a 2% em tampão maleato de sódio 0,05M, pH 5,2 (40 seg). A solução deve ser centrifugada previamente ao uso (1min a 2000 rpm), para deposição de eventuais partículas. Após secagem das telas sobre papel de filtro em recipiente fechado (placa de petri, por exemplo), incubar as telas com solução de citrato de chumbo 0,2% (20 seg), na presença de pastilhas de hidróxido de sódio (NaOH), ao abrigo da luz;

7.7. Revestir os cortes contrastados com película de carbono para observação ao microsocópio eletrônico de transmissão (MET).

7.8. Todas as reações devem ser acompanhadas por uma ou mais telas controles, submetidas a todas as etapas do procedimento, porém suprimindo-se a aplicação do anticorpo primário TRA 54.

<u>Soluções específicas</u>;

❖ tampão fosfato salina (PBS) a 0,02M (pH 7,4):

- dissolver 7,16 g de fosfato dibásico hidratado em 100ml de água destilada;
- dissolver 6,9 g de fosfato monobásico em 250 ml de água destilada;

- com auxílio de aferição por pHmetro, acrescentar pequenas quantidades da solução monobásica na solução dibásica, até que o pH atinja 7,4.

❖ Maleato de sódio 0,5M pH 5,2

- dissolver 16g do sal em 90 ml de água deionizada. Após ajuste do pH, acertar o volume da solução para 100 ml.

❖ Solução de citrato de chumbo 0,2%

- dissolver 0,2 g do sal em 99,8 ml de tampãp PBS 0,01M.

8. SDS-PAGE e imunoblotting com o Amc TRA 54

8.1. Coletar os órgãos de interesse (item 5.1, exceto fixação com solução de Bouin) ou espermatozóides (item 1.1 a 1.6) e homogeneizar (homogeneizador mecânico de rotação) imediatamente em tampão de extração. A homogeneização deve ser conduzida em tubo de ensaio, em banho de gelo, com o menor volume possível para que a amostra esteja totalmente dispersa e homogênea;

8.2. Centrifugar as amostras (10 min, 3000 x g, 4^{o}C) e coletar somente o sobrenadante. Submeter à leitura de dosagem de proteínas pelo método padrão pré-estabelecido de Bradford (Bradford, 1976). Manter congelado a -80^{o}C;

8.3. Preparar o gel de poliacrilamida para eletroforese (7,5%) e esperar gelificar (cerca de 30 min). Em seguida, preparar o gel de amostra, aplicar sobre o gel anterior e encaixar o pente com o número de poços desejados;

8.4. Após gelificar (cerca de 20 min), retirar vagarosamente o pente do gel e inserir o sistema de gel na cuba de eletroforese (sistema Mini-Protean III, Bio Rad) preenchida com tampão de corrida;

8.5. Aplicar a amostra nos poços do gel de acrilamida na concentração de até 100 □g de proteína por poço, diluída em tampão de amostra. Aplicar no primeiro poço de cada gel uma alíquota do padrão de pesos moleculares, nos volumes recomendados nas instruções do fabricante. Como padrões de peso molecular recomenda-se os que variam entre 50 e 250 KDa (BioRad Precision Plus Protein Standard, Dual Color), 48 e 202 kDa (BioRad Prestained SDS-Page Standards, High Range) ou 8 a 213 kDa (BioRad Prestained SDS-Page Standards, Kaleidoscope). O volume da amostra a ser depositado em cada poço varia de acordo com o tamanho do mesmo (ver recomendação do fabricante);

8.6. Fechar o sistema de eletroforese com a tampa acrílica, acoplar o sistema à fonte de eletroforese (BioRad) e iniciar a corrida (corrente de amperagem constante a 30 mA, voltagem variável, em 120 minutos);

8.7. Ao final da eletroforese, desligar a fonte, retirar os sistemas de géis da cuba e imergi-los em tampão de transferência. Desmontar o sistema de géis e retirá-los dos suportes vagarosamente. Separar e desprezar os géis de aplicação de amostra;

8.8. Preparar os sistemas de transferência, sempre imersos em tampão. Colocar os seguintes componentes um sobre o outro, sobre a lateral do sistema de transferência: esponja, conjunto de papéis de filtro, gel de poliacrilamida contendo as amostras, membrana de *polyvinylidene difluoride* (PVDF) (Millipore, Bedford, MA, USA), conjunto de papéis de filtro e

esponja. Fechar o sistema e encaixar na cuba de eletroforese preenchida com tampão de transferência;

8.9. Adicionar compartimento com gelo ao sistema de eletroforese, fechar com a tampa acrílica, acoplar o sistema à fonte de eletroforese (BioRad) e iniciar a corrida (corrente de amperagem constante a 200 mA). Após os primeiros 60 minutos, desligar a fonte e efetuar a troca do gelo. Reiniciar a corrida nas mesmas condições por mais 60 minutos.

