

## The Dominant Humoral Epitope in Phospholipase A<sub>2</sub> Receptor-1: Presentation Matters When Serving Up a Slice of $\pi$

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Phospholipase A<sub>2</sub> receptor-1 (PLA<sub>2</sub>R), the major autoimmune target in primary membranous nephropathy,<sup>1</sup> is one of four mammalian members of the mannose receptor (MR) family, which consists of the MR (encoded by the *MRC1* gene), Endo180 (*MRC2*), DEC205 (*LY75*), and PLA<sub>2</sub>R itself.<sup>2</sup> An additional member, the FcY receptor, is present in avian species and most closely related to PLA<sub>2</sub>R.<sup>3</sup> All are transmembrane glycoproteins with extracellular regions that consist of an N-terminal cysteine-rich (CysR) domain, a fibronectin II-type (FnII) domain, and 8–10 C-type lectin-like domains (CTLDs).

Early studies characterizing the autoantibody–antigen interaction noted that reactivity to PLA<sub>2</sub>R was abolished if the Western blot (WB) was performed under reducing conditions,<sup>1</sup> which suggested that disulfide bonds were crucial for stabilizing the conformation of the humoral epitope(s). The fact that other MR family members underwent conformational shifts in response to changes in pH<sup>2,4</sup> also gave credence to a potential conformational epitope in PLA<sub>2</sub>R. Finally, the identification of several disease-associated single nucleotide polymorphisms within the *PLA2R1* gene<sup>5–7</sup> raised the possibility that a variant sequence might confer an altered configuration to PLA<sub>2</sub>R that might be recognized as foreign by the immune system. However, this speculation has since fallen out of favor, because additional sequence analysis has not identified any nonsynonymous single nucleotide polymorphisms unique to PLA<sub>2</sub>R-associated membranous nephropathy.<sup>8</sup>

In this issue of *JASN*, two groups hone in on the location of the major humoral epitope in PLA<sub>2</sub>R. Kao *et al.*<sup>9</sup> elegantly show that the epitope is within the three most N-terminal domains of the protein and shares the reduction-sensitive

properties initially noted for the entire molecule. Fresquet *et al.*<sup>10</sup> go one step farther to localize the epitope to within the CysR domain. Before these reports, there was limited evidence about the position or number of relevant epitopes within PLA<sub>2</sub>R. It had been shown that preincubation of PLA<sub>2</sub>R with human autoantibodies did not interfere with binding of a well used commercial antibody generated against an epitope in the CTLD2–3 region.<sup>11</sup> Behnert *et al.*<sup>12</sup> identified several linear peptides within PLA<sub>2</sub>R that were reactive with anti-PLA<sub>2</sub>R-positive sera in an SPOT assay; several of these peptides were also able to partially inhibit autoantibody binding to the nondenatured molecule.

The core consensus domain of CysR is the ricin B domain, which was first identified as a repeated sugar-binding region in the B chain of the plant-derived toxin ricin. This evolutionarily conserved domain is thought to have arisen from gene triplication of an ancient galactoside-binding peptide, resulting in its current three-lobed ( $\beta$ -trefoil) structure.<sup>13</sup> The ricin B domain exists in several contexts in mammalian proteins—most notably the N-terminal CysR domains of the MR family members and the C-terminal ricin B domains of the numerous polypeptide N-acetylgalactosaminyltransferases present in the Golgi. Both groups exhibit six conserved cysteine residues as opposed to the four residues in ricin B. The crystal structure of the MR CysR reveals three disulfide bonds, two of which are involved in internally stabilizing the individual lobes and the third links the final lobe to the extended structure of the molecule.<sup>14</sup>

Because of differences in nomenclature between the two works<sup>9,10</sup> and for the purpose of describing the various PLA<sub>2</sub>R constructs used in these articles, I will abbreviate the CysR (or Ricin B) domain as R, the FnII domain as F, and the CTLD repeats as C<sub>x–y</sub>. The full extracellular domain would, therefore, be designated as RFC<sub>1–8</sub>.

