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Co-Purification of Mac-2 Binding Protein with Galectin-3 and Association with Prostasomes in Human Semen

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Abstract

Background—Prostasomes are exosome-like vesicles that are secreted by the prostate and incorporated into semen during ejaculation. Human prostasomes are proposed to function in regulation of sperm function, immunosuppression, and prostate cancer progression. Previously, we identified galectin-3 on the surface of prostasomes. Galectin-3 is a β-galactoside binding protein involved in immunomodulation, cell interactions, and cancer progression, including prostate cancer. Functional characterization of galectin-3 in a given biological environment includes identification of its target glycoprotein ligands.

Methods—Candidate galectin-3 ligands in prostasomes were identified by tandem mass spectrometry of proteins that co-purified with galectin-3 during lactose affinity chromatography. Immunochemical and biochemical methods were used to investigate the association of Mac-2 binding protein (M2BP) with prostasomes.

Results—Proteins identified by tandem mass spectrometry included M2BP, CD26/dipeptidyl peptidase IV, prolactin-inducible protein (PIP), olfactomedin-4 (OLF4), and seminogelins I and II. M2BP is a known galectin-3 ligand that was not previously described in prostasomes. M2BP protein bands were detected in the testis, epididymis, vas deferens, prostate, seminal vesicle, and sperm extracts. In seminal plasma, M2BP was identified in the soluble fraction and in purified prostasomes. Surface biotinylation and immunofluorescence studies indicated that M2BP is present on the prostasome surface and on sperm, respectively.

Conclusions—M2BP, CD26, PIP, OLF4, and seminogelins I and II are candidate glycoprotein ligands for galectin-3 in prostasomes. Given their overlap in functional significance with prostasomes and galectin-3, the identification of these glycoproteins as galectin-3 ligands in prostasomes lays the groundwork for future studies of prostasomes in reproduction and prostate cancer.

Keywords

galectin-3 binding protein; 90K tumor-associated antigen; seminal plasma; lectin

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Introduction

Prostasomes are exosome-like vesicles (40-500 nm) that are secreted by the prostatic epithelium into seminal plasma during ejaculation [1]. Exosomes are membranous vesicles that are secreted by an exocytotic pathway by a variety of cell types and tissues including dendritic cells, lymphocytes, the intestinal epithelium, and the parotid gland [2]. Exosome-like vesicles, termed epididymosomes, are secreted by the epididymis and contribute to sperm maturation [3]. The majority of characterized exosomes, including prostasomes, exhibit immunomodulatory properties and are proposed to mediate intercellular communication [2]. Two main functional roles for prostasomes have been proposed: enhancement of sperm function and immunosuppression in the female reproductive tract [1]. Prostasomes fuse with sperm in vitro, increase sperm motility by delivery of intra-prostasomal calcium stores, and prevent the premature maturation of sperm [4]. Furthermore, prostasomes are proposed to protect sperm from the female immune system by inhibiting the oxidative burst in neutrophils, preventing phagocytosis in leukocytes, inhibiting lymphocyte proliferation, and acting as a reservoir for the complement inhibitor CD59 [1]. Significantly, prostate cancer cell lines continue to secrete prostasomes, and prostasomes have been proposed to play a role in angiogenesis, tumor invasion, and immunosuppression during prostate cancer.

Galectin-3 and a truncated galectin-3 were previously identified on the surface of human prostasomes [5]. Galectin-3 is a member of the galectin family of β-galactoside-binding proteins, and its extracellular functions include immunomodulation, cell-cell and cell-matrix adhesion, and pathogen-host interactions [6-8]. The regulation of immunomodulation, apoptosis, angiogenesis, and metastatic cell adhesion and invasion by galectin-3 is implicated in multiple malignancies, including prostate cancer [6]. The multivalent galectin-3 molecule typically exerts its functions via protein-carbohydrate interactions with target glycoconjugate ligands. These interactions crosslink or alternately prevent the aggregation of galectin-3 binding ligands to induce downstream effects [9,10]. Previously identified galectin-3 binding ligands include functionally diverse glycoproteins such as CD66, matrix metalloprotease-9, cytokeratin, fibronectin, and Mac-2 binding protein (M2BP). Thus, the functional characterization of galectin-3 in a given cell type, body fluid, or exosome, such as prostasomes, includes the identification of its target glycoconjugate ligands.

In the current report, we describe the characterization of candidate galectin-3 binding ligands that co-purified with galectin-3 from prostasomes. The identified glycoproteins included M2BP, a known galectin-3 ligand involved in cell adhesion and tumor progression. Biochemical analyses were performed to examine the association of M2BP with prostasomes. Potential contributions of the candidate galectin-3 binding ligands to prostasome function are discussed.

Materials and Methods

Antibodies and Protein Extracts

Rabbit and goat polyclonal anti-M2BP antibodies were purchased from R&D Systems (Minneapolis, MN) and Atlas Antibodies (Stockholm, Sweden), respectively. R&D anti-M2BP antibodies were generated against full-length, glycosylated M2BP protein expressed in and purified from a mammalian tissue culture cell line. The Atlas anti-M2BP polyclonal antibodies were generated against a non-glycosylated protein epitope sequence tag (PrEST) containing amino acids 114-232 of the M2BP sequence. Both polyclonal antibodies were affinity-purified against immunogen by the manufacturer. Mouse monoclonal antibodies against human CD26 were from Lab Vision (Fremont, CA). Horse radish peroxidase (HRP)-

conjugated goat anti-rabbit, donkey anti-goat and goat anti-mouse secondary antibodies, goat anti-mouse FITC-conjugated secondary antibodies, and HRP-conjugated streptavidin were from Jackson ImmunoResearch Laboratories (West Grove, PA). Protein extracts of human testis, epididymis, vas deferens, seminal vesicle, and prostate were purchased from Biochain Institute, Inc. (Hayward, CA) and Clontech (Mountain View, CA).

