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Mapping the Energetic Epitope of an Antibody/Interleukin-23 Interaction with Hydrogen/Deuterium Exchange, Fast Photochemical Oxidation of Proteins Mass Spectrometry, and Alanine Scave Mutagenesis

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Abstract

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Supplemental experimental procedures, figures and tables.

Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

Epitope mapping the specific residues of an antibody/antigen interaction can be used to support mechanistic interpretation, antibody optimization, and epitope novelty assessment. Thus, there is a strong need for mapping methods, particularly integrative ones. Here we report identification of an energetic epitope by determining the interfacial hot-spot that dominates the binding affinity for an anti-IL-23 antibody by using the complementary approaches of hydrogen/deuterium exchange mass spectrometry (HDX-MS), fast photochemical oxidation of proteins (FPOP), alanine shave mutagenesis, and binding analytics. Five peptide regions on IL-23 with reduced backbone amide solvent accessibility upon antibody binding were identified by HDX-MS, and five different peptides over the same three regions were identified by FPOP. In addition, FPOP analysis at the residue-level reveals potentially key interacting residues. Mutants with 3–5 residues changed to alanine have no measurable differences from wild-type IL-23 except for binding of and signaling blockade by the 7B7 anti-IL-23 antibody. The M5 IL-23 mutant differs from wild-type by five alanine substitutions and represents the dominant energetic epitope of 7B7. M5 shows a dramatic decrease in binding to BMS-986010 (which contains the 7B7 Fab), yet maintains functional activity, binding to p40 and p19 specific reagents, and maintains biophysical properties similar to wild-type IL-23 (monomeric state, thermal stability, and secondary structural features).

Graphical abstract

Keywords

Epitope mapping; Interleukin-23; protein-protein interaction; surface plasmon resonance (SPR); hydrogen/deuterium exchange mass spectrometry; fast photochemical oxidation of proteins; computer modeling; mutagenesis; monoclonal antibody; biotechnology

INTRODUCTION

The purpose of this study is to compare various methods for determining epitopes in antigen-antibody interactions, a subject of high significance for protein therapeutics^{1–3}, and to establish whether they are complementary. Characterizing the binding sites of monoclonal antibodies (mAbs) on target antigens often aids in understanding the mechanism of action for the antibody⁴, particularly for multi-subunit antigens. Certain epitopes can be more desirable than others if they demonstrate improved function, reduce off-target liabilities, aid in securing intellectual property rights, or lead to better antibody properties for later development.

Approaches for epitope mapping vary in their data resolution and in their reagent and resource needs.⁵ Ideally, an approach should map epitopes that are linear (continuous sequence) and conformational (discontinuous residues nearby in tertiary structure) with

sufficient spatial resolution to identify amino acid contact points and assess their relative contribution to the binding energy. The vast majority of natural antigen-antibody interactions have conformational epitopes that are best investigated by combinations of methods.^{6–8} X-ray crystallography can identify the amino acids involved in the interaction and, when coupled with computational methods, can provide the strength of specific amino acid interactions.^{9,10} The amount of protein and resources, however, can be prohibitive. NMR spectroscopy can also provide structure, but it is often limited to smaller proteins. Peptide scanning using synthetic peptides^{5,11} is an inexpensive method to map the epitope although it is typically limited to linear epitopes and does not provide amino-acid resolution.

Site-directed mutagenesis, in combination with other methods, is often the best approach for identifying critical amino acids at the epitope although it is labor-intensive, slow, and often requires iterative rounds of mutagenesis. Hot spot analysis has shown that 80% of the binding affinity is contributed by 3–5 residues on the target protein^{12–14} Given that alanine occurs in both buried and exposed locations and is found in all secondary structures,¹⁵ substitution by alanine often has minimal effect on higher order structure. Alanine-scanning is widely used to determine contributions of specific amino residues to the binding, stability or function.^{13,15,16} Owing to the laborious nature of generating a single-point mutant for each residue, direct substitution of a stretch of residues by alanines, effectively “shaving” the amino acid side chains (alanine shave analysis), will identify regions that have a potential binding epitope.

Mass spectrometry (MS)-based protein footprinting methods also characterize the higher order structure of therapeutic mAbs and other interacting protein-ligand systems with high-throughput and medium structural resolution. The most commonly used approaches are hydrogen/deuterium exchange (HDX)-MS^{17–19} and oxidative footprinting.^{16,20} HDX-MS can probe protein conformation and conformational dynamics in solution²¹ at the peptide level by measuring solvent-accessibility differences that occur upon complex formation between two binding partners^{17,22–24}. The combination of HDX-MS, computational analysis, and site-directed mutagenesis is an integrative approach that can even identify the energetic contribution of specific amino acids.

