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Characterization of novel antigens in membranous nephropathy

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CHARACTERIZATION OF NOVEL ANTIGENS IN MEMBRANOUS NEPHROPATHY

by

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CHARACTERIZATION OF NOVEL ANTIGENS IN MEMBRANOUS NEPHROPATHY

PAIGE COLES

ABSTRACT

Introduction:

Membranous nephropathy is an autoimmune disease that targets glomeruli of the kidney. Previous discoveries in membranous nephropathy include the discovery of megalin as an antigen in the proximal tubular brush border fraction (Fx1A) and glomeruli of Heymann nephritis rats, identification of neutral endopeptidase in alloimmune neonatal nephropathy, and discovery of PLA₂R and THSD7A as causal antigens in approximately 80-85% of primary membranous nephropathy cases. It was then recognized that there must be other antigens responsible for the remaining 15-20% of cases.

Objectives:

The current study aims to screen membranous nephropathy patient serum samples via Western blotting for reactivity with potential antigens in protein extracts of normal human glomeruli, purify potential membranous nephropathy antigens, identify them with mass spectrometry, and validate these identifications with immunoprecipitation and immunohistochemical analysis. Previously, work had been done to identify one novel, 58 kDa antigen. A second novel antigen had been shown in the proximal tubule brush
border. Finally, a third protein, CR1, was shown to contain corresponding antibodies in the antibody preparation used in the rat model of membranous nephropathy, making the antigen a protein of interest in human primary membranous nephropathy.

**Methods:**

Using human glomeruli obtained by detergent extraction, we isolated extracellular domains and identified two novel antigens, called 58-kDa and brush-border, with patient serum. We attempted to further purify the 58-kDa antigen with lectin binding columns and partition phase separation. Upon the identification of a small cohort of cases associated with autoimmune tubulointerstitial nephritis, we set out to determine if these sera recognized a novel antigen. Prior to screening human glomerular extract with these sera, we exposed it to partial proteolysis with trypsin, reducing agent β-mercaptoethanol, and tubular elements to further characterize the antigen before it was pulled down with anti-brush-border antigen+ and control IgG4 and analyzed by mass spectrometry. The third and final antigen we investigated was CR1, which we screened with membranous nephropathy sera and immunoblotted its antibody against different protein preparations.

**Results:**

Labeling of the extracellular portions of the 58-kDa and brush-border antigens with biotin was successful. It was determined that the 58-kDa antigen was not glycosylated due to its inability to bind lectin columns. The 58-kDa antigen was present in the hydrophilic layer when separated with tritonX-114 detergent. Partial proteolysis of the brush-border antigen with trypsin yielded bands at 140 kDa, 120 kDa and 95 kDa. The brush-border
antigen was destroyed under reducing conditions. Candidate proteins for the brush-border antigen as determined by mass spectrometry include megalin and SVEP1. Membranous nephropathy sera were shown to be negative for anti-CR1+ antibody, and anti-CR1+ antibody was reactive with glomeruli and the TBS supernatant fraction.

**Conclusions:**

This study suggests that the 58-kDa antigen which has antibodies in some human primary membranous nephropathy sera contains extracellular portion(s), is not glycosylated, but is membrane-associated. The data indicate that there is also potential for a membranous nephropathy antigen in the tubular brush border with an immunoreactive element around 95 kDa in size, that is sensitive to reducing conditions. Preliminary mass spectrometry information points toward megalin as the identification of this antigen. CR1 does not appear to be a causal antigen in human primary membranous nephropathy.
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LIST OF ABBREVIATIONS

α-58-kDa antigen+................................................. Anti-58-kDa Antigen Positive
α-THSD7A+.................................. Anti-Thrombospondin Type-1 Domain-Containing 7A Positive
α-brush-border antigen+............................ Anti-Brush-Border Antigen Positive
α-PLA₂R+........................................ Anti-Phospholipase A₂ Receptor Positive
μL................................................................................ Microliter
ABBA.......................................................... Anti-Brush-Border Antibody
Con A.............................................................. Concanavalin A
CR1.............................................................. Complement Receptor 1
ECL............................................................ Enhanced Chemiluminescence
ECM.......................................................... Extracellular Matrix
ER.............................................................. Endoplasmic Reticulum
GBM.............................................................. Glomerular Basement Membrane
HGE.......................................................... Human Glomerular Extract
HGTE........................................................ Human Glomerular Extract with Tubular Elements
HTE.......................................................... Human Tubular Extract
IP.......................................................... Immunoprecipitation
kDa.......................................................... kiloDalton
mL........................................................................ milliliter
MN.............................................................. Membranous Nephropathy
NEP.......................................................... Neutral Endopeptidase
PBS…………………………………………………………………Phosphate Buffered Saline
PLA₂R…………………………………………………………………Phospholipase A₂ Receptor
RIPA…………………………………………Radioimmunoprecipitation-Assay Buffer
RPM……………………………………………………………………Rotations per Minute
SDS…………………………………………………………………… Sodium Dodecyl Sulfate
TBS………………………………………………………………………Tris Buffered Saline
TBST…………………………………………………………………..Tris Buffered Saline-Tween
THSD7A ……………………………Thrombospondin Type-1 Domain-Containing 7A
Tx-114………………………………………………………………….Triton-X 114
INTRODUCTION

Nephrotic Syndrome from Membranous Nephropathy

Membranous nephropathy (MN) is an organ specific autoimmune disease of the kidney that targets the specialized blood vessels of the glomerulus that comprise the glomerular filtration barrier. MN is the major cause of nephrotic syndrome of non-diabetic origin in adults (Ronco & Debiec, 2015). Nephrotic syndrome, which can be caused by a number of etiologies that all affect the podocyte and/or glomerular filtration barrier, presents as proteinuria, hypoalbuminemia, hyperlipidemia, and generalized edema (Kemp, Burns, & Brown, 2008). The typical fates of this condition in MN seen clinically are spontaneous remission, or continued proteinuria resulting in either stable renal function or end-stage renal failure (Ronco & Debiec, 2015). Spontaneous remission of MN is seen in 40% of cases, the chances of which are increased by baseline proteinuria of less than 8 g per day, female sex, less than 50 years of age, and preserved renal function at presentation. Nephrotic syndrome is triggered by a fall in osmotic pressure due to loss of protein in the urine, which favors fluid movement out of the intravascular space and into the interstitium or other spaces. This fluid movement, in addition to direct stimulation of sodium absorption due to the effects of filtered proteins, leads the kidneys to progressively retain sodium, followed by water, until a new equilibrium is established between the vascular and interstitial spaces (Perlman, Heung, & Ix, 2013). Hypoalbuminemia also leads to an increase in apolipoprotein from the liver (Kemp et al., 2008).
Nephrotic syndrome in membranous nephropathy is caused by immune-mediated damage to glomeruli at the slit diaphragm of podocyte foot processes which activates the complement system, usually via the classical pathway (Beck, 2014). The damaged foot processes secondarily secrete matrix material and allow calcium influx that cause a less differentiated podocyte phenotype including loss of foot processes and slit-diaphragm structures and expansion of the glomerular basement membrane (GBM, figure 1A), the specialized extracellular matrix between podocytes and the endothelial cells of the glomerulus (Cybulsky, 2011; figure 1), which may still be present after remission due to slow restoration of the GBM (Beck, 2014). The maintenance of the slit diaphragm in healthy individuals is critical to regulating what the GBM is permeable to according to size and charge (Krtíl, Pláteník, Kazderová, Tesař, & Zima, 2007).
The Course of Membranous Nephropathy

MN is a pattern characterized in light microscopy by more thickness to the capillary wall compared to normal (Jones, 1957); in electron microscopy by immune deposits of more electron density below the epithelium (Movat & McGregor, 1959); and, in immunofluorescence by peripheral capillary loop staining for IgG that is fine and granular (Mellors & Ortega, 1956). There are two forms of MN: primary and secondary. Primary (previously known as “idiopathic”) MN describes the 75% of MN cases that show subepithelial IgG deposits by immunofluorescence microscopy (mostly IgG4 with
variable amounts of IgG1) and subepithelial and intramembranous electron dense deposits by electron microscopy in the absence of any other known association (Cybulsky, 2011). Primary MN is more common in men than in women; in fact, when observed in women it can indicate an underlying cause of lupus.