Preparation of Clarified Seminal Plasma, the Seminal Plasma Membrane Fraction and Sperm Protein Extract

Semen samples from healthy human males were obtained following protocols approved by the University of Arkansas for Medical Sciences (UAMS) Institutional Review Board. Semen samples were centrifuged at 1000 × *g* for 20 minutes. Seminal plasma was decanted and clarified at 10,000 × *g*, 4 °C for 30 minutes. To prepare the seminal plasma membrane fraction, clarified seminal plasma was centrifuged at 100,000 × *g*, 4 °C for two hours to prepare a soluble fraction (supernatant) and a membrane-enriched fraction (pellet). In some experiments, the membrane-enriched pellet was treated with 0.5% Triton X-100 at 4 °C for 15 minutes in a volume equal to the original seminal plasma sample and centrifuged at 100,000 × *g*, 4 °C for two hours. The resulting supernatant and pellet contained the detergent-soluble fraction and the detergent-resistant fraction, respectively. The detergent resistant pellet was resuspended in a volume equal to the original seminal plasma sample.

Pelleted sperm were washed twice in Ham's F-10 medium (Sigma Chemical Company, Saint Louis, Missouri), extracted in 1% sodium dodecyl sulfate (SDS) containing 1× Complete® protease inhibitor cocktail (Roche Diagnostics, Indianapolis, Indiana) for 30 minutes at 4 °C and centrifuged at 10,000 × *g*, 4 °C for 30 minutes to remove insoluble material. Protein concentrations were determined using a bicinchoninic acid (BCA) assay (Pierce Chemical Company, Rockford, Illinois).

Electrophoresis and Immunoblot Analysis

Protein samples were separated by one dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [11]. Immunoblot analysis and electroblot analysis of biotinylated protein were performed as described previously [5]. Dilution factors for immunoblots were 1:1000 for anti-CD26 and anti-M2BP antibodies. Secondary antibodies alone were used as a negative control for all experiments. Blots were developed by enhanced chemiluminescence (GE Healthcare) on X-ray film.

β-Galactoside Binding Protein Purification and Sequence Analysis

The membrane fraction containing prostasomes was solubilized in column buffer (10 mM Tris, 130 mM NaCl, 4 mM BME, 1% octyl-β-glucoside [Pierce Chemical Company, Rockford, Illinois], 0.1% SDS, 20 mM methyl-β-cyclodextran [Sigma Chemical Company, Saint Louis, Missouri], 1× protease inhibitors) at the original volume of the seminal plasma sample and subjected to lactose-affinity column chromatography as previously described [5]. Eluted fractions containing putative β-galactoside-binding proteins were pooled, separated by SDS-PAGE and stained with Coomassie blue. Spots were excised from Coomassie blue stained protein bands into a 96-well plate with the ProPic imaging and spot-picking robot from Genomic Solutions, and trypsin digestion was performed with the ProGest in-gel enzymatic digestion robot (Genomic Solutions) using sequencing grade modified trypsin (Promega, Madison, WI). The resulting peptide mixture was loaded using an autosampler onto a trapping column (Symmetry300 C18 5μm NanoEase, Waters Corp., Beverly, MA) using a CapLC XE (Waters) system, a switching valve, and a flow rate of 20 μl/min. Peptides were separated by nanoflow capillary HPLC using a CapLC XE pump (Waters) operating at 12 μL/min; flow rate was controlled with a splitter in front of the switching valve. Peptides were eluted at 300 nL/min onto a self-packed PicoFrit (New

Objective) 75 μm × 10 cm column (Jupiter 4μ Proteo 90A, Phenomenex, Torrance, CA). The eluant was analyzed in-line by ESI-MS/MS using a Micromass Q-Tof Micro (Waters) tandem mass spectrometer operating in the positive ion mode. Data acquisition was performed in a data dependent fashion. Data was processed using ProteinLynx 2.0 (Waters), and the resulting peak list was used to search Mascot protein database (www.matrixscience.com) and assigned a Mascot score. Mascot scores for proteins are a summation of the scores for the individual peptides. Higher scores translate to greater probability for a sequence being correct. A protein interaction map of the identified proteins was built using the Cytoscape software program (www.cytoscape.org) which extracts protein-protein interaction data from the Human Protein Reference Database (HPRD), Biological General Repository for Interaction Datasets (BioGRID), IntAct database, and NCI/Nature Pathway Interaction Database.

Prostasome Isolation and Surface Biotinylation

Prostasomes were isolated from the seminal plasma membrane fraction by size exclusion chromatography on Sephacryl S300 [12]. Prostasomes were collected in the column void volume. In some experiments, proteins on the prostasome surface were labeled with membrane-impermeable sulfo-NHS-LC-biotin (Pierce Chemical Company) [13] and excess biotin was removed by dialysis against PBS. Biotinylated proteins were isolated from prostasomes by avidin affinity column chromatography (Pierce).

Immunofluorescence

Human sperm were isolated from semen by the swim-up method (19), fixed in 4% paraformaldehyde/PBS, and air-dried onto slides. Slides were blocked in 5% normal goat serum (NGS)/PBS and incubated with the R&D anti-M2BP polyclonal antibodies at 1:50 in 1% NGS/PBS overnight at 4 °C. Slides were washed in 1% NGS/PBS, incubated for two hours with goat anti-mouse FITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories), washed in PBS, and mounted with SlowFade (Molecular Probes, Eugene, OR). Secondary antibodies alone were included as negative controls. Results were visualized with a Zeiss Axio Skop2 microscope (Carl Zeiss, Inc., Thornwood, N.Y.) equipped for epifluorescence and phase contrast microscopy.

Results

Purification of β-galactoside-binding proteins and sequence analysis

β-galactoside-binding proteins isolated from the prostasome-enriched, seminal plasma membrane fraction were separated by SDS-PAGE and stained with Coomassie blue (Fig. 1). Four Coomassie stained bands designated Bands 1 - 4 with apparent molecular weights of ~100, 74, 30, and 16 kDa, respectively, were excised and subjected to sequence analysis by tandem mass spectrometry. Protein matches with significant Mascot scores greater than 200 are listed in Figure 1 and Table I. Bands 3 and 4 contained peptides that matched with the reported amino acid sequence of human galectin-3. Band 1 contained peptide matches with lactoferrin and CD26 (dipeptidyl peptidase IV). Band 2 contained peptide matches with lactoferrin, olfactomedin-4 (OLFM4), and M2BP. In addition to galectin-3, Band 4 contained peptide matches with seminogelins I and II, and prolactin inducible protein (PIP).

