Comparing an Epitope Identified from a Crystal Structure with In-Solution Mass Spectrometry-Based Techniques Including HRPF and XL-MS

Zhi Cheng[1], Emily E. Chea[1], Rosa Viner[2], Guang Yang[3], R. Glenn King[3], Todd J. Green[3], John T. Killian[3], Jr., Peter E. Prevelige[3], Frances E. Lund[3], and Scot R. Weinberger[1]

1. GenNext Technologies, Inc., Half Moon Bay, CA
2. Thermo Fisher Scientific, San Jose, CA
3. University of Alabama at Birmingham, Birmingham, AL

GenNext TECHNOLOGIES
www.gnxtech.com

Human Leukocyte Antigen (HLA) Introduction

HLA proteins are essential components of the immune system, playing a critical role in the body's ability to recognize and respond to foreign substances. HLA proteins are categorized into two main classes: Class I and Class II, each with distinct functions and expression patterns. Class I HLA proteins, including HLA-A, HLA-B, and HLA-C, are found on almost all nucleated cells and present peptide fragments from within the cell (including viral peptides if the cell is infected) to cytotoxic T cells. This helps the immune system identify and destroy infected or abnormal cells.

This study focused on epitope / paratope mapping on an HLA-A protein with an antibody Fab domain. Epitope/paratope mapping is crucial to developing new therapeutic antibodies as it offers detailed understanding of the mechanisms of action by the antibody. The group from University of Alabama at Birmingham recently identified the epitope on an HLA-A protein that is bound by an HLA-A specific Fab. While X-ray crystallography provides atomic level information on the protein complex, it reveals a single, albeit most stable, conformation in the crystal state, not in solution. In this study, we expanded the characterization with two complementary in-solution mass spectrometry (MS) techniques: Hydroxyl Radical Protein Footprinting (HRPF) and Chemical Cross-Linking (XL). These methods were applied to characterize the antigen and its epitope, and the findings were then compared with the data acquired from X-ray crystallography.

HRPF Introduction

The Fox® Protein Footprinting System is a novel HRPF method that uses a proprietary flash oxidation lamp to generate hydroxyl radicals (•OH) that irreversibly modify solvent exposed amino acid side chains. As solvent accessibility changes, the •OH modification concordantly changes.

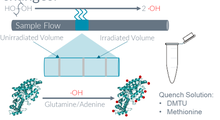


Figure 1: Schematic of an HRPF method, fast Photochemical Oxidation of Proteins (FPOP). With FPOP, protein is mixed with hydrogen peroxide and flowed passed a pulsing light source which photolyzes the hydrogen peroxide into two •OH and modifies solvent exposed amino acids. Following labeling, the sample is deposited into a quench solution of DMTU and methionine. Following labeling, oxidation is detected and quantified using bottom-up proteomics.

HRPF •OH Dosimetry

The Fox System contains a dosimeter module which monitors the effective •OH concentration by detecting the change in UV absorbance at 265 nm. The dosimeter molecule, 1 mM Adenine, absorbs at 265 nm but upon oxidation it decreases in absorbance. The decrease in absorbance happens in a linear fashion. This is important when comparing a protein in multiple conditions that have varying •OH scavenging. By ensuring all conditions are labeled around the same dose response (Δ mAU), we can be confident any change in oxidation was a direct result in the protein's change in solvent accessibility, not simply due to fewer radicals available for labeling. Figure 2 is the dose response data from HLA alone, the Fab domain, and the complex of the two.

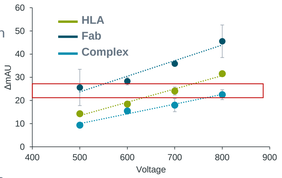


Figure 2: •OH Dosimetry experiment from the Fox® System. As the voltage increases, a larger change in adenine's absorbance (Δ mAU) is detected. The Δ mAU represents the effect •OH concentration. The dose response from HLA alone is in green, Fab domain alone is in dark blue, and the complex of the two is in light blue. The red box represents the overlapping •OH dosage.