8.10. Terminada a corrida, desmontar todo o sistema e imergir a membrana de PVDF em tampão Tris 0,02M contendo 0,05% de tween (TBS-T). Imergir o gel em solução corante de Coomassie Blue e manter sob agitação por 30 min;

8.11. Manter a membrana sob agitação por 5 min e trocar a solução tampão. Repetir o procedimento de lavagem por mais duas vezes;

8.12. Incubar a membrana com leite desnatado (Molico, Nestlé) preparado a 10% em TBS-T (1h a temperatura ambiente), para bloqueio dos sítios inespecíficos;

8.13. Lavar a membrana com TBS-T (3 x 5 min) e incubar com solução do Amc TRA 54 (líquido ascítico) diluído na proporção de 1:3000 em TBS-T (*overnight* a 4^{o}C). Podem ser utilizados para a incubação envelopes de saco plástico, porém é preciso retirar eventuais bolhas formadas sobre a membrana. Em relação ao gel, substituir a solução corante de Coomassie Blue pela solução descorante, que deve ser trocada freqüentemente até a retirada do excesso de corantes e a identificação das bandas protéicas;

8.14. Lavar a membrana com TBS-T (3 x 5 min) e incubar com o anticorpo secundário anti-IgG de rato conjugado com peroxidase (Dako A/S, Glostrup, Denmark), diluído na proporção de 1:200 em TBS-T, à temperatura ambiente, por 1 hora;

8.15. Revelar a reação através de solução de diaminobenzidina (DAB) e peróxido de hidrogênio (Sigma Aldrich, St Louis, MO) nas seguintes proporções: 4,5 ml de TBS sem tween, 500 □l de solução de DAB e 30 □l de peróxido de hidrogênio preparado a 30% em água destilada.

8.16. Parar a reação (após a identificação das bandas antigênicas) imergindo a membrana em água corrente por 10 min;

8.17. Secar a membrana em estufa a 37^{o}C por 20 min.

Soluções Específicas

❖ Tampão de extração:

- Tris-HCl 10 mM, pH 7,4, contendo 10 mM de EDTA, 100mM de fluoreto de sódio, 10 mM de pirofosfato de sódio., 10 mM de ortovonadato de sódio, 2 mM de fenilmetilsulfonil fluoreto (PMSF) e 0,1 mg de aprotinina (Sigma Aldrich).

❖ Tampão Tris-HCl 10mM

- dissolver 30,25 g de tris-base em 900 ml de água deionizada;
- acertar o pH para 7,7 com solução de HCl 1 M;
- completar com água deionizada para volume final de 1000 ml

❖ Tampão de amostra:

- misturar 0,02g de azul de bromofenol, 0,15 g de Tris Base, 2 ml de glicerol, 0,4g de dodecil sulfato de sódio (SDS). Acrescentar água deionizada para volume final de 9 ml. Aliquotar e armazenar a 4º C. No momento do uso, acrescentar 10% de □-mercaptoetanol.

❖ Tampão de Corrida (5 x concentrado):

- misturar 15 g de Tris Base, 72 g de glicina, 2,5 g de SDS, e completar com água deionizada para volume final de 1 litro.
- para utilização, diluir 5 x com água deionizada.

❖ Tampão de Transferência:

- misturar 4,8 g de Tris Base, 23,6 g de glicina, e completar com água deionizada para volume final de 1,6 L.
- para utilização, acrescentar 20% de metanol absoluto.

❖ Gel de poliacrilamida a 7,5%:

- 4,82 ml de água deionizada;
- 2,49 ml de solução de acrilamida 30% em água;
- 2,55 ml de Tris-HCl pH 8,8 1,5M;
- 90 µl de solução de SDS a 10% em água deionizada;
- 200 µl de solução de persulfato de amônia (APS, Sigma) a 10% em álcool etílico (manter o estoque a - 20ºC);

- 15 µl de TEMED (Sigma)

(os dois últimos reagentes devem ser adicionados juntos. Imediatamente após a adição dos mesmos, transferir a solução com seringa para o sistema de géis, para solidificação).