When the other 25% of MN cases are caused by other systemic autoimmune processes like diseases, medications, or exposures in this way, they are classified as secondary MN (Ma, Sandor, & Beck, 2013). Secondary MN can be identified by secondary causes and histopathological features, frequently with atypical lesions (Ma et al., 2013). Subepithelial and mesangial extraglomerular IgG1 and IgG2 deposits are seen by immunofluorescence microscopy and electron microscopy, which also shows intramembranous electron dense deposits and, in the case of lupus, tubuloreticular inclusions (Cybulsky, 2011). Subendothelial and mesangial deposits are often found in lupus-associated MN in addition to subepithelial and paramesangial deposits. In both types of MN, staging of the disease is done according to the extent to which subepithelial immune deposits are surrounded by GBM.

**Causal Antigens in Membranous Nephropathy**

Experimental studies into the pathophysiology of MN date back to the Heymann nephritis model initially developed in 1959 (Heymann, Hackel, Harwood, Wilson, & Hunter, 1959). The model involved immunizing Lewis rats intraperitoneally using homologous renal homogenate and complete Freund’s adjuvant to induce nephropathy identical to human MN clinically and histopathologically, complete with presence of subepithelial immune deposits via light, immunofluorescence, and electron microscopy.
Over time, antigenic activity in this model was narrowed down to a tubule rich (brush-border) fraction called Fx1A that was thought to form immune complexes in the blood. A seminal study performed at Boston University in 1979, however, revealed that the anti-Fx1A antibodies instead bound in situ to a fixed antigen present within the glomerular filtration barrier. Years later this fixed antigen was identified on podocytes as megalin and its chaperone, receptor-associated protein (Kerjaschki & Farquhar, 1982). However, once it was discovered that megalin was not present on human podocytes (Lundgren et al., 1997), and thus could not cause human primary MN, the search for a causal antigen in humans was back on.

Fortunately, in 2002, neutral endopeptidase (NEP) was discovered by Ronco, Debiec, and colleagues as the antigenic target of circulating antibodies in alloimmune neonatal nephropathy. While this antigen was only causal in this rare disease, it did inspire researchers to continue the search for antigens responsible for MN. Then in 2009, Beck et al. discovered M-type phospholipase A$_2$ receptor-1 (PLA$_2$R) as the antigenic target in autoimmune adult MN (Figure 2A).

![俍](image-url)
Figure 2. Phospholipase A2 Receptor. A shows the comparable results of Western blot with guinea pig anti-PLA₂R and membranous nephropathy serum against human glomerular extract and recombinant PLA₂R. Each sample is shown deglycosylated (+ PNGase F) and in its natural state (- PNGaseF), illustrating a difference in size between the two conditions. B illustrates the structure of PLA₂R, including the N-terminal cysteine-rich region (CysR), a fibronectin type II domain (FNII), eight C-type lectin-like domains (CTLD), a transmembrane domain (TM), and an intracellular C-terminal tail (After Beck et al. 2009).

PLA₂R is a type 1 transmembrane receptor that constitutes one of four members of the mannose-receptor family in mammals (Figure 2B). It demonstrates a conserved domain structure and undergoes endocytic recycling so that there is a constant source of it at the podocyte membrane for immune complex formation (Beck et al., 2009). In 2009, Beck et al. found that when incubated with reactive serum samples from MN patients, human glomerular extract (HGE) yield the same 185-kiloDalton (kDa) band via gel electrophoresis that is also detected by guinea pig polyclonal anti-PLA₂R positive (α-PLA₂R+) serum via Western blot. Also, immune-precipitates from HGE with human MN
sera could be Western blotted with the polyclonal anti-PLA$_2$R antibody, showing that the autoantibodies in the sera identified the same protein as monospecific anti-PLA$_2$R antiserum. This established the target antigen as PLA$_2$R.

It has been shown that about 70% of primary MN patients have antibodies to the antigen PLA$_2$R, (Beck et al., 2009). Beck et al. discovered that anti-PLA$_2$R antibodies are most often of the IgG4 subclass. In fact, PLA$_2$R co-localizes with IgG4 in the fine, granular pattern that is typical of MN (Beck et al., 2009). However, IgG4 does not activate the classical pathway of complement although there is emerging evidence that it may activate the lectin pathway. The discovery of PLA$_2$R allowed for retrospective diagnosis of PLA$_2$R-related MN in archival kidney biopsies (Ronco & Debiec, 2015). Some studies show that the concentration of α-PLA$_2$R+ serum correlates with urinary protein output and disease activity. Therefore, a high titer signifies a decreased possibility of spontaneous or immunosuppressant-induced remission, increased risk of nephrotic syndrome in non-nephrotic patients, deterioration in renal function, and an increased time interval between the start of immunosuppressive treatment and remission (Ronco & Debiec, 2015).

There are other antigens responsible in the remaining cases, such as the recently discovered thrombospondin type-1 domain-containing 7A, a 250 kDa, type-1 transmembrane protein identified by Tomas et al. in 2014 via mass spectrometry. Thrombospondin type-1 domain-containing 7A (THSD7A) shares several features with PLA$_2$R, including N-glycosylation, membranous location, reactivity with serum only under non-reducing conditions, and the predominant IgG subclass of autoantibodies to it.
being IgG4 (Tomas et al., 2014). THSD7A consists of a large extracellular region of eleven thrombospondin type-1 repeats and one arginine-glycine-aspartic acid (RGD) motif; a single transmembrane domain; and, a short intracellular tail (Figure 3). Autoantibodies to THSD7A recognize one or more conformation-dependent epitopes in native and recombinant THSD7A in 5-10% of primary MN patients negative for α-PLA₂R. Furthermore, it was found to be located on or close to the podocyte foot processes, not the glomerular basement membrane (Tomas et al., 2014).

**Figure 3. Thrombospondin type-1 domain-containing 7A.** Pictured are: the thrombospondin domain (TSD) repeats and arginine-glycine-aspartic acid (RGD) motif of the extracellular domain; the transmembrane (TM) region; and, the short intracellular tail (as well as a signal peptide, in blue; after Tomas et al., 2014).

**Characterizing Three Novel Antigens Associated with Autoimmune Kidney Disease**

As PLA₂R and THSD7A do not account for all cases of primary MN, the search continues for minor antigens in this disease. Iwakura et al. showed that 38% of all primary MN biopsies were negative for both PLA₂R and THSD7A. Hence, it is very likely that there are antigens yet to be identified (Iwakura, Ohashi, Kato, Baba, & Yasuda, 2015). However, these antigens have yet to be found using existing techniques. This could be due to the very small numbers of patients reactive with the other antigens.
It could also be that these minor proteins are not extractable using detergent, i.e. they are possibly components of the GBM. Furthermore, these marginal antigens may react with non-IgG4 autoantibodies, and thus are not detected on Western blot by anti-IgG4 secondary antibody, which is the predominant immunoglobulin subclass in glomerular deposits. Finally, it could be that the conformation of the epitope of these proteins is destroyed by the Western blot procedure. Overcoming these hurdles will be key in classifying the unidentified antigens responsible in the remaining 15-20% of primary MN cases.