Eight unique peptides matched with M2BP with a significant Mascot score of 466 and represented 14% of the 567 amino acid sequence of the mature M2BP protein (Table I), which contain four major protein domains. Peptide matches were restricted to a 423 amino acid M2BP polypeptide fragment that contains the second, third, and fourth domains of M2BP (M2BP-2,3,4; Fig. 2). M2BP-2,3,4 is produced by removal of a 144 amino acid C-terminal region (M2BP-1) by plasmin cleavage [14]. The identified peptides represented

18.7% of the 423 amino acid M2BP-2,3,4 polypeptide fragment, which is reported to have an apparent molecular weight of ~67-75 kDa.

Protein-Protein Interactions

A protein-protein interaction network for the identified proteins was drawn using Cytoscape based on interaction data extracted from protein-protein interaction databases (Fig. 3). Direct protein interactions were identified between galectin-3 and M2BP and between seminogelins I and II. Galectin-3 and M2BP were both found to interact with galectin-1. Lactoferrin, PIP, CD26, and OLM4 were not found to interact directly with any of the other identified proteins. However, M2BP and lactoferrin; lactoferrin and PIP; and PIP and CD26 interact in common with CD14, glucocorticoid-induced protein kinase, and CD4, respectively.

M2BP immunoreactivity in male reproductive tract samples

To examine M2BP in the human male reproductive tract, tissue and sperm protein extracts and clarified human seminal plasma were separated by SDS-PAGE under reducing conditions and evaluated by immunoblot analysis (Fig. 4A). In the tissue extracts, immunoreactive bands of ~100 and ~74 kDa were identified with the R&D and Atlas anti-M2BP antibodies in the testis, epididymis, vas deferens, and prostate. In the prostate, the Atlas antibodies identified additional bands at ~50 and ~37 kDa. The R&D anti-M2BP antibodies detected an additional ~24 kDa band in the epididymis and prostate. In the seminal vesicle, the R&D antibodies identified immunoreactive bands of ~100 and ~90 kDa, with additional ~74, ~67 and ~50 kDa bands detected by the Atlas antibodies. In seminal plasma and sperm, the Atlas antibodies detected an ~74 kDa immunoreactive band, and the R&D anti-M2BP antibodies also detected an ~24 kDa band.

M2BP association with the soluble and membrane fractions of seminal plasma was investigated by differential ultracentrifugation. Following SDS-PAGE and immunoblot analysis, the Atlas antibodies detected an ~74 kDa M2BP immunoreactive band and the R&D anti-M2BP antibodies detected ~74 kDa and ~24 kDa M2BP bands in the soluble and membrane fractions of seminal plasma (Fig. 4B). Strong M2BP immunoreactivity was detected in the seminal plasma soluble fraction with weaker immunoreactivity in the whole, non-ionic detergent-soluble, non-ionic detergent-resistant membrane fractions. Furthermore, the Atlas anti-M2BP antibodies detected an ~74 kDa protein band in the seminal plasma membrane fraction that contained prostasomes and in the pooled galactoside-binding proteins purified from the seminal plasma membrane fraction by lactose-affinity chromatography (Fig. 4C).

M2BP immunoreactivity in purified prostasomes

Prostasomes were purified from seminal plasma using a standard size exclusion column chromatography method for prostasome isolation [12]. Prostasomes are larger in mass than the exclusion limit of the Sephacryl S300 column and were collected in the column void volume (fractions 14-19) as demonstrated by total protein staining and immunoblot analysis for the positive control CD26 (Fig. 5). Anti-M2BP immunoblot analysis demonstrated M2BP immunoreactivity in fractions 14-19 with a minor amount of immunoreactivity in fractions 20 and 21.

Purification of biotinylated proteins from surface-biotinylated prostasomes

Purified prostasomes were surface-biotinylated with membrane-impermeable sulfo-NHS-LC-biotin and biotinylated proteins were purified by avidin-affinity chromatography. Electroblot analysis detected multiple biotinylated proteins in purified fractions 2 to 6 with

the highest relative amount in fractions 3 and 4 (Fig. 6). Immunoblot analysis of the same material identified M2BP immunoreactivity in fractions 2-5 with the highest relative amount of immunoreactivity in fractions 3 and 4.

Sperm immunofluorescence

Paraformaldehyde-fixed, air-dried human sperm were incubated with R&D anti-M2BP antibodies followed by FITC-conjugated secondary antibodies. M2BP immunoreactivity was detected over the entirety of the mid and principal pieces of the sperm tail with intense staining of the neck region and minimal staining of the head (Fig. 7).

Discussion

The multiple functions of galectin-3 are exerted through ligand binding and the consequential effects are dependent on the specific galectin-3 ligands involved [15]. Lactose-affinity column chromatography is a standard purification method for β-galactoside binding proteins such as galectin-3. Previously, we identified galectin-3, a truncated form of galectin-3, and lactoferrin in the β-galactoside binding protein fraction following lactose-affinity chromatography of human prostasomes [5]. The known lectin-like properties of lactoferrin suggested that it bound to immobilized lactose directly. In the current study, lactose-affinity purification of galectin-3 from prostasomes resulted in the co-purification and identification of additional non-galectin proteins: CD26 (dipeptidyl peptidase IV), OLMF4, PIP, seminogelins I and II (SgI and SgII), and M2BP. These co-purified proteins represent potential galectin-3 binding ligands that interacted with galectin-3 bound to the lactose affinity matrix. CD26, OLMF4, PIP, SgI, and SgII were previously identified in prostasomes using proteomic approaches [16,17]. The identified candidate galectin-3 binding ligands and their potential relevance to prostasome function are discussed.