❖ Gel de amostra:

- 3,6 ml de água deionizada;
- 0,9 ml de solução de acrilamida 30% em água;
- 1,5 ml de Tris-HCl pH 6,8 0,5M;
- 50 µl de solução de SDS a 10% em água deionizada;
- 200 µl de solução de persulfato de amônia (APS, Sigma) a 10% em álcool etílico (manter o estoque a – 20ºC);
- 15 µl de TEMED (Sigma)

(os dois últimos reagentes devem ser adicionados juntos. Imediatamente após a adição dos mesmos, transferir a solução com seringa para o sistema de géis, para solidificação).

❖ Tris-HCl 0,5M pH 6,8:

- adicionar 3g de tris-base a 40 ml de água deionizada. Utilizar gotas de solução de HCl 0,2 M para acertar o pH (6,8). Completar com água para o volume final de 50 ml.

❖ Tris-HCl 1,5M pH 8,8:

- adicionar 18,5 g de tris-base a 70 ml de água deionizada. Utilizar gotas de solução de HCl 0,2 M para acertar o pH (8,8). Completar com água para o volume final de 100 ml.

❖ Solução corante para gel de poliacrilamida (Coomassie Blue):

- dissolver 1g de Brilliant Blue R (Sigma) em 200 ml de ácido acético glacial;
- adicionar 200 ml de álcool isopropílico;
- adicionar 1300 ml de água deionizada.

❖ Solução descorante para gel de poliacrilamida:

- misturar 100 ml de ácido acético glacial em 900 ml de água deionizada.

9. Coloração de gel de poliacrilamida com nitrato de prata (detecção de quantidades pequenas de proteína)

9.1. O gel de poliacrilamida a ser corado deve ser obtido como descrito previamente nos itens 8.1 a 8.9;

9.2. Após desmontado o sistema de géis de eletroforese (item 8.10), lavar o gel por 5 min em 200 ml de água deionizada (manter sob agitação);

9.3. Manter o gel em agitação mergulhado em solução fixadora por no mínimo 2h (ou *overnight*);

9.4. Desidratar o gel: efetuar três banhos de 20 min, sob agitação, em solução de etanol 50% (200 ml / banho);

9.5. Reidratar o gel com banho de 1 min de solução de tiossulfato de sódio 0,2% (200 ml). Reservar 5 ml do total desta solução;

9.6. Efetuar três banhos de 20 seg cada com água deionizada;

9.7. Corar o gel por 20 min em solução de nitrato de prata 2%, sob agitação e ao abrigo da luz;

9.8. Efetuar três banhos de 20 seg cada com água deionizada;

9.9. Incubar o gel com 200 ml de solução reveladora, à meia luz. Observar atentamente o surgimento e escurecimento das bandas;

9.10. Imediatamente após a observação das bandas, desprezar a solução de nitrato de prata em béquer e parar a reação incubando o gel com 200 ml da solução “stop”, por 10 min;

9.11. Conservar o gel em solução de glicerol 8,7%.

<u>Soluções Específicas:</u>

❖ Solução fixadora:

- 200 ml de metanol absoluto;
- 48 ml de ácido acético;
- 100 µl de solução de formaldeído previamente diluída em água na proporção de 150 □l para 100 ml de água deionizada;

❖ Solução de tiossulfato de sódio 0,2% (Reservar 20 ml para a solução reveladora)

- dissolver 60 mg de tiossulfato de sódio em 300 ml de água deionizada.

❖ Solução de nitrato de prata 2%

- dissolver 600 mg de nitrato de prata em 200 ml de água deionizada;
- adicionar 450 µl de formaldeído;
- completar com água para volume final de 300 ml. Manter a solução ao abrigo da luz.

❖ Solução reveladora

- dissolver 24 g de carbonato de sódio (Na_2CO_3) em 200 ml de água deionizada;
- adicionar 200 µl de formaldeído;
- adicionar 16 ml da solução de tiossulfato de sódio 0,2%;
- completar a solução com água deionizada para o volume final de 400 ml.

❖ Solução “stop”

- misturar 15 ml de ácido acético glacial em 285 ml de água deionizada.