One such potential antigen (so-called “58 kDa-antigen”) was originally identified by the serum of patient MN 07-15 which was shown to positively bind to an antigen in the 58-kDa region on Western blot. The gel region corresponding to the Western blot signal at 58 kDa was excised and analyzed by mass spectrometry after immunoprecipitation with patient serum. Previous work in the lab (Beck, unpublished data) that was done with our mass spectrometry collaborators at the University of Louisville, analyzed the excised band. Biochemical features of these proteins were compared to the characteristics of the novel 58 kDa-antigen (Table 1). While some of the proteins share characteristics with the novel antigen, we decided to search for proteins with extracellular components that were more likely to be targeted by autoantibodies. The 58-kDa antigen has been identified as a membrane-associated protein by making “membrane preps” via cell lysis and high-speed ultracentrifugation of the cellular membranes (Eric Eisenberg, unpublished data). This protein remains unidentified, but we
have sporadically found reactivity for this antigen in other patients with primary MN.

Thus we repeated efforts to try to identify this antigen.

**Table 1. List of best candidate proteins for 58-kDa antigen from mass spectrometry analyses.** Characteristics that fit the protein of interest are marked in red. (Data from “Human Protein Reference Database,” n.d.)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Size</th>
<th>Glycosylation</th>
<th>Tissue Expression</th>
<th>Cellular Location</th>
</tr>
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<tbody>
<tr>
<td>Annexin A11</td>
<td>54</td>
<td>No</td>
<td>White blood cells, ovary, semen</td>
<td>Cytoplasm and nucleus</td>
</tr>
<tr>
<td>ATP synthase, alpha</td>
<td>60</td>
<td>No</td>
<td>Heart, pancreas, white blood cells, ovary, plasma, saliva, serum</td>
<td>Mitochondrion, extracellular, zymogen granule</td>
</tr>
<tr>
<td>ATP synthase, beta</td>
<td>57</td>
<td>No</td>
<td>Heart, pancreas, <strong>kidney</strong>, liver, ovary, muscle, urine</td>
<td>Mitochondrion, plasma membrane, nucleolus, extracellular</td>
</tr>
<tr>
<td>Complement component C1q, B chain</td>
<td>27</td>
<td>No</td>
<td>Brain, nervous system, plasma, spinal cord, umbilical vein endothelial cell</td>
<td>Extracellular</td>
</tr>
<tr>
<td>Hydroxysteroid (17-beta) dehydrogenase 4</td>
<td>80</td>
<td>No</td>
<td>Liver, heart, prostate, testes</td>
<td>Peroxisome, cytoplasm, nucleosome, mitochondrion</td>
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<td>PDIA3</td>
<td>57</td>
<td>N</td>
<td>Liver, colon, <strong>kidney</strong>, testis, lymph node, cerebral cortex, pancreas</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>Prenylcysteine oxidase 1</td>
<td>57</td>
<td>No</td>
<td>Brain, colon, heart, <strong>kidney</strong>, liver, lung, ovary, pancreas, muscle, spleen, testis, urine</td>
<td>Lysosome</td>
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<tr>
<td>Properdin</td>
<td>51</td>
<td>No</td>
<td>White blood cells, plasma, endothelial cells,</td>
<td>Extracellular</td>
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<tr>
<td>Vimentin</td>
<td>54</td>
<td>O</td>
<td>Lacrimal gland, white blood cells, muscle, ovary, plasma, saliva, urine</td>
<td>Intermediate filament, ECM fraction, Golgi apparatus, ER</td>
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Complement receptor 1 (CR1) is another potential antigen of interest in membranous nephropathy in that in the Heymann nephritis model, antibodies that neutralized the effect of complement receptor 1-related protein y (Crry), a rodent complement regulatory protein related to CR1, were found in the sheep anti-Fx1A preparation that were necessary for causing disease. Complement receptors modulate the effects of the complement system by binding complement proteins (Lee, 2012). CR1 is involved in phagocytosis, clearance of immune complexes, and downregulation of complement. It also regulates complement pathways in human cells to protect them from damage. The only epithelial site of expression of CR1 in the kidney is at the podocyte, where it has been shown to act as a potent regulator of the alternative pathway, in addition to the classical pathway (Java, Liszewski, Hourcade, Zhang, & Atkinson, 2015). The regulatory activity of CR1 is mediated by decay accelerating factor, and only works intrinsically (only on the cell on which it was expressed). The cleavage of these complement factors also occurs with Factor I as a cofactor. It regulates the alternative pathway via a rapid initial response with decay accelerating activity, followed by degradation via cofactor activity.

Yet another autoantigen of interest was identified in a recent publication. The authors noted the existence of a potentially novel antigen associated with minor glomerular subepithelial deposits as in MN but whose major site of immune complex formation appears to be at the base of the proximal tubule, leading to an immune-mediated tubulointerstitial nephritis that caused end-stage kidney disease (Rosales et al., 2015). This condition is characterized by tubular injury and interstitial inflammation on
light microscopy; granular tubular basement membrane staining for IgG, C4d, and C3 on immunofluorescence; and electron-dense tubular basement membrane deposits staining for IgG1, but no endocapillary hypercellularity or GBM abnormality nor staining for PLA2R. Small, sparse subepithelial deposits are also observed in the glomeruli. The fact that the disease rapidly occurred after kidney transplantation, and that serum from the patient - both pre- and post-transplantation - reacted with normal human brush border on indirect immunofluorescence provide evidence for circulating autoantibodies to a brush border protein. Sera from similar cases have been made available to us, and preliminary data from another member of the laboratory (figure 4; unpublished data) has shown that it also is reactive with brush border proteins in normal neonatal rat kidney. What exactly this antigen is has yet to be determined, but megalin, CD10, and maltase (three known brush border proteins) have been ruled out based on immunofluorescence data (Rosales et al., 2015).

Figure 4. Examples of positive & negative staining for the brush border antigen (200x). Negative staining of newborn rat glomeruli for the brush border antigen with normal human serum (1:100) (left) and positive staining with patient serum (1:100) (right) are shown. Exposure time was 90 milliseconds. From Youngsoo Song, unpublished data.
OBJECTIVES

Methods developed in our laboratory have thus far utilized patient-derived autoantibodies to identify two important antigens in membranous nephropathy. Antigens targeted by patient antibodies in MN are starting to be discovered with the established protocols in place. However, the existing antigens only explain, at best, 80-85% of cases of MN and there remains a significant proportion of cases in which we do not have useful biomarkers of disease activity. There are still other antigens that could be targeted. The aim of this study is to shed light on the process of characterizing these antigens by applying the current discovery process to two such potential antigens. The specific objectives of the study are: to screen serum samples via Western blotting; to purify potential MN antigens; to identify them with mass spectrometry; and to validate their identity with immunoprecipitation and immunohistochemical analysis.
METHODS

Preparation of Human Glomerular and Tubular Extracts

Glomeruli and tubules were obtained by sieving (Fisher Scientific Nos. 140, 80, and 200) cortical portions of human kidneys that had been deemed unsuitable for transplantation from the New England Organ Bank with cold phosphate buffered saline (PBS). Glomeruli and a small proportion of intact tubular segments were retained on the final #200 sieve and were collected as the glomerular fraction (figure 9). Tubular and interstitial cortical elements that passed through the #200 sieve were also collected for use in later experiments. Proteins were extracted with radioimmunoprecipitation-assay buffer (RIPA; Boston BioProducts), and contaminating IgG was removed via incubation with Immobilized Protein G Plus (Fisher Scientific). The resulting preparations are termed human glomerular extract (HGE) or human tubular cell extract (HTE). A glomerular tris buffered saline (TBS) supernatant fraction was also prepared in which glomeruli were incubated at 37°C for 1 hour in TBS and the resultant supernatant, after centrifugation for 10 minutes at 14,000 rotations per minute (rpm), was collected. This fraction is known to contain a subcellular “shed” membrane fraction and contains both PLAr2R and THSD7A.