A complementary MS method is oxidative labeling²⁵ for protein footprinting that uses hydroxyl radicals to detect changes in solvent accessibility of protein side chains. Hambly and Gross²⁶ expanded the method with “fast photochemical oxidation of proteins” (FPOP) that utilizes photolysis of hydrogen peroxide to generate OH radicals, which have a comparable size to water and react to give detectable products with at least 14 of 20 amino acid side chains. By introducing a radical scavenger (usually an amino acid like Gln or His), the lifetime of the primary radicals can be controlled to ~1 μ s. The approach affords stable, irreversible modifications, removing the constraints of back exchange to permit the use of a wide range of proteases and buffer conditions. The method probes the protein-protein binding interface at the peptide level and sometimes the residue level.

Here, we compare the use of HDX-MS, FPOP, alanine shave mutagenesis, and quantitative binding studies to determine the specific residues involved in the energetic epitope of interleukin-23 (IL-23)²⁷, a pro-inflammatory heterodimeric cytokine composed of two

disulfide-bridged subunits, a unique p19 subunit with a four-helical core, and a p40 subunit in common with IL-12²⁸. IL-23 is secreted predominantly by activated dendritic cells (DCs) and macrophages and owing to a shared subunit, has activity similar to, but distinct from IL-12.²⁹ IL-23 signals through a high-affinity receptor complex, IL-12R β 1 and IL-23R³⁰. The main immunologic role of IL-23 is differentiation of naïve CD4⁺ cells into Th17 cells³¹, which produce IL-17, a pro-inflammatory cytokine that stimulates the production of IL-1, IL-6, TNF- α , NOS-2, and the chemokines that are important in defense against infection. Indeed, IL-23 regulation of Th17 cells plays a critical role in the pathogenesis of autoimmune diseases: psoriasis³², Crohn's disease (CD)³³, arthritis³⁴, and inflammatory bowel disease (IBD)³⁵.

Antibodies against IL-23 have the potential to treat patients with autoimmune inflammatory diseases.^{36–39} Several antibodies and other therapeutic proteins targeting one or both subunits of IL-23 have been developed for clinical use.^{40–47} For example, ustekinumab, a monoclonal antibody (mAb) targeting the p40 subunit of IL-12 and IL-23, is approved against plaque psoriasis and psoriatic arthritis.⁴⁸ In addition, there are several IL-23 p19 specific antibodies in phase I and II clinical testing for the treatment of rheumatoid arthritis, psoriasis, and Crohn's disease.^{49,50} The 7B7 Fab used here is a known p19 binder that is specific for IL-23 over IL-12; this Fab exists in both the BMS-986010 and BMS-986113 antibody-type therapeutics included in this study. There are no differences in binding IL-23 between the two 7B7-containing therapeutics.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification

BMS-986010 is an anti-human IL-23 p19 antibody and the Fab 7B7 represents the active binding region of the antibody. BMS-986113 is a bispecific antibody-type protein⁵¹ that contains two copies of the 7B7 Fab and also two copies of a Fab that binds IL-17 (Stevens et al., manuscript in preparation). Fab 7B7, BMS-986010 and biAb BMS-986113 were used in previous binding studies at various stages because they are interchangeable as far as their recognition of the epitope on IL-23p19.⁵² Antibodies were generated in-house, expressed from a stable CHO cell line, and purified by affinity or ion-exchange chromatography. His-tagged wild-type and alanine shave mutant constructs of IL-23 p19 were generated by PCR or gene synthesis, cloned into the transient expression vector, and confirmed by sequencing. Non-tagged wild-type IL-23 p40 subunit was co-expressed with His-tagged wild-type p19 subunit transiently in HEK293-6E cells at 4L scale for IL-23 heterodimer purification. HEK supernatants of IL-23 variants were harvested then concentrated and buffer exchanged by tangential flow filtration. Proteins were purified by immobilized nickel affinity chromatography, buffer exchanged to PBS, and purity assessed by SDS-PAGE.

Hydrogen/Deuterium Exchange-Mass Spectrometry

Equilibrium buffer was prepared as 10 mM potassium phosphate in 100% H₂O, pH 7.0. Labeling buffer was prepared as 10 mM potassium phosphate in 100% D₂O, pD 7.0 (all measured pH values of D₂O solutions (also known as pH*) were all adjusted to the corresponding pD values^{53,54}). The details are given in Supporting Information.