CD26 (dipeptidyl peptidase IV) is a 110 kDa ectopeptidase that is expressed by most human epithelial cells as an intrinsic membrane protein and/or as a soluble enzyme [18]. This enzyme has also been identified in exosomes secreted by salivary and intestinal epithelia [2,19]. CD26 has diverse roles in immunity, metabolism, endocrinology, and cancer, suggesting that CD26 may be involved in the immunomodulatory activities and cancer-associated functions of prostasomes [18]. Galectin-4 was shown to recognize the carbohydrate on the CD26 glycoprotein [20], but the ability of galectin-3 to bind CD26 has not been reported.

The olfactomedin family, including OLFM4, is a group of proteins with diverse functions with their common characteristic being conserved, C-terminal olfactomedin domains [21]. OLFM4 is a secreted glycoprotein that is expressed in the prostate, bone marrow, and gastrointestinal tissues. OLFM4 is implicated in cell proliferation and cell adhesion, and thus, may be involved in prostasome interactions with sperm and leukocytes. Neither carbohydrate binding, galectin-3 binding, or immunomodulatory activity has been reported for OLFM4.

Prolactin-inducible protein (~17 kDa; PIP), also known as seminal actin binding protein (SABP), gp17, and gross cystic disease fluid protein-15 (GCDFP-15), is present in body fluids such as seminal plasma, saliva, and sweat [22-24]. PIP is also expressed in breast tumors [25] and is over-expressed in prostate carcinomas [26]. Multiple PIP functions have been proposed including inhibition of T cell apoptosis following interaction with CD4 on the lymphocyte surface [25]. PIP was also demonstrated to bind to sperm [27]; however, the PIP receptor on sperm has not been identified conclusively. Thus, PIP may contribute to prostasome interactions with sperm and lymphocytes. The potential for PIP-galectin-3 binding is unknown.

SgI and SgII are the major proteins that form the human semen coagulum following ejaculation and are proteolytically cleaved by prostate specific antigen during semen liquefaction [28]. Prior to cleavage, association of SgI with the sperm surface inhibits sperm motility [29]. The seminogelins are secreted by the seminal vesicle and apparently associate with prostasomes following ejaculation; however, a role for the seminogelins in prostasome function has not been described. Only SgII appears to be glycosylated, thus, galectin-3 may interact with SgI and SgII through its non-lectin binding domain rather than the carbohydrate recognition domain.

The co-purified proteins described above represent candidate galectin-3 binding ligands in prostasomes that were apparently immobilized on the lactosyl-affinity column via interaction with galectin-3. Conversely, the co-purified proteins may themselves be carbohydrate binding proteins that bound directly to immobilized lactose. Therefore, the identification of the described co-purified proteins as galectin-3 binding ligands must be directly confirmed in future studies. However, M2BP is a known galectin-3 binding ligand that was co-purified with galectin-3 from the prostasome-enriched membrane fraction and was identified in prostasomes for the first time in this study.

M2BP is a secreted glycoprotein normally present in human body fluids, epithelia, and the extracellular matrix (ECM) [30]. Originally identified as a binding ligand for galectin-3 (formerly Mac-2), M2BP is also known as galectin-3-binding protein, 90K tumor-associated antigen, L3 antigen, and alpha-2-seminoglycoprotein. The M2BP glycoprotein also interacts strongly with galectins-1 and -7; collagen IV, V, and VI; fibronectin; nidogen; and β1 integrins [31]. Although the function of M2BP has not been completely elucidated, M2BP appears to be involved in cell–cell and cell–ECM adhesion and is a member of the scavenger receptor cysteine-rich (SRCR) protein superfamily, a family of proteins that function in host defense. Multiple investigators have proposed a role for M2BP as an immunomodulator via interaction with macrophages and monocytes [32,33]. M2BP is also implicated in tumor progression and metastasis, and is thought to mediate homotypic tumor cell aggregation in the blood stream via interactions with cell surface galectin-1 and -3 [30].

The mature M2BP glycoprotein contains four domains (M2BP-1,2,3,4) including an N-terminal SRCR domain (M2BP-4) [14,31]. The remaining three domains comprise a large mucin-like domain containing the majority of the putative glycosylation sites. These domains include a BTB/POZ domain (M2BP-3), which contains a zinc finger motif, an intervening region (M2BP-2), and a C-terminal domain (M2BP-1) delineated from the IVR by a plasmin cleavage site. In the native protein, M2BP-1 remains non-covalently associated with M2BP-2,3,4 following plasmin cleavage and has no function independent of M2BP-2,3,4. M2BP dimers form large, oligomeric ring structures (>1,000 kDa) that present multiple binding sites for its ligands. Under denaturing conditions, the M2BP monomer can be detected as a variable combination of polypeptide bands of ~90–100 (M2BP-1,2,3,4), ~70 (M2BP-2,3,4), and ~25 kDa (M2BP-1), depending on the source of the M2BP, the extent of proteolytic cleavage, and epitope specificity of the anti-M2BP antibodies used.

In the male reproductive tract, M2BP expression was previously identified in human seminal plasma [34], prostate secretions, and prostate cancer cells [35]; and in the mouse testis [31]. In the current study, we used two different anti-M2BP polyclonal antibodies to confirm identification of M2BP. M2BP was identified as a variable combination of immunoreactive bands in extracts of human testis, epididymis, vas deferens, seminal vesicle, prostate and sperm, and in seminal plasma consistent with the identification of intact M2BP monomer (M2BP-1,2,3,4) and the ~67-75 kDa (M2BP-2,3,4) and ~25 kDa (M2BP-1) plasmin cleavage products. The R&D anti-M2BP antibody reacts with epitopes in all domains of M2BP and detects M2BP-1,2,3,4, M2BP-2,3,4, and M2BP-1 immunoreactive bands. The

Atlas anti-M2BP antibodies only reacts with M2BP-2,3,4 and therefore, only detects M2BP-1,2,3,4 and M2BP-2,3,4, but not M2BP-1. The ~50 and ~37 kDa immunoreactive bands identified in the seminal vesicle and prostate extracts may represent uniquely glycosylated and/or cleaved forms of M2BP. Nevertheless, these experiments identified M2BP in the male reproductive tract tissues tested.