Western Blot

Western blotting was done on 4-15% Mini-PROTEAN TGX gels from Biorad with 25 microliters (μL) of sample loaded per lane (or 10 μL of Invitrogen See Blue Plus 2 Prestained Standard). Gels were run in 1x running buffer (Boston BioProducts). 1x non-reducing gel loading buffer (Boston BioProducts) was added to sample, and then
boiled at 95°C for five minutes, before being loaded into the gel. We also added beta-
mercaptoethanol to a final concentration of 2% prior to boiling when experiments called
for reducing conditions. Gels were run and then transferred onto nitrocellulose
membranes. Blots were then blocked in 10% milk/TBS-tween (TBST) for one to four
hours at room temperature and incubated in patient serum diluted in 10% milk/TBST at
4°C overnight.

The next day, blots were incubated for one hour at room temperature in sheep
anti-human IgG4 at 1:3000 or sheep anti-human IgG1 at 1:4000 (both from The Binding
Site) and donkey anti-sheep IgG at 1:10,000 (Jackson ImmunoResearch) with washes in
TBST in between incubations. Blots were quickly rinsed with distilled water and rocked
at room temperature in enhanced chemiluminescence (ECL) solution (20 mL Tris, pH
8.5; 100 μL luminol; 44 μL p-coumaric acid; and 5.5 μL hydrogen peroxide) for two or
three minutes. Then the blots were routinely exposed for ten seconds, thirty seconds, and
two minutes before being developed in a Kodak X-Omat developer.

Biotinylation of 58-kDa Antigen

EZ-Link™ Sulfo-NHS-Biotin (Thermo Scientific) was incubated for 30 minutes
with isolated human glomeruli in PBS. Then the biotin was washed off three times with
PBS. Next, 500 μL of 2x RIPA + protease inhibitor was added and the mixture was
incubated on ice for 40 minutes with intermittent vortexing to extract the proteins. After
centrifugation, the supernatant containing the extracted biotinylated cell-surface proteins
was incubated with streptavidin beads. Then we used a streptavidin bead pull-down to
enrich these biotinylated proteins. The streptavidin beads were washed twice with one
milliliter (mL) of PBS at 10,000 rpm, incubated for one hour at 4°C with intermittent inversion, and centrifuged to separate the beads from the product that was not pulled down. Beads were then boiled with loading buffer and loaded onto a gel for Western blotting as above. Original, pull-down, and after pull-down fractions were run on a gel for Western blotting. Samples were blotted against streptavidin-HRP (to confirm that the biotinylation worked) and anti-58-kDa positive (α-58-kDa+) serum. Streptavidin-HRP strongly reacted with biotinylated HGE pull-down only, and not HGE pull-down that was not biotinylated (data not shown).

**Biotinylation of Brush-border Antigen**

EZ-Link™ Sulfo-NHS-Biotin (Thermo Scientific) was incubated for 1 hour with an isolated human tubular fraction obtained by sieving (Fisher Scientific Nos. 140, 80, and 200) kidneys from the New England Organ Bank with cold PBS. The biotinylated tubules were resuspended in TBS, spun down, and the supernatant was removed. Hypotonic tris plus protease inhibitors was added to the tubules and frozen at -80°C for one hour. After thawing, the mixture was Dounce homogenized and 100 μL were saved and spun down for several minutes. Next, 3 mL of 2x RIPA + protease inhibitors were added and the mixture was incubated on ice for 40 minutes with intermittent vortexing. The extraction was then spun down and saved for Western blot and immunoprecipitation.

**Lectin Binding Assay**

To determine if a lectin column could be used to purify the 58-kDa antigen, approximately 300 μL of HGE was added to 500 μL of immobilized Wheat Germ Agglutinin (WGA; Vector Laboratories) and rotated at 4°C for one hour. Then the HGE
and beads were spun down, 75 μL of the supernatant were saved for a gel, and the remaining supernatant was run over concanavalin A (ConA) beads (Vector Laboratories) as above. The starting HGE fraction, HGE after the WGA column, and HGE after the ConA column fractions were run on a gel for Western blot analysis and each incubated against α-PLA₂R+, anti-THSD7A positive (α-THSD7A+), and α-58-kDa-antigen+ diluted serum samples. The HGE fractions obtained after the lectin assays represents non-lectin bound protein, thus any lectin-bound protein was expected to be on the column.

**Ion Exchange Columns**

Three different types of mini Vivapure Ion Exchange Columns (strong cation exchange, weak cation exchange, and weak anion exchange; Vivascience Sartorius Group) were used in an attempt to purify and characterize the 58-kDa antigen. First, columns were equilibrated with 400 μL of 25 mM tris/hydrogen chloride (HCl), pH 8.0 (anion exchanger) or sodium acetate, pH 5.5 (cation exchanger). Columns were centrifuged for five minutes at 2000 g at 4°C. Next, columns were loaded twice with 300 μL of HGE diluted 1:2 in the respective equilibration buffers above and centrifuged at 2000 g for five minutes. Then, columns were washed twice with 400 μL of 25 mM Tris/HCl, pH 8.0 or sodium acetate, pH 5, as above at room temperature. Finally, the columns were eluted with 400 μL of 0.5 M sodium chloride in 25 mM Tris/HCl, pH 8.0 (anion exchanger) or in 25 mM sodium acetate, pH 5.5 (cation exchanger) and centrifuged at 2000 g for five minutes. The flow through after sample loading and the final eluate were saved and run on a gel for Western blotting as above. Primary antibody to the 58-kDa antigen and THSD7A was from two different patient sera at concentrations
of 1:100 and 1:200, respectively. The blots were then detected for IgG1 and IgG4, respectively, as described above.

**Affinity Purification**

Twenty-five µL of HGE were loaded to each of eight lanes of 4-15% Mini-PROTEAN TGX gels from which a strip corresponding in size to the 58-kDa antigen was cut. Nitrocellulose membranes were incubated overnight at 4°C in α-58-kDa antigen+ serum. The following day, the membrane was washed three times for ten minutes in TBST, quickly rinsed twice with 150 nM NaCl, and eluted of antibody with 0.1 M glycine, pH 2.5, ten times in succession. The elution step was then repeated and the acid was neutralized with 1x TBS, pH 7.4, and 50 mM Tris, pH 8.0. Next, the same membranes were blocked in 10% milk/TBST for 15 minutes and then they were incubated in 1:50 dilutions of each of the two eluates for one hour at room temperature. Both blots were then incubated and developed according to the Western blot protocol above.