FPOP Mass Spectrometry

Interleukin-23 and the Fab were held at a 1:1 molar ratio for 1 h at 4 °C to form the complex followed by dilution in PBS buffer (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4), and storage on ice as 40 μ L aliquots (10 μ M complex). Just prior to FPOP, 5- μ L aliquots of histidine and H₂O₂ each were added to give 50 μ L of 0.5 and 15 mM of histidine and H₂O₂, respectively. The sample was then flowed in fused silica tubing for labeling as previously described⁵⁵. Both FPOP and no-laser control experiments were performed in triplicate. Labeled solutions were snap-frozen in liquid nitrogen and stored in a freezer (−80 °C) before digestion. Details for this experiment are in Supporting Information.

Computational Epitope Prediction and Design of Alanine Shave Mutants

The residues contained in the regions identified by HDX-MS were mapped onto the sequence of the IL-23p19 domain and three linear regions of common residues were identified as Regions A, B and C (Figure 1). Regions are defined as residue numbers in the mature sequence which begins with residue 20 of UniProt KB ID #Q9HBE5. Region A corresponds to amino acid residues 14–40; Region B is residues 70–106; Region C is residues 125–154. In order to calculate the residues whose side chains are exposed (solvent accessible surface area, SASA)⁵⁶ and would therefore be located on the protein surface of the p19 domain of IL-23, an in-house structure of the IL-23 heterodimer was used. For each residue in the p19 domain of IL-23 the ratio of accessible surface to the standard exposed surface for the amino acid type was calculated and residues were grouped into bins. Residues were placed in accessibility bins as follows: <30%, 30–40%, 40–50%, 50–60%, 60–70%, 70–80%, > 90% exposed. The standard residue accessibilities for each amino acid type were calculated in the extended tripeptide Gly-X-Gly. The second calculation performed was ODA (Optimal Docking Area) which is useful for predicting likely protein-protein interaction surfaces (Molsoft LLC).⁵⁷ The method identifies optimal surface patches with the lowest docking desolvation energy.

Residues were then prioritized based upon a high score in both the Optimal Docking Area and Solvent Accessible Surface Area calculations and also weighted based on the extent of hydrogen-deuterium exchange relative to the uncomplexed IL-23 (HDX peptide #1 >#2>#3>#4>#5) and the FPOP protection ranking (FPOP peptide #1 >#2>#3>#4>#5). Residues were not selected from the sequence identified in FPOP #2 peptide because it is involved in packing against the P40 domain. Residues were not selected from the sequence identified in FPOP #5 and HD #5 because that helix is mostly buried and could disrupt IL-23-p19 helical bundle folding. With the exception of M7 which contains a linear sequence of residues in an extended loop, residues were then combined into non-linear epitopes based on mapping them to the X-ray crystallographic structure of IL-23 (M5, M6, M8) as shown in Figure 2. Additional backup mutants were generated with the sub-epitopes of predominantly linear residues (M9, M10, M11).

Surface Plasmon Resonance Binding

Binding affinity of mutants was compared with that of wild-type IL-23 by surface Plasmon resonance using a Biacore T100 in PBS with 0.05% Tween 20 at 25°C. Control antibodies, 7B7 antibody and receptor-Fc fusions were captured on immobilized protein A before

flowing each IL-23 variant titrated from 25nM to 1.5nM at 30uL/min and regenerating with 10 mM glycine pH 2.0. Results for M5 were simulated using BIAsimulation software 2.1 using the 1 RU of reference subtracted M5 signal and the average Rmax determined from kinetic analysis of mutants M9 and M10. Biacore energetics calculations are in Supporting Information.

Cellular Assays

A BaF3 cell line stably transfected with human IL-23R α and IL-12R β 1 full length receptors was used for an IL-23 dose-dependent STAT3 phosphorylation (pSTAT3) assay to assess antibody inhibition of IL-23 signaling. An EC50 concentration of IL-23 (20 pM) was premixed with 3-fold serial dilutions of the 7B7 antibody and two control antibodies from 3.7 to 0.56 pM and incubated at 37 °C for 15 minutes to stimulate pSTAT3. After quenching with ice-cold wash buffer and lysing the cells (Bio-Rad Cell Lysis kit), pSTAT3 was determined by ELISA (Bio-Rad Phospho-STAT3 (Tyr705) kit) for IC50 calculations. Details are in Supporting Information.