To further investigate M2BP in semen, seminal plasma was fractionated by differential centrifugation. Although M2BP immunoreactivity was identified predominately in the soluble fraction, M2BP was also detected in the membrane fraction of seminal plasma, a portion of which remained associated with the non-ionic detergent-resistant membrane fraction. These results are consistent with an association with cholesterol-rich membranous vesicles such as prostasomes. Furthermore, M2BP immunoreactivity was detected on an electroblot of the β-galactoside-binding proteins that were isolated from the prostasome-enriched, seminal plasma membrane fraction. The association of M2BP was demonstrated by the identification of M2BP in prostasomes purified by size exclusion chromatography from the membrane fraction of seminal plasma.

Prostasomes are too small for adequate visualization by light microscopy and immunofluorescence [1]. Therefore, biotinylation of the prostasome surface combined with avidin-affinity purification was used to examine the localization of M2BP on the prostasome surface. Sulfo-NHS-LC-biotin is not membrane permeable; only molecules on the prostasome surface would be biotinylated. M2BP was identified in the fractions of surface-biotinylated proteins purified from prostasomes. Definitive confirmation of M2BP localization to the prostasome surface will require electron microscopy. Nevertheless, these results indicate that M2BP is present on the surface of human prostasomes. Furthermore, immunofluorescence microscopy identified M2BP immunoreactivity over the tail and neck of human sperm. Collectively, these results suggest that M2BP interacts with binding ligands on the prostasome surface and sperm.

The function of M2BP in semen is unknown. However, based on the function of M2BP in cell adhesion [14,30,31], M2BP may be involved in the binding of prostasomes to sperm and prostasome regulation of sperm function. Furthermore, the cell adhesion and immunomodulatory functions of M2BP [31-33] suggest that M2BP may participate in prostasomes-leukocytes interactions in the female reproductive tract that are proposed to suppress leukocyte function and protect sperm from the female immune system [1]. Significantly, the immunosuppressive factors of seminal plasma, including prostasomes, are also thought to facilitate the transmission of sexually-transmitted pathogens [36]. Conversely, M2BP is implicated in host defense [33,37] and prostasomes have been shown to exhibit anti-bacterial properties [1], indicating that M2BP may participate in the host defense functions of prostasomes. The roles of M2BP in tumor cell adhesion and metastasis in multiple malignancies [30] suggest that M2BP may contribute to the proposed function of prostasomes in invasion and immunosuppression [1] during prostate cancer progression. Moreover, the co-purification of M2BP with galectin-3 from prostasomes suggests that galectin-3 is involved in M2BP interactions with prostasomes and is involved in M2BP's role in prostasome function. For example, M2BP potentially binds to ligands, such as galectin-3, on prostasomes and sperm to facilitate prostasome-sperm interactions.

Conclusions

The co-purification of CD26, OLFM4, SgI, SgII, and PIP with galectin-3 from prostasomes implicates these proteins as galectin-3 binding ligands. Interactions between galectin-3 and these proteins have not been identified previously; therefore, the investigation of their interactions with galectin-3 will provide new avenues of exploration for the characterization

of galectin-3 function in prostasomes. Furthermore, identification of the galectin-3 binding protein M2BP on the surface of prostasomes, together with its cell adhesive and immunomodulatory properties, implicates a functional role for M2BP in prostasome regulation of sperm function, immunosuppression in the female reproductive tract, and prostate cancer progression. Therefore, we anticipate that the future study of the interactions of galectin-3 with its binding ligands in prostasomes will provide new insights into the intercellular signals communicated from the male genital tract to the female genital tract during normal reproductive function and in molecular mechanisms of intercellular communication in prostate cancer.

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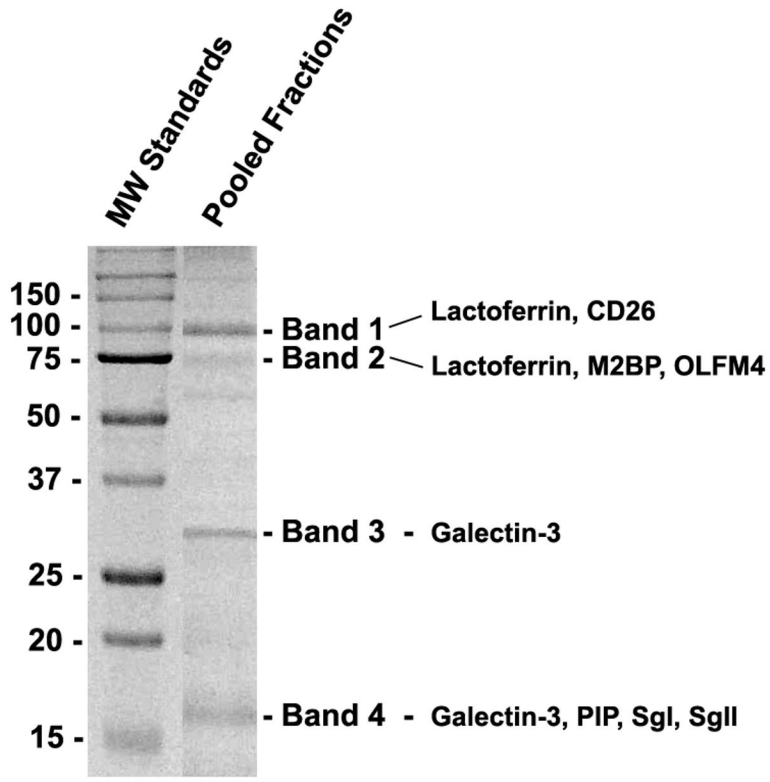

Figure 1.

Proteomic analysis of β-galactoside binding proteins and co-purified proteins from the prostasome-enriched, seminal plasma membrane fraction. Lactose affinity-purified proteins were separated by SDS-PAGE and stained with Coomassie blue. Specific protein bands were excised and microsequenced by tandem mass spectrometry. The proteins identified for each band are indicated. Molecular weight markers are indicated in kDa. Mac-2 binding protein (M2BP), olfactomedin-4 (OLFM4), prolactin inducible protein (PIP), seminogelin I (SgI), seminogelin 2 (SgII).