In order to see if we had actually been successful in eluting IgG from the membrane, we repeated a Western blot with the two eluates overnight at 4°C. In addition, each of the eluates were run on a gel and Western blotted against IgG1, 1:4000 for one hour. Finally, the affinity purification protocol was repeated with minced nitrocellulose strips incubated with acid heated to 60°C. Part of the eluted antibody was then used for Western blot, as before, and any IgG of the other part was removed with protein G beads. We used the purified antibody for Western blotting.
Partition Phase Separation

We pre-condensed the detergent Tx-114 according to established protocols (Ausubel et al., 2000) and incubated it in the presence of protease inhibitors with either whole human glomeruli or glomerular TBS supernatant, in order to separate the starting glomerular materials into a detergent and aqueous phase. The established separation process involves incubation on ice for 30 minutes, centrifugation for 10 minutes at 4°C (supernatant removed and replaced by an equal volume of ice cold TBS), incubation at 37°C until the contents turn cloudy, and centrifugation for eight minutes at room temperature. The supernatant is then removed and some was saved to run on a gel, while the rest was added to 1/5 volume of Tx-114 before repeating the process to generate a second aqueous layer. Any IgG in the aqueous layers was removed with protein G beads. Then we ran a Western blot with the original glomerular TBS supernatant fraction and the aqueous fractions against α-PLA$_2$R$^+$ and α-58-kDa antigen$^+$ sera.

Immunoprecipitation of the Brush-border Antigen and Mass Spectrometric Analysis

Incubation at room temperature for one hour of an excess of appropriate patient serum with either Protein G beads or Capture Select IgG4 (BAC BV/ThermoFisher Scientific/Life Technologies) preceded immunoprecipitation (IP) to concentrate the immunoglobulins or specifically IgG4, respectively. Then, the beads were washed with PBS to remove excess sera components and incubated with human kidney extract (glomerular or tubular) for two hours at 4°C. Following another set of washes, PBS was removed and the IP pellet was frozen at -20°C until it could be run on a gel for Western
Once the location of the appropriate band was determined with one Western blot, we cut out and sent the corresponding 250-350 kDa region of another identical gel with the immuno-precipitates from tubular extract for mass spectrometry analysis of the peptides in each patient serum and normal control serum (looking for proteins present in the IP from the case patient, and not in the normal).

**Screen of MN Sera for Reactivity with CR1**

Reagents for human CR1 were kindly provided by John Atkinson at Washington University, St. Louis, MO. CR1 lysate (in a ratio of 1:4:2 with PBS and 4x loading buffer), glomerular TBS supernatant, and HGE were run on 4-15% Mini-PROTEAN TGX gels as above and incubated in primary antibody overnight. For the lanes containing CR1 lysate, various MN sera served as the source of primary antibody (with one additional case from a patient with dense deposit disease, a glomerular disease associated with severe complement dysregulation), and sheep-anti-human IgG4 served as the source of secondary antibody. Anti-CR1 rabbit polyclonal antibody (1:5000) was incubated with the glomerular TBS supernatant and one HGE blot. The IgG4-containing blots were incubated with donkey-anti-sheep IgG as usual, and the lanes blotted with the rabbit polyclonal antibody were next incubated in anti-rabbit -HRP, 1:5000. All blots were then treated with ECL solution and exposed as above.
RESULTS

Biotinylation of the 58-kDa Antigen

According to our hypothesis that relevant target antigens in primary MN should have epitopes exposed on the cell surface, we biotinylated cells isolated from kidneys prior to extraction to label cell structures that existed on the outside of cells. Western blotting of streptavidin bead pull-downs from a biotinylated HGE and a control non-biotinylated HGE with α-58-kDa antigen+ serum specifically detected the antigen in the biotinylated reaction (Figure 5). Anti-58-kDa antigen+ serum reacted with only the biotinylated HGE pull-down as well (figure 5).

Figure 5. Biotinylation of 58-kDa Antigen. Lane 1: HGE. Lane 2: biotinylated-HGE pulled down with streptavidin beads. Lane 3: Non-biotinylated-HGE incubated with streptavidin beads. Blotted with α-58-kDa antigen+ serum primary antibody, and sheep-anti-human IgG1 secondary antibody. Arrow: 58-kDa antigen. The strong bands at 140 kDa and the weak bands at 50 kDa represent non-specific binding.
**Lectin Binding Assay**

To determine whether the 58-kDa antigen might be a glycoprotein that could potentially be purified by means of a lectin column, we sequentially ran HGE first through a wheat germ agglutinin (WGA) column and then a concanavalin A (ConA) column. The flow-through fractions after the first (WGA) and then both (WGA + ConA) types of lectin columns (i.e. what did not bind either type of column) was run on a gel and Western blotted as above against sera from three MN patients, each reactive with one of the three known autoantigens. In contrast to PLA$_2$R and to THSD7A, both of which were completely removed from HGE after incubation with the immobilized WGA beads, the 58-kDa antigen was detected in both flow-through fractions (figure 6). The successive decrease in band intensity is more likely reflective of the small amount of dilution that occurred throughout the experiment, rather than a small amount of binding to the column. This result suggests that the 58-kDa antigen does not possess glycan chains capable of binding to the WGA or ConA lectins.
**Figure 6. 58-kDa Antigen over WGA and ConA columns.** The starting HGE fraction, the flow-through from the WGA column (After WGA), and the flow-through from the ConA column (After ConA) were immunoblotted with patient serum and detected for the relevant subclass (parentheses). The α-PLA2R+ and α-THSD7A+ sera were used at 1:200; the α-58-kDa antigen+ serum was used at 1:100.

**Ion Exchange Assays**

To determine whether we could utilize the charge of the 58-kDa antigen for further purification of the protein, we ran HGE over a variety of ion exchange columns and collected flow-through and eluate fractions to run on gels for Western blot with α-58-kDa+ serum. There was early indication that the strong cation exchange column might be most effective. The experiment was run on strong cation exchange columns because it was believed that the protein was negatively charged from previous experiments (unpublished data). The experiments yielded inconsistent results, which might have been due to the age of the reagents; this line of experimentation was not pursued further.
**Affinity Purification**

To specifically immunoprecipitate the 58-kDa antigen for mass spectrometry analysis, we attempted to purify autoantibodies to the antigen via elution with acid. On the first attempt at affinity purifying autoantibodies to the 58-kDa antigen, it was found to be present with both eluates from affinity purification and milk alone. However, upon blotting the eluates with IgG1 only, the IgG was not reactive with the eluted fractions. These results were supported by repeating the experiment with mincing of the nitrocellulose strips to completely immerse the nitrocellulose in the acid solution and vigorously vortex and heat for elution. We were not able to elute the antibody from the nitrocellulose membrane in any of our attempts.

**Partition Phase Separation**

Tx-114 is a detergent with unique properties that allow it to separate out of the aqueous phase when warmed. We used phase partitioning with pre-condensed Tx-114 to assess if the 58-kDa antigen would associate with the detergent (Tx-114) phase or with the aqueous phase, to determine the relative hydrophobicity vs. hydrophilicity of the antigen. The aqueous phases, after phase partitioning extracts from whole human glomeruli or from a TBS glomerular supernatant starting preparation, were Western blotted with \( \alpha \)-PLA\(_2\)R+ and \( \alpha \)-58-kDa antigen+ sera. Anti-PLA\(_2\)R+ serum reacted with the aqueous phase (figure 7A). Anti-58-kDa antigen+ serum did as well, but the reaction was stronger against the glomerular aqueous layer than it was against the glomerular TBS supernatant aqueous layer (figure 7B). For \( \alpha \)-58-kDa antigen+ serum, the reaction was
stronger in the aqueous layer that was partitioned after using the aqueous phase of the first step with fresh pre-condensed Tx-114 (figure 7B).