10. Protocolo para investigação da integração de proteínas à membrana do espermatozóide através de SDS-PAGE e imunoblotting com o Amc TRA 54

10.1. Coletar espermatozóides conforme previamente descrito nos itens 1.1 a 1.6. Podem ser utilizadas também amostras de espermatozóides capacitados ou "reagidos" (itens 2.3 e 2.5, respectivamente);

10.2. Acrescentar ao eppendorff contendo o sedimento final de espermatozóides 600 µl de PBS 1x (Gibco) e centrifugar por 5 min a 450 X g;

10.3. Recolher o sobrenadante em outro eppendorff, identificando-o como "passo de lavagem 01". Armazenar a - 20ºC até o momento da utilização;

10.4. Repetir os passos descritos nos itens 10.2 e 10.3 por mais seis vezes, para obtenção das amostras de lavagem dos passos 2 a 7;

10.5. Para acessar a extratibilidade do antígeno da membrana do espermatozóide, ressuspender o *pellet* do passo final de lavagem em tampão de extração e sonicar a amostra por 8 ciclos de 15 seg em intensidade alta;

10.6. Centrifugar a amostra por 5 min a 450 x g;

10.7. Separar o sobrenadante e recentrifugá-lo por 10 min a 10.000 x g, a 4^{o}C. Armazenar a - 20ºC até o momento da utilização;

10.8. Submeter todas as amostras obtidas à dosagem de proteínas e realizar SDS-PAGE e immunoblotting conforme itens 8.3 a 8.17.

<u>Soluções Específicas:</u>

❖ Tampão de Extração

- tampão Tris-HCl 10mM, pH 7,4 contendo 0,5% de Igepal (Sigma); 10mM de Hepes (Sigma); 0,15M de NaCl e 0,2mM de aprotinina (Sigma)

❖ Tampão Tris-HCl 10mM pH 7,4

- dissolver 1,21 g de tris-base em 900 ml de água deionizada;
- acertar o pH para 7,4 com solução de HCl 1 M;
- completar com água deionizada para volume final de 1000 ml

.

11. Protocolo de preparação de amostra para isolamento do antígeno epididimário por cromatografia

11.1. Coletar a região da cabeça do epidídimo de 8 10 camundongos C57Bl6 adultos, conforme descrito no item 5.1 (exceto etapa de fixação com solução de Bouin);

11.2. Centrifugar as amostras (10 min, 3000 x g, 4^{o}C) e coletar somente o sobrenadante;

11.3. Centrifugar o sobrenadante obtido por 20 min a 25000 x g, 4^{o}C. Coletar o sobrenadante;

11.4. Centrifugar o sobrenadante obtido por 25 min a 30000 x g, 4^{o}C. Coletar o sobrenadante;

11.5. Submeter o sobrenadante obtido à leitura de dosagem de proteínas pelo método padrão pré-estabelecido de Bradford (Bradford, 1976). Manter congelado a -80^{o}C até o momento da utilização;

12. Parâmetros para realização de cromatografia de afinidade

12.1. Aplicar de 5 a 10 mg de proteínas totais do extrato epididimário obtido conforme protocolo 11.1 a 11.5;

12.2. Utilizar coluna de Concavalina A (17-0404-03, Com-A Sepharose 4B, Amersham, Buckinghamshire, United Kington), equilibrada com tampão o acetato de sódio 0,1M pH 6,0;

12.2. Monitorar a cromatografia por leitura das frações utilizando absorbância de 280 nm;

12.3. Realizar eluição das frações com tampão acetato de sódio 0,1M pH 6,0 contendo 0,5M de glicose, a fluxo constante (1 ml / min); manter as frações em banho de gelo durante a coleta e congeladas a -80^{o}C até o momento da utilização;

12.4. Analisar a presença do antígeno em todas as frações coletadas por metodologia de *dot blotting* (protocolo 13).

Soluções Específicas:

- Tampão acetato de sódio 0,1M pH 6,0

- adicionar 0,1M (8,203 g/L)de acetato de sódio, 1M (58,5 g/L) de NaCl, 1M (95,21 g/L) de $MgCl_2$, 1M (111,17 g/L) de $CaCl_2$ para volume final de 1 L de solução em água deionizada.

- Tampão acetato de sódio 0,1M pH 6,0 com 0,5 M de glicose

- adicionar 0,1M (8,203 g/L)de acetato de sódio, 1M (58,5 g/L) de NaCl, 1M (95,21 g/L) de $MgCl_2$, 1M (111,17 g/L) de $CaCl_2$ e 0,5 M (180 g/L), para volume final de 1 L de solução em água deionizada.