HRPF Results to Identify the Paratope

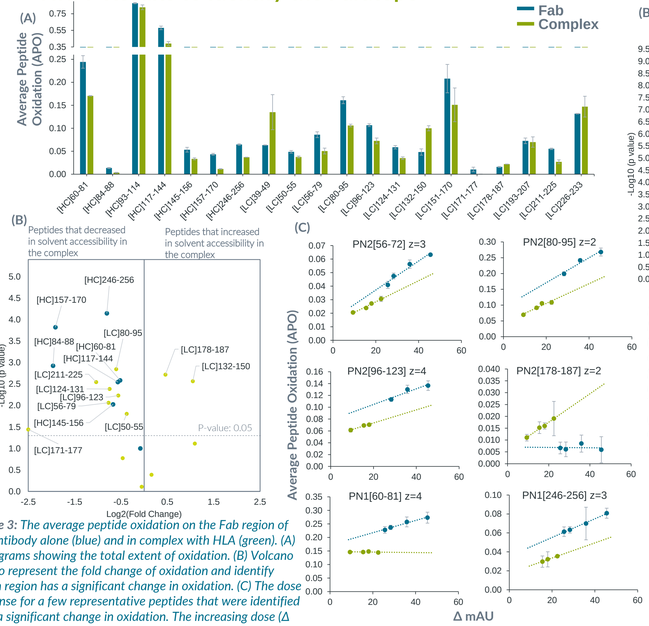
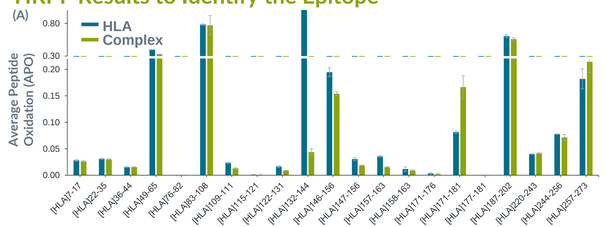


Figure 3: The average peptide oxidation on the Fab region of the antibody alone (blue) and in complex with HLA (green). (A) Histograms showing the total extent of oxidation. (B) Volcano plot to represent the fold change of oxidation and identify which region has a significant change in oxidation. (C) The dose response for a few representative peptides that were identified with a significant change in oxidation. The increasing dose (Δ mAU) results in a linear increase in oxidation.

HRPF Results to Identify the Epitope



HRPF Results to Identify the Epitope, Continued

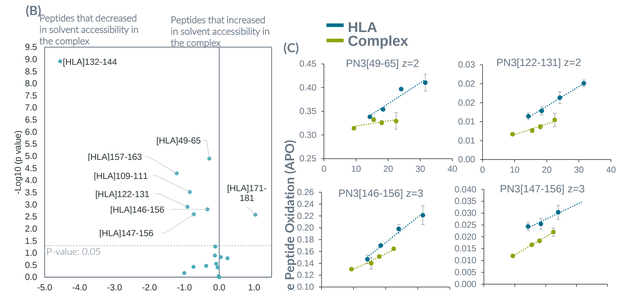


Figure 4: The average peptide oxidation on HLA alone (blue) and in complex with HLA (green). (A) Histograms showing the total extent of oxidation. (B) Volcano plot to represent the fold change of oxidation and identify which region has a significant change in oxidation. (C) The dose response for a few representative peptides that were identified with a significant change in oxidation. The increasing dose (Δ mAU) results in a linear increase in oxidation.

FAB and HLA HRPF Coverage Map

Fab HC: 46% Coverage

```
  1 MPLLLLLPLLWAGALAQVQLQESGGGVVQPGGSLRLSCAASGFNFSNYGM  50
 51 HWVRQTPGKGLEWVASIPYDGSHQWHADSVKGRFTISRDNSKNTLYLQIN 100
101 SLRPEDTAMYYCSKARISYLSAPAWWFDPWGQGTLVTVSSASTKGPSVFP 150
151 LAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS 200
201 GLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTDY 250
251 KDDDDK
```

Fab LC: 75% Coverage

```
  1 MGWSCIILFLVATATGSWAQSVSTQPPSVSVAPGQTARITCGGNNIGSKS  50
 51 VHWYRQKPGQAPVLVVYDNNARPSGIPERISGSNFANTATLTISRVEAGD 100
101 EADYYCHVWDSSSDHVVFGGGTKLTVLGQPKAAPSVTLFPPSSEELQANK 150
151 ATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLS 200
201 LTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS                  233
```