Kao *et al.*<sup>9</sup> have masterfully engineered a full panel of PLA<sub>2</sub>R constructs and show that anti-PLA<sub>2</sub>R autoantibodies seem to exclusively recognize a reduction-sensitive epitope localized to the three most N-terminal domains, RFC<sub>1</sub>. Kao *et al.*<sup>9</sup> could not further delimit the epitope-containing region because of the instability of the individual domains. However, deleting the first CTLD from the otherwise intact extracellular domain (*i.e.*, RFC<sub>2–8</sub>) was sufficient to abolish all autoantibody binding by WB, suggesting an important role for this domain in stabilizing the structure of the humoral epitope. Of interest, introduction of the M292V or H300D risk alleles into CTLD1 did not, within the limits of the assay, affect binding. By artificially introducing proteolytic cleavage sites between the domains of RFC<sub>1</sub>, Kao *et al.*<sup>9</sup> could selectively remove domains on either end (CysR or CTLD1); however, the smaller pieces did not retain antigenicity by WB. This line of experimentation also provided

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evidence that FnII and CTLD1 might be linked by an interdomain disulfide bridge. Kao *et al.*<sup>9</sup> conclude that the RFC<sub>1</sub> structure is the smallest portion of PLA<sub>2</sub>R that allows accurate presentation of the epitope by WB because of an ill-defined interdependency of the three domains. This result was strengthened by the fact that soluble RFC<sub>1</sub> could block autoantibody binding to the full extracellular domain.

The article by Fresquet *et al.*<sup>10</sup> reveals that there are limitations to a comprehensive characterization of antibody–antigen interactions imposed by the typical WB protocol. Before SDS-PAGE, proteins are denatured with heat and detergents, disrupting tertiary structure. This is in contrast to other assay systems, such as ELISA, that allow detection of antigen in a more physiologic native state. Just as the routine performance of WB under reducing conditions may have delayed identification of PLA<sub>2</sub>R as the major antigen in membranous nephropathy, the reliance on WB alone to detect discrete epitopes in PLA<sub>2</sub>R may have resulted in an incomplete story.

Fresquet *et al.*<sup>10</sup> use a variety of experimental systems to assess autoantibody binding to PLA<sub>2</sub>R and ultimately, define the major humoral epitope within the CysR domain proper. Like Kao *et al.*,<sup>9</sup> they were unable to find reactivity by WB in small N-terminal constructs; in their hands, RFC<sub>1–3</sub> was the smallest construct identified under denaturing WB conditions, because reactivity was lost with RFC<sub>1–2</sub>. However, when these same constructs were assayed under the nondenaturing conditions of a slot blot, anti-PLA<sub>2</sub>R autoantibodies detected RFC<sub>1–2</sub> reactivity and more importantly, the isolated CysR domain. The larger constructs RFC<sub>1–3</sub> and RFC<sub>1–8</sub> were able to maintain the structure of the epitope, even in the face of denaturation or reduction, hinting at interdomain interactions within the context of the larger molecule. Experiments using surface plasmon resonance (SPR) reveal remarkably consistent binding affinities of purified anti-PLA<sub>2</sub>R to CysR and the larger constructs, strongly suggesting that, for the majority of sera tested by SPR, the autoantibody binds primarily to CysR and not to other locations within the extracellular domain.

In a parallel set of experiments, Fresquet *et al.*<sup>10</sup> perform mass spectrometric analysis of the smallest common proteolytic fragments of PLA<sub>2</sub>R reactive with autoantibodies by WB. Fresquet *et al.*<sup>10</sup> find that only two of the identified peptides, both from a similar region of CysR, can significantly inhibit anti-PLA<sub>2</sub>R binding to RFC<sub>1–3</sub>. When they add the intervening sequence of PLA<sub>2</sub>R to synthesize a 31-residue stretch roughly corresponding to the first lobe within the  $\beta$ -trefoil structure of CysR, Fresquet *et al.*<sup>10</sup> discover that they have recreated the dominant epitope of PLA<sub>2</sub>R. It is remarkable that not only can this 31-mer inhibit anti-PLA<sub>2</sub>R binding to RFC<sub>1–3</sub> by 85% but that it also exhibits a binding affinity identical to that of the larger constructs as determined by SPR and has reduction-sensitive properties because of an internal disulfide bond.

Fresquet *et al.*<sup>10</sup> additionally use two techniques to show that, similar to its MR family counterparts, PLA<sub>2</sub>R has pH-dependent changes in configuration, behaving as a more

compact structure at acidic pH. Although this change does not affect binding of anti-PLA<sub>2</sub>R, it is possible that such conformational change, possibly in conjunction with one or more disease-associated sequence variants, could influence processing of PLA<sub>2</sub>R within the acidic endosomes and lysosomes of an antigen-presenting cell. Finally, Fresquet *et al.*<sup>10</sup> use transmission electron microscopy and single-particle averaging to generate low-resolution structural models of PLA<sub>2</sub>R. There is evidence that CysR and CTLD3 make contact to form a ring-like structure and that the overall PLA<sub>2</sub>R structure resembles the Greek character  $\pi$ .