Figure 7. Partition Phase Separation of PLA2R and 58-kDa Antigen. Lane 1: Glomerular TBS Supernatant, Lane 2: Glomerular TBS Supernatant Aqueous Layer 1, Lane 3: Glomerular TBS Supernatant Aqueous Layer 2, Lane 4: Glomeruli Aqueous Layer 1, Lane 5: Glomeruli Aqueous Layer 2, Lane 6: HGE.

**Proteolysis of Brush-border Antigen via Trypsin, Sensitivity to Reducing Conditions, and Content of HGE**

During our experiments designed to characterize the 58-kDa antigen, we began collaboration on a project that employed similar methods in an attempt to identify another kidney antigen targeted in an autoimmune process. Namely, our collaborators had identified a small cohort of cases associated with glomerular subepithelial and tubular basement deposits in the setting of autoimmune tubulointerstitial nephritis. Of note, all these sera contained IgG that localized to normal proximal tubular brush border, suggestive of autoantibodies to a putative brush border protein. We set out to determine if these patient sera could identify a common target antigen. Anti-brush-border antigen+ sera from four different patients were shown to interact with what appeared to be a similar antigen in an HGE preparation that was known to contain tubular elements (figures 8 and 9). However, that antigen was not detected by control or α-PLA2R+ sera.
Figure 8. Common brush border antigen amongst ABBA sera but not negative controls. Lanes 1-4: HGTE blotted with four different α-brush-border antigen+ patient sera. Lanes 5-9: Negative controls. Lane 1 incubated with sheep-anti-human IgG1 secondary antibody; others incubated with sheep-anti-human IgG4.

Figure 9. Photomicrograph of human glomerular extract with tubular elements (100x). Glomeruli are indicated with black arrows. Tubules are indicated with black arrow heads. Blue arrow heads indicate cell ghosts. From Laurence Beck, unpublished data.
In addition, experiments were run to determine the sensitivity of the brush border antigen to reducing conditions, as well as the reactivity of the antibodies with standard HGE (without contaminating tubular elements). Running HGE with tubular elements (HGTE) on a gel under reducing and non-reducing conditions and blotting against anti-brush-border antibody (ABBA)-4 serum yields a band only under non-reducing conditions (figure 10). Furthermore, there is not a visible band present on Western blot of HGE without tubular elements against ABBA-4 serum (data not shown), suggesting that the candidate antigen we have characterized is indeed from tubular cells.

**Figure 10. Brush border antigen reducing vs. non-reducing conditions.** Lane 1: HGTE, non-reducing conditions. Lane 2: HGTE, reducing conditions. Blotted with α-brush-border antigen+ patient serum, 1:100, primary antibody and sheep-anti-human IgG4 secondary antibody.
To narrow down the immunoreactive element that reacted with α-brush-border antigen+ sera, HGTE was treated with increasing amounts of trypsin ranging from 0-25% (Note: the starting (“100%”) trypsin was a cell culture preparation containing only 0.05% trypsin) and incubated at 37°C for 15 minutes before heat denaturation prior to Western blotting. When immunoblotted with ABBA-4 serum, reactive bands are observed at about 140 kDa, 120 kDa, and 95 kDa (figure 11). The band at 95 kDa appears to be stable as it is present in all the trypsinized conditions. There are no visible bands smaller than the 95 kDa band with patient serum.

Figure 11. Partial proteolysis of brush border antigen with trypsin. HGTE proteolyzed with increasing amounts of trypsin was immunoblotted with α-brush-border antigen+ patient serum (1:100) and detected for IgG4.
**Biotinylation and Immunoprecipitation of Brush Border Antigen**

Separate extracts of tubular cells, with and without biotinylation, were prepared as described in the Methods. Western blotting with streptavidin-HRP confirmed robust biotinylation of the human tubular extract (HTE). Biotinylated- and non-biotinylated-HTE were then separately immunoprecipitated with ABBA-4 serum using reagents to pull down either total IgG (protein G beads) or the IgG4 subclass alone (Capture Select anti-human IgG4 beads). The IP pellet was electrophoresed under non-reducing and reducing conditions and Western blotted with streptavidin-HRP. The antigenic band of interest appeared to be destroyed under reducing conditions when pulled down with total IgG, and became more concentrated when pulled down specifically by IgG4 (figure 12A). Biotinylated-HTE was also immunoprecipitated with purified α-PLA2R+ and normal human IgG4 as controls, and α-megalin+ IgG. Western blot with streptavidin-HRP determined that a single biotinylated band was specifically pulled down by the ABBA-4 serum, but not the α-PLA2R+ serum, the normal human serum, or the α-megalin+ antibody (figure 12B).

IPs of biotinylated and non-biotinylated HTE with ABBA-4 IgG4 as well as an IP of non-biotinylated HTE with control IgG4 were run on identical gels as in the Methods. Then, the band corresponding to the brush-border antigen was localized with streptavidin on one gel and the gel regions corresponding to the exact position of the band (from all three lanes shown in figure 12C) were excised from the other gel. Upon sending the gel regions to our collaborators at the University of Louisville for mass spectrometry analysis, several proteins were considered as candidates for the brush-border antigen.
based on the ratio of the amount of peptides identified in the patient IP to the control IP (table 2). Low-density lipoprotein receptor-related protein 2 (megalin) and sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1 (SVEP1) were the strongest candidates based on size and tissue expression. Glycosylation and cellular location were also considered.
**Figure 12. Brush border antigen IP.** Western blot with Streptavidin-HRP, 1:2500. A. Initial IP of HTE (either biotinylated and non-biotinylated) immune-precipitated with α-brush-border antigen+ IgG, or specifically, IgG4. Each IP was run in duplicate, under reducing and non-reducing conditions. B. IP of biotinylated HTE & controls. Lane 1: IP with α-brush-border antigen+ serum purified by Capture Select IgG4; (relevant band indicated with arrow) Lane 2: IP with α-PLA₂R+ serum purified by Capture Select IgG4; Lane 3: IP with normal human serum purified by Capture Select IgG4; Lane 4: IP with α-megalin+ antibody purified by Protein G. C. Identical gel of preparative IP of HTE for mass spectrometry with sera purified by Capture Select IgG4 that was blotted and detected with streptavidin; excised bands were from the corresponding lanes of a gel that was not transferred.
Table 2. List of best candidate proteins for brush-border antigen from mass spectrometry analyses (Data from “Human Protein Reference Database,” n.d.)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Size</th>
<th>Ratio ABBA+/Control</th>
<th>Glycosylation</th>
<th>Tissue Expression</th>
<th>Cellular Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha-1-antitrypsin</td>
<td>47</td>
<td>ABBA+ only</td>
<td>No</td>
<td>White blood cells, liver, mammary gland, ovary, placenta</td>
<td>Extracellular, ER, lysosome, lysosome, cytoplasm</td>
</tr>
<tr>
<td>Cluster of Ig heavy chain V-III region BRO</td>
<td>13</td>
<td>13.46</td>
<td>No</td>
<td>Plasma, saliva, urine</td>
<td>Extracellular, plasma membrane</td>
</tr>
<tr>
<td>Isoform 14 of Fibronectin</td>
<td>249</td>
<td>109.56</td>
<td>N</td>
<td>Liver, white blood cells, plasma, serum, skin, urine</td>
<td>Extracellular, ER</td>
</tr>
<tr>
<td>Isoform 2 of Fibrinogen alpha chain</td>
<td>70</td>
<td>ABBA+ only</td>
<td>N</td>
<td>Blood, liver, plasma, urine</td>
<td>Extracellular</td>
</tr>
<tr>
<td>Isoform C of Proteoglycan 4</td>
<td>141</td>
<td>ABBA+ only</td>
<td>No</td>
<td>Bone, heart, liver, lung, serum, urine</td>
<td>Extracellular</td>
</tr>
<tr>
<td>Low-density lipoprotein receptor-related protein 2 (Megalin)</td>
<td>522</td>
<td>277.36</td>
<td>No</td>
<td>Brain, fetus, kidney, lung, parathyroid, placenta, proximal convoluted tubule, urine</td>
<td>Plasma membrane, endosome</td>
</tr>
<tr>
<td>Protein IGHV3-30 (fragment)</td>
<td>13</td>
<td>ABBA+ only</td>
<td>No</td>
<td>Plasma, saliva, urine</td>
<td>Extracellular, plasma membrane</td>
</tr>
<tr>
<td>Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1</td>
<td>390</td>
<td>ABBA+ only</td>
<td>N and O</td>
<td>Placenta, marrow</td>
<td>Cytoplasm, membrane, extracellular</td>
</tr>
</tbody>
</table>
Screen of MN Sera for Reactivity with CR1

