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Molecular interactions in the retinal basement membrane system: A proteomic approach

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ABSTRACT

Basement membranes (BMs) are physiologically insoluble extracellular matrix sheets present in all multicellular organisms. They play an important role in providing mechanical strength to tissues and regulating cell behavior. Proteomic analysis of BM proteins is challenged by their high molecular weights and extensive post-translational modifications. Here, we describe the direct analysis of an *in vivo* BM system using a mass spectrometry (MS) based proteomics approach. Retinal BMs were isolated from embryonic chick eyes. The BM macromolecules were deglycosylated and separated by low percentage gradient SDS PAGE, in-gel digested and analyzed by LC-MS/MS. This identified over 27 extracellular matrix proteins in the retinal BM. A semi-quantitative measure of protein abundance distinguished, nidogens-1 and -2, laminin subunits α1, α5, β2, and γ1, agrin, collagen XVIII, perlecan, FRAS1 and FREM2 as the most abundant BM protein components. Laminin subunits α3, β1, γ2, γ3 and collagen IV subunits α5 and α6 were minor constituents. To examine binding interactions that contribute to the stability of the retinal BM, we applied the LC-MS/MS based approach to detect potential BM complexes from the vitreous. Affinity-captured nidogen- and heparin-binding proteins from the vitreous contained >10 and >200 proteins respectively. Comparison of these protein lists with the retinal BM proteome reveals that glycosaminoglycan and nidogen binding interactions play a central role in the internal structure and formation of the retinal BM. In addition, we studied the biomechanical qualities of the retinal BM before and after deglycosylation using atomic force microscopy. These results show that the glycosaminoglycan side chains of the proteoglycans play a dominant role in regulating the thickness and elasticity of the BMs by binding water to the extracellular matrix. To our knowledge, this is the first large-scale investigation of an *in vivo* BM system using MS-based proteomics.

1. Introduction

Basement membranes (BMs) are extracellular matrix sheets present in all multicellular organisms. They serve as an adhesive substrate for epithelial and muscle cells, sequester growth factors and provide mechanical strength to tissues and organs (Sanes, 2003; Yurchenco et al., 2004). The importance of BMs in stabilizing tissue walls under stress is particularly evident by the frequent ruptures of blood vessels in mice and humans with mutations of BM proteins (Costell et al., 1999; Halfter et al., 2002; Gould et al., 2005). The high biomechanical strength is reflected in the composition of BMs, which comprise proteins that polymerize and form mega-Dalton supramolecular complexes (Timpl and Brown, 1996; Erickson and Couchman, 2000; Sanes, 2003). Direct biochemical analysis of BM proteins has mostly been limited to tumor-derived BM protein complexes (Kleinman and Martin, 2005), and much of our current understanding of *in vivo* BM composition is based on immunocytochemistry approaches (Kabosova et al., 2007). These studies have shown that laminin 111, nidogen-1, collagen IV and the proteoglycan perlecan are dominant BM proteins (Timpl and Brown, 1996). Additional BM proteins belonging to the laminin, nidogen, collagen and proteoglycan families have been identified using monoclonal antibodies and homology cDNA cloning (Erickson and Couchman, 2000). Recently, Manabe et al.

Abbreviations: BM, basement membrane; ILM, inner limiting membrane; SDS PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; emPAI, exponentially modified protein abundance index; IPI, International Protein Index; mAb, monoclonal antibody; LC-MS/MS, liquid chromatography nano electrospray tandem mass spectrometry; QqToF MS, Quadrupole quadrupole time of flight mass spectrometry; FRAS1, FREM2, Fraser syndrome 1 protein, Fraser syndrome-related extracellular matrix protein 2; HSPG, Heparan sulfate proteoglycan; GAG, Glycosaminoglycan.

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(2008) used a transcriptome-based search to identify new ECM proteins, followed by a large-scale immunocytochemistry mapping project that employed antibodies developed against these proteins. Drawbacks of using immunocytochemistry are the dependence on antibodies that may vary in quality and specificity, the restriction to proteins that have previously been identified as potential candidates, the inability to infer protein stoichiometries, and failure to detect type, site and extent of post-translational modifications.

Mass spectrometry based proteomics presents an unbiased approach for directly analyzing proteins from complex mixtures. Typically, proteins are separated with 1D/2D gels, in-gel trypsin digested, and proteolyzed peptides are analyzed with MALDI TOF-MS/MS or LC-MS/MS. Alternatively, a complex protein mixture may be in-solution trypsin digested and fractionated by multidimentional chromatographic techniques before MS analysis. High-throughput protein identification is achieved by database searching of the tandem mass spectra (MS/MS). These 'shotgun' proteomic approaches require efficient protein solubilization and digestion, both of which pose a challenge in the case of ECM proteins (Hansen et al., 2009; Wilson et al., 2009). Another obstacle to BM proteomics is the dynamic range issue, where, proteins present at a higher abundance in a complex mixture are detected while those at a lower abundance go undetected.

BMs are present as 50–200 nm thin sheets sandwiched between layers of epithelial tissue and connective tissue and therefore crude mixtures of BM tissues are dominated by the presence of cellular and non-basement membrane connective tissue proteins. Thus, any strategy for proteomic analysis of *in vivo* BMs would need to incorporate some type of enrichment technique for BM proteins, fractionation to reduce complexity of the resulting protein mixture and an efficient method for proteolytic digestion. We addressed this by a) isolating an *in vivo* BM, b) deglycosylating the BM proteins to remove GAG side chains, and c) separating the BM proteins with low percentage gradient SDS PAGE, followed by in-gel trypsin digestion and analysis of the proteolyzed peptides by LC-MS/MS.