Biophysical and Functional Assessment of Mutants

Mutants were characterized biophysically to confirm that they retained the properties of the wild-type IL-23, except for specific binding interactions impacted by the mutated residues. The biophysical methods are described in Supporting Information.

RESULTS

Hydrogen/deuterium exchange mass spectrometry

IL-23 epitope mapping by HDX-MS involves two steps: mapping of peptic peptides in the absence of D₂O and then HDX with deuterium labeling.²¹ To probe binding epitopes, we performed mapping on the free and bound IL-23: IL-23/BMS-986113 and IL-23/anti-IL-23 7B7 Fab. We found 32 peptic peptides in triplicate runs of all three samples, covering 97% of the linear sequence of IL-23p19, with overlapping peptides present in multiple regions. We then monitored the HDX of these peptides from all three samples as a function of time. The HDX levels for these peptides are similar in the IL-23/BMS-986113 and IL-23/anti-IL-23 7B7 Fab. The HDX differences between the free antigen and bound complexes are similar in multiple regions of IL-23 p19 (Figure S1).

We assigned significant differences in HDX levels from triplicate analysis of the labeling experiments for each sample and a difference value of 0.5 Da based on three times the averaged standard deviation of the deuterium uptake level of each IL-23 peptic peptide at each time point. We used the same criteria to determine that a summed HDX level for any peptide greater than 1.2 Da (either positive or negative difference) is a significant difference. Upon binding to BMS-986113 or anti-IL-23 7B7 Fab, five regions of IL-23 p19 had significantly decreased deuterium uptake, suggesting that IL-23 p19 has a discontinuous epitope comprised of the five peptide regions: (1) 98 PDSPVGQL 105, (2) 89 IFTGEPSLL 97, (3) 143 KILRSLQAF 153, (4) 15 QQLSQKLCTLAWSAHPLVGHMD 36, (5) 70 CLQRIHQGLIFYEKLLG 86 (see highlights in Figure 1, and representative HDX kinetic curves in Figure S1).

Fast photochemical oxidation of proteins (FPOP)

FPOP is a relatively new protein footprinting approach that provides epitope information at both peptide and residue levels^{55,58}. Knowing that efficient proteolytic digestion and high coverage of an antigen/antibody complex is important for peptide and residue-level FPOP to achieve certain epitope mapping, we designed combination digestion strategies because IL-23p19 has a very low arginine/lysine content (11 out of 176 amino acids), and the average length of its tryptic peptides is 18. Although trypsin digestion yields a complete set of peptides covering 95% of protein sequence, the peptide segments in regions 21–64 and 74–148 are too large for our instrument. Others reported the use of multiple enzymes for improved sequence coverage in bottom-up proteomics where mostly trypsin is used in combination with an endoprotease (Lys-C, Lys-N or Glu-C), or less specific proteases to achieve better sequence coverage⁵⁹. The N-terminal half of IL-23p19 is rich in aspartic acid (D), allowing us to digest the protein sequentially with trypsin and Asp-N. This combination ensures efficient cleavage at R, K, and D sites with high specificity and minimum missed cleavages. For regions that are hydrophobic or lacking in cleavage sites for specific enzymes, we also utilized separately chymotrypsin and pepsin. This combined protocol enabled a near complete coverage map (Figure S2). We identified 20 peptide segments with a range of 8–22 amino acids within each peptide, covering 98% of IL-23 p19 subunit.

Peptide level—To establish a basis for comparing free and 7B7 Fab-bound states of IL-23, we first evaluated the labeling efficiency of IL-23 p19 in the absence of antibody. Up to 14 of the 20 amino acids are candidates for FPOP modification including aromatic, sulfur containing, and positively charged amino acids.⁶⁰ We found up to 50% modification for regions that are solvent-exposed or lack rigid secondary structure (loops; residues 32–53, 91–110, and 124–139), whereas the labeling is attenuated (less than 10%) in regions with limited solvent accessibility and rigid structure. The overall high extent of FPOP labeling in loop regions that often contain epitopes facilitates the epitope mapping.