Both articles provide evidence that, in the majority of cases, the epitopic region in the N-terminal portion of PLA<sub>2</sub>R explains all of the reactivity of anti-PLA<sub>2</sub>R–positive sera.<sup>9,10</sup> In constructs lacking the three N-terminal domains, Kao *et al.*<sup>9</sup> fail to detect reactivity to the residual domains of the molecule. In a cohort of serum samples from patients with primary membranous nephropathy, reactivity to RFC<sub>1</sub> was almost entirely concordant with reactivity to the whole molecule. Although this analysis was performed by WB with the caveats above, it is reassuring that Fresquet *et al.*<sup>10</sup> provide confirmatory data, with one exception: 10% of the subjects in the cohort described by Fresquet *et al.*<sup>10</sup> do not exhibit inhibition by RFC<sub>1–3</sub> of anti-PLA<sub>2</sub>R reactivity with the full molecule, suggesting that one or more epitopes may exist in the more C-terminal portion of the molecule. The location of these minor epitopes and the percentages of PLA<sub>2</sub>R-associated cases associated with antibodies to these minor epitopes will need to be better determined. For now, existing immunoassays containing the entire extracellular domain should be sufficient to detect all anti-PLA<sub>2</sub>R antibodies. Neither work shows any strong evidence for epitope spreading (*i.e.*, additional subdominant epitopes that might appear later in the course of disease), although this has not been specifically investigated in either of the articles.<sup>9,10</sup>

The end result of these studies is that there is a dominant humoral epitope in the very N-terminal region of PLA<sub>2</sub>R with exposure that is clearly affected by experimental conditions and potentially, physiologic conditions as well. Such results move the field forward at a number of levels. By identifying a discrete region that seems to confer the majority of reactivity with anti-PLA<sub>2</sub>R autoantibodies, hypotheses about the initial events in the autoimmune process can better be proposed and studied. Fresquet *et al.*<sup>10</sup> note that the nine-amino acid peptide within their 31-mer that itself is capable of inhibiting 47% of binding shares homology with a bacterial enzyme, thus invoking molecular mimicry as a potential initiator of autoimmunity. Although humoral (B cell) epitopes are not equivalent to T cell epitopes, Fresquet *et al.*<sup>10</sup> propose a hypothetical model of how B cells, initially reactive with a specific bacterial antigen, might subsequently internalize and process a similar-appearing PLA<sub>2</sub>R, such that specific peptides are loaded into class II HLA molecules encoded by specific risk alleles (*e.g.*, HLA-DQA1) in membranous nephropathy. In addition, if the identified region turns out to be the primary and dominant humoral epitope responsible for disease, a small molecular inhibitor of antigen binding might

be sufficient to neutralize anti-PLA<sub>2</sub>R antibodies and render them unable to bind their target antigen.

Both groups are to be congratulated for their meaningful contributions to our evolving understanding of PLA<sub>2</sub>R-associated membranous nephropathy, and we look forward to future studies that build on this exciting research.<sup>9,10</sup>

## DISCLOSURES

None.

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See related articles, “Identification of the Immunodominant Epitope Region in Phospholipase A<sub>2</sub> Receptor-Mediating Autoantibody Binding in Idiopathic Membranous Nephropathy,” and “Identification of a Major Epitope Recognized by PLA2R Autoantibodies in Primary Membranous Nephropathy,” on pages 291–301 and 302–313, respectively.

## A Breath of Fresh Air for Diabetic Nephropathy

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Diabetic nephropathy (DN) is a leading cause of CKD and a common complication in patients with type 1 or type 2 diabetes mellitus. The disease, as it progresses through defined morphologic and clinical stages, frequently leads to ESRD. Despite certain therapeutic interventions that slow its progression (such as blockade of the renin-angiotensin axis and strict BP, lipid, and glycemic control), the risk of advancing to ESRD remains high. Innovative therapeutic interventions are urgently needed to halt the development and progression of this devastating disease, which is responsible for approximately 40% of all new ESRD cases in the United States.<sup>1</sup>

In this issue of *JASN*, Nordquist and colleagues report that activation of the hypoxia-inducible factor (HIF) pathway with cobalt chloride (CoCl<sub>2</sub>) protects kidneys from DN in a rat model of type 1 diabetes.<sup>2</sup> The investigators used streptozotocin to induce diabetes mellitus and began to treat animals with CoCl<sub>2</sub> at the time of streptozotocin administration for 4 weeks. Their study demonstrates that CoCl<sub>2</sub> treatment has strong beneficial effects on a functional and morphologic level. By the end of the study, diabetic rats had normal GFR and showed significant

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