13. Dot Blotting (para avaliação da presença do antígeno reconhecido pelo Amc TRA 54 nem frações obtidas após cromatografia)

13.1. Descongelar as amostras obtidas por cromatografia;

13.2. Recortar um retângulo de membrana de nitrocelulose (162-0115, Trans Blot Transfer Medium, Pure nitrocellulose Membrane, Bio Rad);

13.3. Colocar a membrana com a face da frente voltada por sobre uma placa acrílica de cultura de células de 96 poços (TPP). Deslizar firmemente um bastão de vidro por sobre o verso da membrana de nitocelulose, para demarcar áreas circulares na face da frente da membrana (áreas circulares correspondem às bordas dos poços da placa de cultura celular);

13.4. Ativar a membrana por banho de 20 seg, sob agitação, em 50 ml de tampão carbonato / bicarbonato de sódio 0,1M pH 9,5;

13.5. Deposita a membrana sobre papel de filtro e aguardar completa secagem (aproximadamente 6 a 8 min);

13.6. Aplicar 3µl de cada fração protéica obtida nas cromatografias individualmente em cada uma das áreas circulares demarcadas na membrana de nitrocelulose;

13.7. Congelar as frações imediatamente após o uso;

13.8. Aguardar novamente a secagem da membrana (10 min após a adição do último volume de fração);

13.9. Proceder imunoquímica com o Amc TRA 54 conforme previamente descrito nos itens 8.12 a 8.17.

<u>Soluções Específicas:</u>

❖ Tampão carbonato / bicarbonato de sódio 0,1M pH 9,5

- preparar solução 0,1M de carbonato de sódio (Na_2CO_3) diluindo 10,58 g de sal para 100 ml de água deionizada;
- preparar solução 0,1M de bicarbonato de sódio ($NaHCO_3$) diluindo 8,4 g de sal para 100 ml de água deionizada;
- com auxílio do pHmetro, ajustar o pH a 9,5 da solução de carbonato de sódio com a solução de bicarbonato de sódio (utilizar o volume necessário para atingir o pH).

14. Parâmetros para realização de cromatografia de troca iônica aniônica

14.1. Aplicar frações com concentração total de proteínas de no mínimo 1mg/ml;

14.2. Utilizar coluna HiTrap Q 1mL, Sepharose ™ HP (17 1153 01, Amersham, Buckinghamshire, United Kington) previamente equilibrada com tampão Tris-HCl 50mM pH 7,4. Resina empacotada com amina quaternária carregada positivamente (grupos – $N^+(CH_3)_3$).

14.3. Realizar eluição das frações através da aplicação de gradiente linear de tampão Tris-HCl 50mM com 1M de NaCl, em fluxo constante de 1ml / min (*salting-in*). Manter as frações em banho de gelo durante a coleta e congeladas a -80°C até o momento da utilização;

14.4. Analisar a presença do antígeno em todas as frações coletadas por metodologia de *dot blotting* (protocolo 13).

Soluções Específicas:

❖ Tampão Tris-HCl 50mM pH 7,4:

- dissolver 6,55 g de tris-base em 900 ml de água deionizada;
- acertar o pH para 7,4 com solução de HCl 1 M;
- completar com água deionizada para volume final de 1000 ml

❖ Tampão Tris-HCl 50mM pH 7,4 com 1 M de NaCl

- dissolver 12,1 g de tris-base em 900 ml de água deionizada;
- acertar o pH para 7,4 com solução de HCl 1 M;
- completar com água deionizada para volume final de 1000 ml
- em outro recipiente, adicionar 117 g e NaCl para volume final de 1000 ml de água deionizada;
- misturar 500 ml da solução de Tris-base e 500 ml da solução de NaCl.

15. Excisão de banda protéica do gel de poliacrilamida e digestão tríptica para geração de peptídeos

15.1. Identificar a banda protéica de interesse em gel corado com Coomassie Blue (protocolo 8);

15.2. Utilizando bisturi cirúrgico estéril, cortar a região de interesse do gel em fragmentos de 2mm^3, sobre placa de vidro estéril. Armazenar os fragmentos em eppendorff a -80 ^oC até o momento da utilização;

15.3. Para a redução das pontes dissulfeto dos resíduos de cisteína da proteína, tratar os fragmentos de gel com 50µl de solução 100 mM de bicarbonato de amônio e 45mM de ditiotreitol (DTT, 43817, Sigma-Fluka, St Louis, MO) a 60^oC por 30 min.;

15.4. Para alquilação dos resíduos de cisteína, tratar os fragmentos de gel com 50µl de solução 100mM de bicarbonato de amônio e 100mM de solução de iodoacetoamida (A3221, Sigma-Aldrich, St Louis, MO), no escuro, por 30 min.;

15.5. Para digestão das proteínas, encobrir os fragmentos com 100µl de acetonitrila por 10 min e levar o eppendorff para secar em concentrador à vácuo (*speed vacuum*, Biotron, Modul 3180C, Puchon, Korea) pelo tempo necessário.