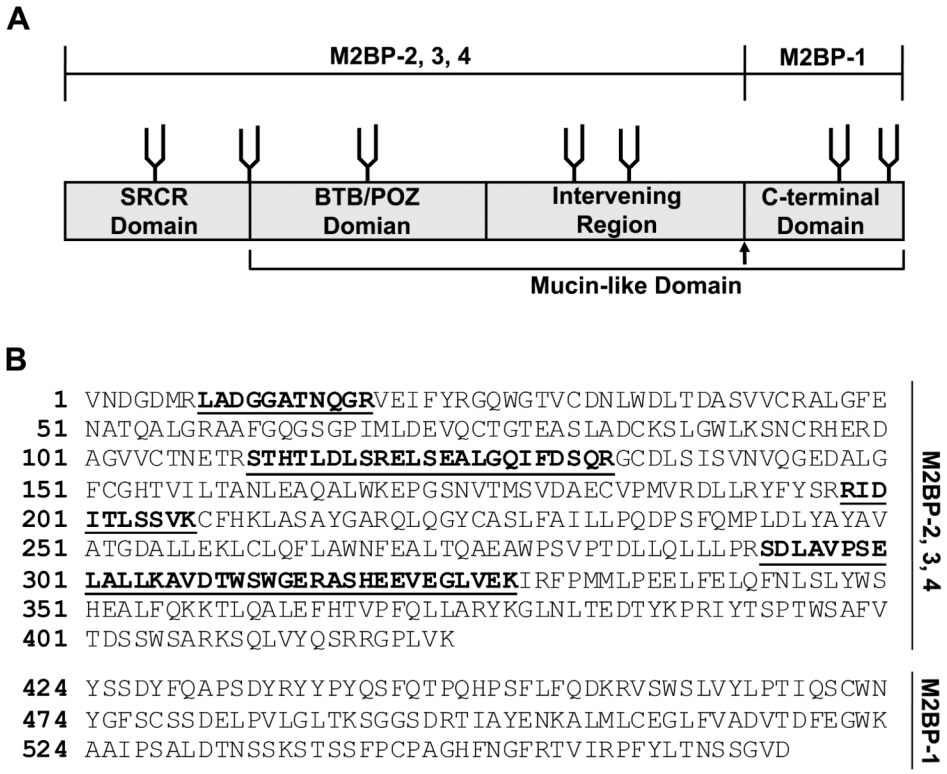

Figure 2.

A: Structure of the M2BP monomer. The mature M2BP glycoprotein contains four domains (M2BP-1,2,3,4) including an N-terminal SRCR domain (M2BP-4). The remaining three domains comprise a mucin-like domain containing the majority of the putative glycosylation sites. These domains include a BTB/POZ domain (M2BP-3), which contains a zinc finger motif, an intervening region (M2BP-2), and a C-terminal domain (M2BP-1) delineated from the IVR by a plasmin cleavage site. In the native protein, M2BP-1 remains non-covalently associated with M2BP-2,3,4 following plasmin cleavage. Under denaturing conditions, the M2BP monomer can be detected as a variable combination of polypeptide bands representing M2BP-1,2,3,4 (~90-100 kDa), M2BP-2,3,4 (~70 kDa), and M2BP-1 (~25 kDa), depending on the source of the M2BP and the extent of proteolytic cleavage. **B:** Sequence coverage of M2BP peptides identified by tandem mass spectrometry. Following lactose-affinity chromatography of the seminal plasma membrane fraction, tandem mass spectrometry of an ~74 kDa protein band identified peptides (Bold Underline) that spanned 79 amino acids of the reported sequence for human M2BP (GenBank Accession number EAW89543). The identified peptides represented 18.7% of the 423 amino acid M2BP-2,3,4 polypeptide fragment generated by plasmin cleavage (A).

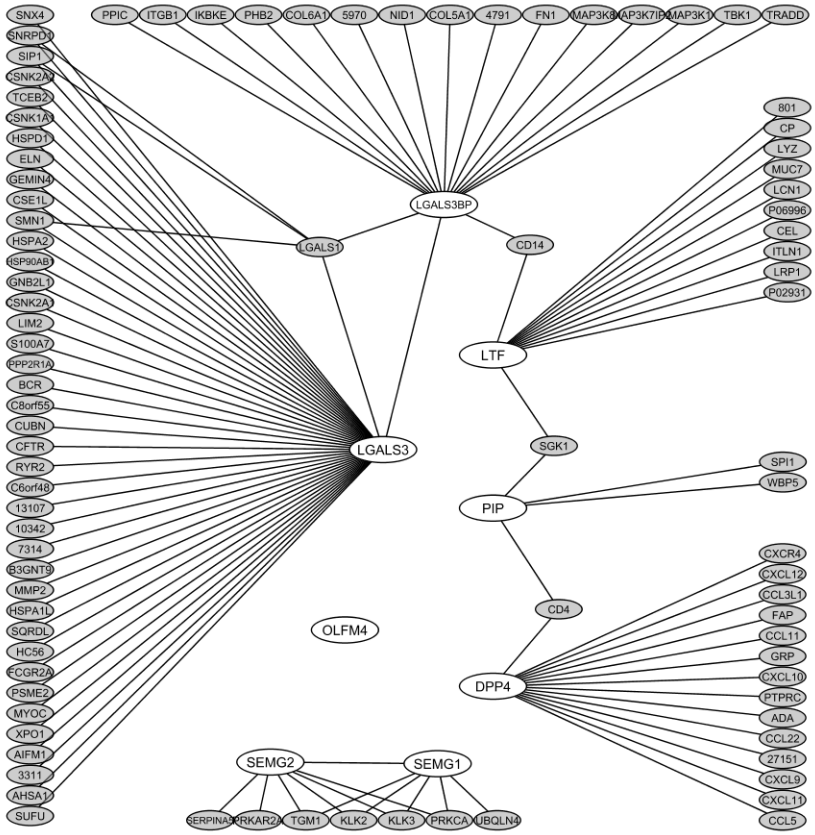

Figure 3.