Finally, we took a candidate antigen approach to screen for other potential autoantibodies in MN. Because antibodies to Crry (similar to human CR1) were found in the sheep anti-Fx1a preparation and were necessary for causing disease in the Heymann nephritis model, we hypothesized that CR1 might be a relevant target antigen in human primary MN that might exacerbate complement-mediated injury. We assayed serum from patients with PLA2R-associated and non-PLA2R-associated MN for reactivity with recombinant CR1. Upon Western blotting α-PLA₂R+ sera against CR1 lysate, none of the serum reacted with the lysate (figure 13). In addition, α-CR1+ antibody did react with glomerular TBS supernatant fraction and HGE (figure 13).

![Figure 13. CR1 reactivity screen.](image)

*Figure 13. CR1 reactivity screen.* Lane 1: glomerular supernatant fraction. Lane 2: HGE. Lanes 3-11: recombinant CR1 cell lysate. Lanes 1 & 2 blotted with α-CR1+ antibody; lanes 3-10 blotted with MN sera; lane 11 blotted with serum from a patient with dense deposit disease.
DISCUSSION

Using lines of experimentation similar to those used to identify PLA₂R and THSD7A as the first two target antigens in adult primary MN, we have been able to better characterize other kidney-derived antigenic targets of human autoantibodies and have made significant headway into identifying one of them. It was determined that one antigen of interest, a 58-kDa antigen that is recognized by sera from several patients with MN, was biotinylated, implying that the antigen is likely to be extracellular. Our work on the 58-kDa antigen suggests that it is extracellular, not heavily glycosylated, and not a transmembrane protein. We made a novel discovery that cases of autoimmune tubulointerstitial nephritis with tubular basement membrane deposits all seem to have autoantibodies to a common antigen. We have made significant progress in identifying the actual antigen, with only a small number of likely candidate proteins, including megalin and SVEP1.

Antigens found on the outside of cells, such as PLA₂R and THSD7A, are of interest because they would be interacting with antibodies, so we first chose to confirm the extracellular location of these putative autoantigens via biotinylation. Biotinylation of extracellular structures was successful as shown by the reactive binding of streptavidin-HRP to biotinylated HGE only, as opposed to HGE that was not biotinylated and thus not reactive with streptavidin-HRP. It was determined that the antigen of interest (58-kDa) was indeed biotinylated by the reaction of the α-58-kDa+ patient serum with the biotinylated HGE only. From the successful biotinylation of the 58-kDa antigen, we concluded that this antigen in fact contained portions on the outside of cells, and was
therefore potentially interesting as an extracellular epitope capable of being targeted by autoantibodies.

Wheat germ agglutinin and concanavalin A are lectins that bind membrane proteins that are N-glycosylated (Pohleven et al, 2012). The lack of binding of the 58-kDa antigen to either type of lectin binding column tested suggests that it is not highly glycosylated. The validity of this experiment comes from the fact that neither PLA2R nor THSD7A (both of which are known N-glycosylated glycoproteins) were present in the flow through as detected by Western blot with the appropriate human serum. This experiment allowed for further characterization of the 58-kDa antigen, as we now know that it is not likely to be N-glycosylated, and offers a method for partial purification of the antigen by separating it from the bulk of glomerular glycoproteins.

Our inconsistent results with ion exchange columns did not add to the characterization of the 58-kDa antigen or offer a potential avenue for further partial purification. These contradictory results could be due to the columns used being past their expiration date or an inappropriate equilibration buffer being used. After attempting to affinity purify the α-58-kDa antigen+ autoantibody, which would have been able to specifically IP the antigen for mass spectrometry purposes, it was suspected that the antigen-bound antibody was not eluted off the nitrocellulose membrane from the reactivity of both α-58-kDa antigen and milk only with the eluted fractions. This was confirmed by the lack of reactivity in the presence of IgG1 only. Additionally, because immunoblotting is a very sensitive technique and may overestimate the mass of antigen and/or autoantibody present, the actual amounts of eluted, affinity purified autoantibody
may have been miniscule and therefore undetectable using the small-scale preparation that we used.

Our TX-114 phase separation experiments were intended to distinguish hydrophobic transmembrane proteins that would partition in the detergent phase, from hydrophilic soluble proteins that would partition into the aqueous phase. The mild, non-ionic Tx-114 detergent gently solubilizes membrane proteins and separates them from hydrophilic proteins via phase partitioning above 20°C (the detergent is homogeneous at 0°C; Qoronfleh, Benton, Ignacio, & Kaboord, 2003). This results in an aqueous layer of hydrophilic (peripheral and cytosolic) proteins and a detergent layer of integral membrane proteins. The fact that α-PLA₂R+ serum reacted with the aqueous layer obtained by partition phase separation indicates that the protocol for this experiment may need to be enhanced with additional partitioning steps. It is known that PLA₂R is a transmembrane protein, and thus α-PLA₂R+ serum should not have reacted with the aqueous layer. However, the fact that α-58-kDa+ serum reacted more strongly against the second phase partitioned aqueous phase than it did with the first may indicate that the phase partition separation was concentrating the 58-kDa antigen into the aqueous layer and that it is not a trans-membranous protein. This is consistent with previous work in the laboratory (unpublished data) suggesting that the 58-kDa antigen was a membrane-associated protein that could be solubilized using pH 9.5 sodium carbonate buffer. The fact that the antigen signal is stronger in the glomerular sample than in the glomerular TBS supernatant sample via Western blot may simply be due to the antigen being more
concentrated in the glomeruli itself and not shed in the membrane particles thought to comprise the TBS glomerular supernatant fraction.

The next steps in the identification of the 58-kDa antigen may include an initial partial purification with lectin columns, followed by phase partitioning (since that was shown to contain the 58-kDa antigen and it is purer than the original HGE product), and then immunoprecipitation. We could also try adding an extra partitioning step to this purification experiment for added purity. Then we could remove any contaminating IgG with Protein G beads and have a suitable product to send for analysis via mass spectrometry.