15.6. Após desidratação total dos fragmentos, incubar a amostra com 20 ul de solução de bicarbonato de amônio 45mM e 12,5 ng/µl de tripsina bovina (*overnight*, 37^oC), sob agitação vibratória constante;

15.7. Extrair dos fragmentos de gel os peptídeos resultantes da digestão ressuspendendo os fragmentos em 50□l de solução de acetonitrila 50% com 0,1% de ácido trifluoroacético (TFA), por 20 min. Reservar o sobrenadante. Ressuspender os fragmentos com 50 □l de acetonitrila absoluta por mais 10 min. Adicionar ao sobrenadante armazenado;

15.8. Levar o eppendorff com a solução de peptídeos para secagem em concentrador à vácuo;

15.9. Após secagem completa, ressuspender em 10 µl de solução 1% de ácido trifluoroacético;

15.10. Dessalinizar a amostra por passagens consecuitvas em mini-colunas Zip-Tip com resina fixa C18 (ZTC18M96, Millipore, Zip Tip C18 Pipette Tips, Bedford, MA, USA) previamente equilibradas, de acordo com as recomendações do fabricante;

15.11. A amostra deve ser imediatamente utilizada para análise por espectrometria de massas.

Soluções Específicas:

- ❖ Bicarbonato de amônia 100 mM e ditiotreitol (DTT) 45mM;

- dissolver 1,58 g de bicarbonato de amônia em 100 ml de água deionizada;
- em outro recipiente, dissolver 1,38 g de DTT em 100 ml de água deionizada;
- misturar as duas soluções (v/v).

- ❖ Bicarbonato de amônio 100 mM e solução de iodoacetoamida 100mM;

- dissolver 1,58 g de bicarbonato de amônia em 100 ml de água deionizada;
- em outro recipiente, dissolver 3,68 g de iodoacetoamida (C_2H_4INO) em 100 ml de água deionizada;
- misturar as duas soluções (v/v).

- ❖ Bicarbonato de amônio 45 mM e tripsina bovina 12,5 ng/µl

- dissolver 0,35 g de bicarbonato de amônia em 100 ml de água deionizada;
- dissolver 0,008 de tripsina bovina em 40 ml da solução de bicarbonato supra-citada.

- ❖ Acetonitrila 50% com 0,1% de ácido trifluoroacético (TFA),

-dissolver 200 ul de TFA em 99,8 ml de água deionizada;
- misturar a solução acima em igual volume de acetronitrila pura.

16. Parâmetros para realização de espectrometria de massas e sequenciamento

16.1. A amostra, previamente preparada como descrito no item 15, pode ser analisada:

- por espectrometria por "Dessorção de Matriz Assistida por Laser com Analisador por Tempo de Vôo" (matrix-assisted laser desorption ionization mass spectrometer fitted with a time-of-flight analyzer - MALDI-TOF-MS), utilizando o aparelho Micromass Maldi (Waters, Milford, MA, EUA), operado no modo "positive ion";
- por um sistema de cromatrofrafia líquida bidimensional em capilar (CapLC system, Waters, Manchester, UK) acoplada a espectrômetro de massa com quadrupolo ortogonal, analisador por tempo de vôo e hexapolo de colisão / dissociação (Q-TOF hybrid mass spectrometer with a collision induced dissociation hexapole), utilizando o aparelho QTOF (Waters-Micromass, Manchester, UK), operado no modo "positive ion".
- ambos equipamentos foram utilizados no presente trabalho, no Laboratório Thomson de Espectrometria de Massas do Instituto de Química da Unicamp.

16.2. A calibração deve ser conduzida com peptídeos conhecidos. A tolerância de erro deve ser de 300 ppm;

16.3. Avaliar os dados para identificação dos peptídeos com o software Mascot Wizard (Matrix Science, London, UK, http://www.matrixscience.co.uk), utilizando o algorítimo MaxEnt3 para decodificar os dados.

16.4. Alinhar os peptídeos com seqüências de bancos de dados pelo Mascot utilizando o algorítimo MOWSE scoring.

16.5. Utilizar a ferramenta BLAST (Basic Local Alignment Search Tool) para comparar a sequência de peptídeos obtida com as sequenciaas derivadas de RNAm de outras moléculas (http://www.ncbi.nlm.nih.gov).