In the present study, we used the embryonic chick retinal BM as the model system for comprehensive proteome analysis using mass spectrometry. The retinal basement membrane, also referred to as the inner limiting membrane (ILM), is structurally, compositionally and functionally a typical BM: it shares the ultrastructure and major protein constituents with other BMs (Halfter et al., 2000; Libby et al., 2000; Candiello et al., 2007), and it ruptures, like other BMs do, in mice, zebrafish and humans with mutations of BM proteins (Halfter et al., 2005a,b; Lee and Gross, 2007). The ILM is located at the border between the neural retina and the vitreous body, the gel-like extracellular matrix structure that fills the vitreous chamber of the eye. A unique experimental advantage for proteome analysis and biophysical measurements is that the ILM can be separated from the vitreous and, thus, the ILM is the one of the few BMs that can be prepared without being contaminated with extracellular matrix proteins from the adjacent connective tissue (Halfter and Von Boxberg, 1992). The mass spectrometry-based proteomics and Western blot analysis, as presented here, allowed us, for the first time, to calculate the relative abundance of individual proteins in BMs *in situ*. Since most ILM proteins have heparin-binding sites and originate from the lens and ciliary body and are secreted into the vitreous (Sarthy and Fu, 1990; Dong and Chung, 1991; Sarthy, 1993; Halfter et al., 1997; Halfter et al., 1998; Dong et al., 2002; Balasubramani et al., 2004), the proteome of heparin-binding proteins from vitreous was established as well. Further, to determine if ILM proteins occur as protein complexes in the vitreous for rapid ILM assembly as the eye expands during development, we also determined the proteome of a nidogen-affinity fraction from the vitreous. While specific functions have been assigned for most of the BM proteins, it is unclear why BMs include multiple proteoglycans. By using atomic force microscopy (AFM), we found that proteoglycans play a major role in determining the elasticity and thickness of BMs by binding large quantities of water.

The current study shows that an MS-based approach can be applied for a comprehensive compositional analysis of BMs. It is technically feasible to apply our approach to mouse or human tissues. MS-analysis of BMs provides a catalog of proteotypic peptides that is useful in developing targeted MS-based protein assays to monitor protein levels in body fluids and tissues. Such quantitative measurements of BM proteins will allow detection of BM protein changes associated with disease.

2. Results

Fig. 1 depicts the proteomic approach taken to analyze the retinal BM and its binding interactions. Four compartments were investigated in the embryonic chick eye: (i) isolated retinal BMs, (ii) vitreous body, and two subproteomes of the vitreous, (iii) affinity-purified heparin binding proteins and (iv) immunoisolated nidogen complexes.

2.1. Retinal basement membrane preparation and mass spectrometry

ILMs (Fig. 2A) were prepared by detergent treatment of dissected embryonic retinae (Halfter and Von Boxberg, 1992). In contrast to the retinal cells, BMs are insoluble in detergent, and the ILMs were collected from the Triton X-100 solution under dark field illumination (Fig. 2B). The isolated ILMs were brightly labeled by staining for laminin, a protein that is abundant in all BMs (Fig. 2C). Transmission electron microscopy (TEM) revealed that the isolated ILMs were 70 nm-thin sheets of ECM that were cell and organelle-free (Fig. 2 D) and ultra-structurally identical to ILMs *in situ* (Fig. 2E). To experimentally verify that detergent does not cause a loss of BM proteins or a degradation of the BMs we resorted to human lens capsules that can be obtained by dissection alone. Coomassie-stained SDS gels and Western blots showed that detergent-treatment of lens capsule BMs did not solubilize BM proteins. Further, we used the detergent-treated and non-treated lens capsules as substrate for axonal outgrowth to test whether the detergent treatment denatures the BM by destroying its cell adhesive properties. Results showed that this was not the case, since there was the same extensive neurite outgrowth on the detergent-treated and the non-treated lens capsule substrates (Supplemental data, Fig. S1).

To make the BMs suitable for proteomic analysis, heparan sulfate and chondroitin sulfate lyases were used to digest the GAG side chains of proteoglycans. Deglycosylation did not result in a significant loss of proteins nor alter the macroscopic appearance of BMs (Fig. 7D–G), rather resulted in a greater solubility of the BM proteins in SDS sample buffer, as evident from the greater staining intensity of electrophoresed protein bands. Further, proteoglycans appeared as discrete bands corresponding to their core protein mass, instead of a diffuse smear due to GAG heterogeneity. To reduce sample complexity for LC-MS/MS, the entire gel lane was fractionated into 10–20 slices. Each slice was in-gel digested with trypsin. An aliquot of the proteolyzed peptides was analyzed by online liquid chromatography nanoelectrospray using a hybrid quadrupole time-of-flight mass spectrometer (QqTOF MS, QSTAR Elite, ABI/Sciex). To obtain a representative BM proteome list, we used four separate preparations of BMs for analysis by SDS PAGE and LC-MS/MS (QqTOF MS). Each preparation used BMs isolated from ~200 embryonic chick retinae. One sample preparation was also analyzed on another analytical platform using a 3D ion trap mass spectrometer (QIT MS, LCQ Deca, Thermo).

2.2. Database searching and ProteinCenter analysis

The LC-MS/MS data were analyzed with database search programs for peptide and protein identification: QqTOF MS data were submitted to ProteinPilot software, and analyzed with two independent database search programs, Paragon-ProGroup and Mascot,