Protein-protein interaction network of the identified proteins. Cytoscape was used to display the protein-protein interaction map generated from interaction database data pulled down from the HPRD, BioGRID, IntAct, and NCI/Nature Pathway Interaction databases. White nodes indicate the proteins identified in this study following β-galactoside affinity purification. Gray nodes indicate interacting proteins retrieved from the databases. Connecting lines indicate identified protein interactions. Each protein is shown by its gene symbol or entrez identification number. Galectin-3 (LGALS3), M2BP (LGALS3BP), lactoferrin (LTF), PIP (PIP), CD26 (DPP4), seminogelin I (SEMG1), seminogelin 2 (SEMG2), olfactomedin 4 (OLFM4).

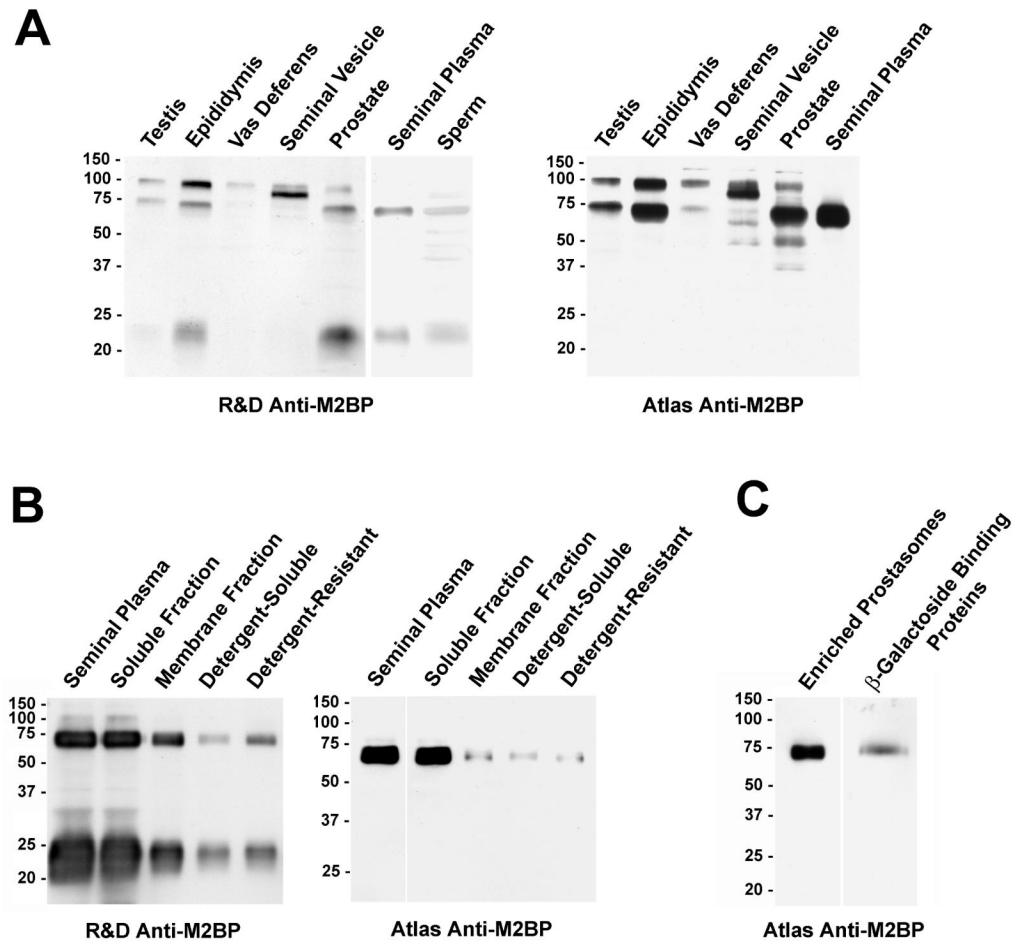

Figure 4.

M2BP immunoblot analysis of male reproductive tract extracts and fractionated seminal plasma. **A:** Human testis, epididymis, vas deferens, seminal vesicle, prostate and sperm extracts and clarified seminal plasma were separated by SDS-PAGE and electroblots were evaluated for M2BP immunoreactivity with R&D and Atlas anti-M2BP polyclonal antibodies. **B:** Clarified seminal plasma was subjected to differential ultracentrifugation as described in the Materials and Methods section. Clarified seminal plasma, soluble fraction, membrane fraction, detergent-soluble membrane fraction, and detergent-resistant membrane fraction were evaluated by immunoblot analysis with the R&D and Atlas anti-M2BP antibodies to compare the relative amount of M2BP in each fraction. **C:** Immunoblot analysis with the Atlas anti-M2BP antibodies of the prostasome-enriched, seminal plasma membrane fraction and β-galactoside binding proteins purified from the seminal plasma membrane fraction. Molecular weight markers are indicated in kDa.

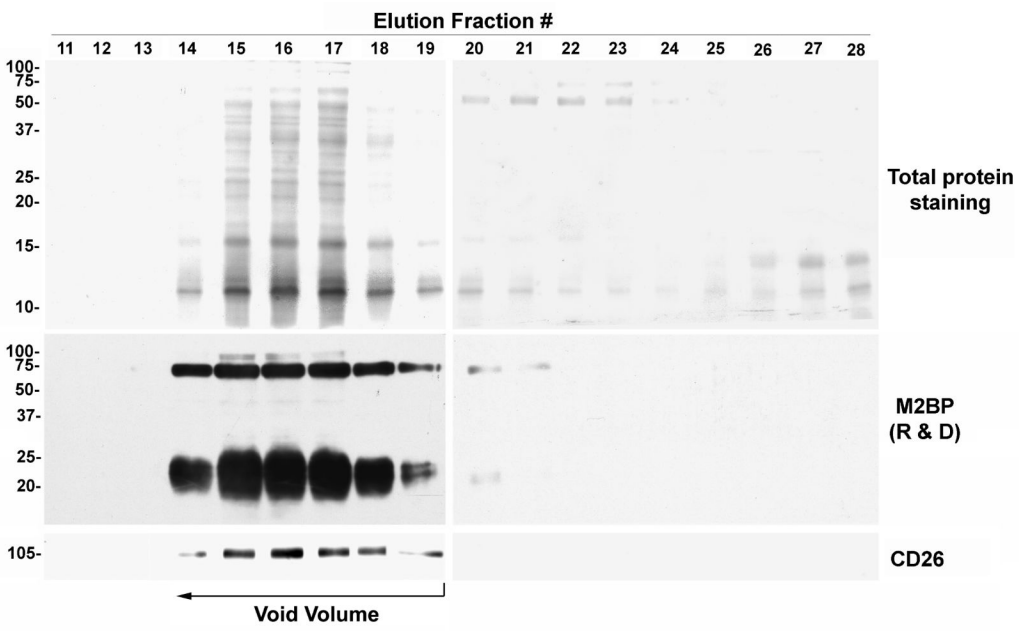

Figure 5.