HLA: 75% Coverage

```
  1 GSHSMRYFFTSVSRPGRGEPRFIAVGYVDDTQFVRFDSDAASQKMEPRAP  50
 51 WIEQEGPEYWDQETRNMKAHSQTDRANLGTLRGYYNQSEDGSHTIQIMYG 100
101 CDVGPDGRFLRGYRQDAYDGKDYIALNEDLRSWTAADMAAQITKRKWEAV 150
151 HAAEQRRVYLEGRCVDGLRRYLENGKETLQRTDPPKTHMTHHPISDHEAT 200
201 LRCWALGFYPAEITLTWQRDGEDQTQDTELVETRPAGDGTFQKWAAVVVP 250
251 SGEEQRYTCHVQHEGLPKPLTLRWELSSQPGSLHHIILDAQKMVWNHR   297
```

Figure 5: Coverage map for the Fab region of the antibody and HLA. Peptides colored green were identified in both protein alone and in complex. Fab heavy chain (HC) has 46% coverage, Fab light chain (LC) has 75% coverage, and HLA has 75% coverage. Peptides colored blue showed a significant decrease in oxidation following complex formation. Peptides colored orange showed a significant increase in oxidation following complex formation.

Epitope/Paratope Mapping by XL-MS

The crosslinking experiments used two lysine specific mass- spec cleavable cross-linkers (DSBU and DSSO). The crosslinked peptides were identified using the XlinkX node in Thermo Scientific Proteome Discoverer 3.1 software. A total of 70 crosslinks were identified with 2 unique inter-crosslinked. From those 2 inter-crosslinked regions of HLA, 5 peptides were identified with significant protection from the HRPF data set(Fig.4).

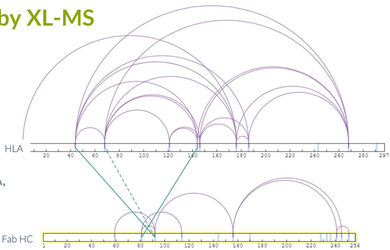


Figure 6: DSBU/DSSO crosslink mapping of HLA-Fab HC complex. Crosslinking map was generated using xiNET.

HRPF and XL-MS mapped on crystal structure

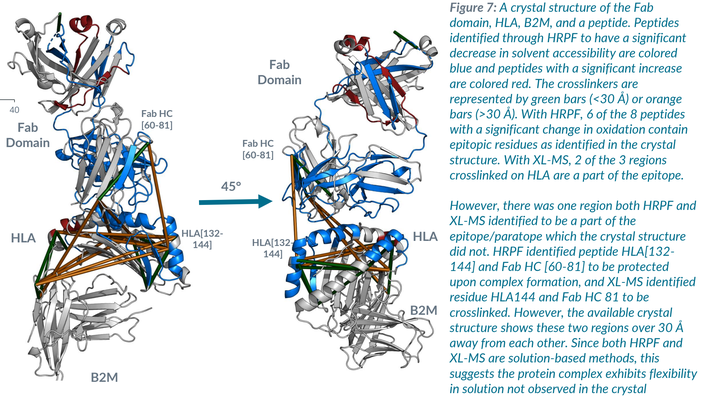


Figure 7: A crystal structure of the Fab domain, HLA, B2M, and a peptide. Peptides identified through HRPF to have a significant decrease in solvent accessibility are colored blue and peptides with a significant increase are colored red. The crosslinkers are represented by green bars (<30 Å) or orange bars (>30 Å). With HRPF, 6 of the 8 peptides with a significant change in oxidation contain epitopic residues as identified in the crystal structure. With XL-MS, 2 of the 3 regions crosslinked on HLA are a part of the epitope.

However, there was one region both HRPF and XL-MS identified to be a part of the epitope/paratope which the crystal structure did not. HRPF identified peptide HLA[132-144] and Fab HC [60-81] to be protected upon complex formation, and XL-MS identified residue HLA144 and Fab HC 81 to be crosslinked. However, the available crystal structure shows these two regions over 30 Å away from each other. Since both HRPF and XL-MS are solution-based methods, this suggests the protein complex exhibits flexibility in solution not observed in the crystal structure.

Conclusions

- HRPF and XL-MS provide complementary insights
- Epitope/ Paratope on HLA and a Fab domain were identified with HRPF and XL-MS
- Protein complex exhibits flexibility in solution observed from HRPF and XL-MS.
- These findings underscore the importance of using multiple techniques for comprehensive protein characterization. PO003514