During my experiments designed to characterize the 58-kDa antigen, we were fortunate enough to collaborate on a project that employed similar methods in an attempt to identify another kidney antigen targeted in an autoimmune process. A case report describing autoantibodies to a brush border antigen demonstrated that these antibodies were associated with tubular immune deposits as well as minor subepithelial glomerular deposits (Rosales et al., 2015). We decided to pursue the identification of this new candidate antigen based on techniques that had been successful in the identification of PLA$_2$R and THSD7A were identified. Our first significant finding was that there is a common antigen (according to banding on Western blot) that is recognized by 100% of the ABBA sera. This antigen is even more prominent among ABBA sera than PLA$_2$R is among MN sera, with only 75-80% of cases having reactivity.

The proteolysis of the brush border antigen with trypsin yielded an apparently stable band at about 95 kDa that was theorized to contain the immunoreactive epitope to
be immunoprecipitated for mass spectrometry. The lack of reactive bands on Western blot under reducing conditions and with HGE indicates that the brush border antigen is sensitive to reducing conditions as well as present in tubules. The sensitivity of the antigen under reducing conditions could be explained by the biotin chains becoming less available to streptavidin when the polypeptide chain is linearized by boiling and reduction. Or perhaps reduction makes the antigen more “sticky” and it gets entangled in the beads, and does not make it onto the gel. The single biotinylated band specifically pulled down by the α-brush-border antigen+ serum, but not the α-PLA2R+ serum nor the normal human serum, was determined to be useful for preparative IP and subsequent mass spectrometry analysis.

Mass spectrometry analysis of HTE immune-precipitated with ABBA-4 serum provided a list of potential candidates for the brush-border antigen. Out of the proteins with peptides that were only found in the immune-precipitates from the patient, and not in the control, SVEP1 was the only one with a molecular mass close to that of the band we observed on Western blot. However, SVEP1 is not detected in kidney brush border; but rather, it is a ligand for an integrin (Sato-Nishiuchi et al., 2012). It is also unlikely that the antigen is an isoform of fibronectin, because that is not expressed in kidney tissue. So, that leaves the other antigen within a reasonable size range: megalin. Megalin was predominantly found in the immune-precipitates from the patient rather than in that from the control. While megalin is in fact larger than the protein band we identified, it was originally thought to be a 330 kDa protein, but is in fact closer to 600 kDa (Saito, Pietromonaco, Loo, & Farquhar, 1994). Although megalin was initially ruled out as the
brush-border antigen by immunofluorescence, there could be a distinct form or portion of megalin recognized by patient serum but not commercial antibody, as is the case with THSD7A. Patient serum also failed to detect an antigenic band immune-precipitated with the commercial antibody, but it has not actually been confirmed that the antibody works for IP, similar to how it does not work in Western blot. Therefore, while megalin is the strongest candidate for the brush-border antigen at this point, this and other potential candidates will need to be tested further.

With the mass spectrometry results analyzed, we can proceed with the next steps in identifying the brush border antigen. These next steps may include Western blotting with commercial antibodies of proteins determined to be present by mass spectrometry or transfecting cells with cDNA clones of the protein sequence and extracting proteins from the cells grown up expressing the antigen. In the meantime, we may also want to run HTE over WGA or ConA columns and blot with α-brush-border antigen+ patient serum to determine whether the brush border antigen is a glycoprotein that may be bound by a lectin column. We could also do more IPs with serum from other patients with this disease to get material for mass spectrometry. Looking forward, the positive staining of rat glomeruli with human α-brush-border antigen+ serum is promising for potential use of an animal model of membranous nephropathy elicited by autoimmunity to brush border antigens.

Since anti-Crry antibodies were present in the anti-Fx1A preparation necessary to cause Heymann nephritis in the rat model, we decided to screen a group of MN sera to determine whether or not they contained antibodies to CR1. In fact, none of the sera
reacted to the CR1 lysate run on a gel for Western blot. In addition, anti-CR1+ antibody reacted with HGE and the glomerular TBS supernatant fraction. However, autoantibodies to CR1 in podocytes does not appear to be the case in the human primary MN cases we tested.

Here we have applied a technique that has been shown to be instrumental in discovering new autoantibody-autoantigen systems in renal disease. This technique involves screening serum samples via Western blotting; purifying potential MN antigens; identifying them with mass spectrometry; and validating their identity with immunoprecipitation and immunohistochemical analysis. This process affords the possibility of new biomarkers, insight into pathomechanisms of human diseases, and therapy that targets antigens responsible for disease.
REFERENCES


VITA

PAIGE COLES
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EDUCATION
Boston University, Boston, MA
Masters of Science in Medical Sciences May 2016
Thesis: Characterization of Novel Antigens in Membranous Nephropathy
Bachelor of Arts in Biology May 2014
Specialization in Cell Biology, Molecular Biology, and Genetics
Awards/Achievements:
Cardinal Medeiros Full-Tuition Merit Scholarship recipient September 2010-May 2014
Undergraduate Research Opportunity Program award recipient June 2012-May 2014
Saint Clement High School, Medford, MA May 2010
Academic Awards/Achievements: Class valedictorian

SKILLS
Lab skills Design, execution, and analysis of experiments; data analysis; Western blotting; mammalian cell culture; immunoprecipitation; ELISA; histochemical analysis/microscopy; gel electrophoresis; PCR; nucleic acid purification/column chromatography; DNA miniprep; preparation of solutions and reagents; oral presentation of progress with experiments at weekly lab meetings

LAB EXPERIENCE
Boston University School of Medicine/Boston Medical Center Research Assistant, Nephrology Department May 2015-present
Applied immunology techniques:
- Western blot execution and analysis
- SDS-PAGE gel electrophoresis
- Protein purification
- Immunoprecipitation
- ELISA
- Preparation of solutions and reagents
Mammalian Cell Culture:
- Cell types: Human podocytes and inner medullary collecting duct cells
- Cytotoxicity assays and cell staining/microscopy
Boston University, Boston, MA
Undergraduate Research Opportunity Program
June 2012- May 2014
Applied biochemistry/molecular biology techniques:
- PCR
- Gel electrophoresis
- Nucleic acid purification/column chromatography and DNA miniprep
- Preparation of solutions and reagents

CLINICAL EXPERIENCE
Boston University/Massachusetts General Hospital
Study Monitor, Biomedical Engineering Department
May 2014-August 2014
Monitored bionic pancreas devices for patients with Type I diabetes

Boston University School of Medicine/ Boston Medical Center
Research Assistant, Rheumatology Department
May 2013- May 2014
Assisted study coordinators with consenting scleroderma patients into research studies
Centrifuged blood samples for processing and assisted physicians with skin biopsies

VOLUNTEER EXPERIENCE
Brigham and Women’s Hospital, Boston, MA
Volunteer, Medical Career Exploration Program
January 2011-October 2013
Supported nurses in the Neonatal ICU and cradled newborn infants
Escorted patients to surgery and through security
Provided companionship to patients
Shadowed a physician for morning rounds

OTHER EXPERIENCE
Boston University, Boston, MA
Mentor
September 2012- May 2013
Met with freshmen on a weekly basis to give them guidance

Learning Assistant, Introductory Biology Department
September 2011-May 2012
Applied knowledge of STEM education to weekly biology lab sections

Camp Six Acres, Medford, MA
Counselor
Summers June 2008-August 2011
Provided leadership for a group of approximately 25 girls
Taught and supervised campers in various sports and activities
Served as a role model and built the campers’ confidence and self-esteem

ACTIVITIES/ LEADERSHIP
Boston University, Boston, MA
Vice President and Physician Mentoring Program Coordinator, Premedical Society
September 2012-May 2014
Worked with fellow e-board members to organize events including Specialty Nights, Information Sessions, Volunteer Opportunities;
Organized the Physician Mentoring Program