Prostasome purification by size exclusion chromatography and M2BP immunoblot analysis. The membrane fraction from seminal plasma was subjected to size exclusion column chromatography on Sephacryl S300 and prostasomes were collected in the void volume (fractions 14-19). Electroblots of collected fractions were stained for total protein with Ponceau S and for M2BP (R&D polyclonal antibodies) and CD26 immunoreactivity. Molecular weight markers are indicated in kDa.

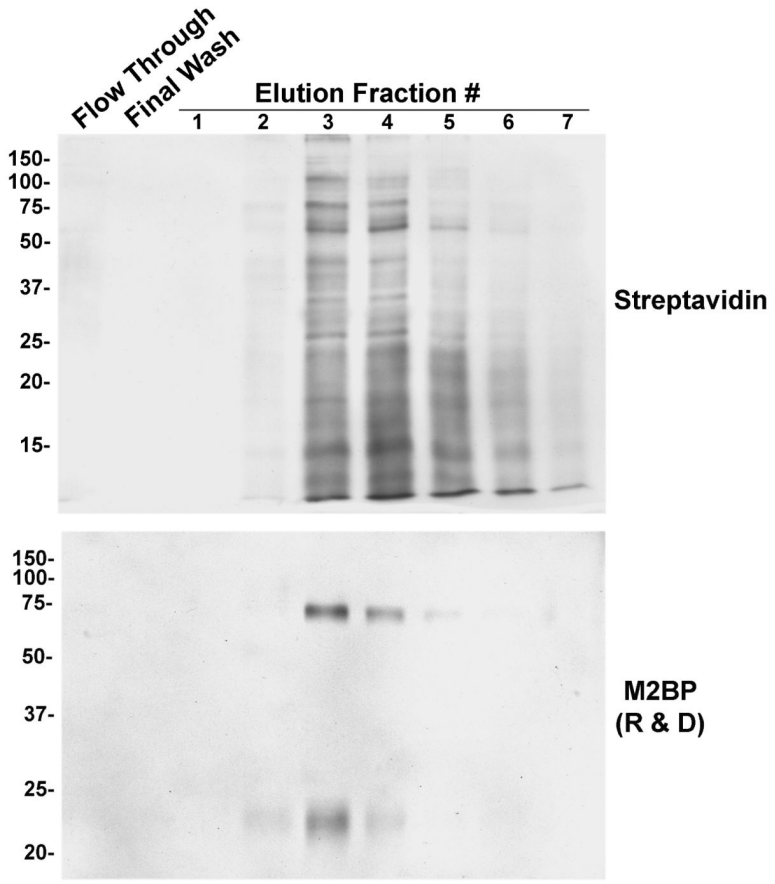

Figure 6.

Purification of biotinylated proteins from purified, surface-biotinylated prostasomes. The surface of isolated prostasomes was biotinylated with membrane-impermeable sulfo-NHS-LC-biotin. Biotinylated proteins were affinity purified on immobilized avidin. Non-bound material in the column flow through and purified biotinylated proteins were subjected to electroblot analysis with the R&D anti-M2BP antibodies to identify M2BP. Molecular weight markers are indicated in kDa.

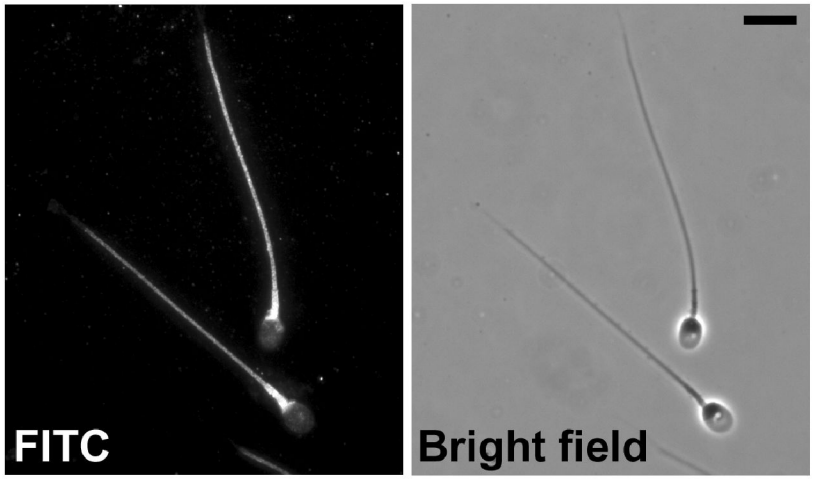

Figure 7.
Localization of M2BP in human sperm by immunofluorescence with the R&D anti-M2BP polyclonal antibodies. M2BP immunoreactivity was detected with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies. Scale bar = 10 μm.

Table I

Peptide Matches from Tandem Mass Spectrometry of Proteins Purified by Lactose Affinity Chromatography of Human Prostasomes

	Mascot score	# Unique Peptides	% Sequence Coverage
Band 1			
Lactoferrin (Lactotransferrin)	1485	26	43
CD26/Dipeptidyl peptidase IV	201	5	6
Band 2			
Lactoferrin (Lactotransferrin)	640	14	22
Mac-2 binding protein	466	8	14
Olfactomedin-4	318	4	19
Band 3			
Galectin-3	265	6	32
Band 4			
Galectin-3	423	9	31
Semenogelin I	262	6	14-17
Prolactin inducible protein	215	5	43
Semenogelin II	205	5	10