We compared the FPOP labeling between free and 7B7 Fab-bound IL-23 p19 by performing Student's t-test on the set of triplicate experiments after correction for non FPOP-induced in-air oxidation. This approach allows us to discriminate between Fab-binding and nonbinding peptides based on p-values (p-value less than 0.01). In total, we found five discontinuous epitope regions with pronounced protection in the FPOP labeling of the 7B7 Fab-bound state, located at the loops at the “tip” of the four-helix bundle (Figure S3). We then ranked the strength of all epitopes by calculating the relative protection in labeling between free- and Fab bound-states of IL-23 p19 (Table S1). The ranking order from dominant to weak is: (1) 21 LCTLAWSAHPLVGHM 35, (2) 32 VGHMDLREEGDEETTNDVPHIQ 53, (3) 91 TGEPSLLPDSPVGQLHASLL 110, (4) 124 ETQQIPSLSPSPWQR 139, and (5) 74 IHQGLIFYEK 83. Other peptides either show similar oxidation in both states, serving as negative controls, or have no detectable oxidation. Furthermore, regions with over 50% FPOP protection are considered major epitopes, whereas regions with less than 50%, yet significant, FPOP protection are ranked as minor (Figure 3).

Residue level—The ranking of epitope binding sites at the peptide level highlights a few dominant regions that prompted us to investigate further the epitopes at the residue level.

When two or more residues were modified within a peptide, we used only those represented by well-separated chromatography peaks with good signal-to-noise (S/N) product-ion spectra, achieving reproducible quantification. Residue analysis facilitates higher spatial discrimination between free and bound forms of the antigen, especially for regions with low oxidation yield or yields that are dispersed over multiple residues. Overall, for the 13 peptides that show modification, a total of 14 specific residues were modified at a sufficient level to afford good S/N ratios and good precision in their quantification even for the very-low-yield modified residues P4, F80, H106, W123, and F154 (Figure 4). Of these, the decreased modifications in the Fab-bound form of IL-23 are notable for nine residues (the comparisons are with p-values less than 0.01) (Figure 4). We then selected only residues with more than 2-fold changes (more than 50% protection) in FPOP labeling for residue-level analysis and identified amino-acid residues W26, M35 and L96/L97/P98 as potentially key interacting residues with the 7B7 Fab. The complicated product-ion spectrum of peptide 91–110 does not reveal definitively which residue is modified; however, it does show that the modification occurs at either residue L96, L97, or P98 or at some combination. Five responsive residues are highlighted in the crystal structure of IL-2 (Figure 5).

Modeling and Mutant Design

We utilized well established computational methodology for estimating useful surface-related properties, principally solvent-accessible surface area (SASA) and ODA.⁵⁶ ODA (Optimal Docking Areas) is a newer method to predict protein-protein interaction sites based on lowest desolvation energies.⁵⁷

The residues contained in the regions identified by HDX and FPOP were mapped onto the sequence of the IL-23p19 domain, and three linear regions of common residues were identified as Regions A, B and C (Figure 1). The residues within these regions whose side chains are exposed (as determined by their SASA) and have a high ODA value were prioritized for mutagenesis (shown as underlined residues in Figure 1). Three to five residues that are nearby in the 3-Dimensional structure were selected for each mutant to cover the relevant possible epitopes as shown in Table 1.

Binding and functional analysis of mutants

We then used Biacore analysis to screen HEK293-6E supernatants expressing the mutants (Figure S4), determine binding affinities and G values for purified mutants (Table 2), and provide quality control for mutants based upon binding to control antibodies and receptors (Figures 6A, 6B). To triage mutants, we used supernatant Biacore binding studies to determine that three mutants (M5, M9, M10) showed significant reductions in BMS-986010 binding while maintaining control protein binding similar to that of the WT. The IL-23 alanine shave mutants maintain binding to the p40 specific antibody and the p19 specific IL-23 receptor (except for M8 which potentially describes the receptor binding site). We prioritized mutants M5, M9, and M10 for scale-up. Mutant M7 was also selected for scale-up as a control mutant that maintained WT binding interactions.

We determined binding affinities and G values for purified mutants using the same Biacore assay format (Table 2). Biacore analysis of single alanine mutants confirmed the

non-linear epitope residues contained in the M5, M9, and M10 alanine shave mutants. Most single alanine mutants in the three linear regions A, B, and C show no change in binding to the BMS-986010 or BMS-986113. Only 4 of the 14 single alanine mutant of IL-23 tested yield a significant decrease in binding affinity greater than 0.5 kcal/mole (these mutants and two control mutants are shown in Table 2). The affinity and the ΔG values for the key residues overlapping between alanine shave mutants M5, M9, and M10 and demonstrate that one major residue in linear region B and two residues in linear region A contribute predominantly to the binding energy of the BMS-986010-IL-23 complex.

The pSTAT3 assay EC50 results demonstrate that IL-23 Alanine shave mutants designated M5, M7, M9 and M10 are all active and equal potent to WT IL-23 (Table 3). BMS-986010 neutralizes the biological activity of WT IL-23 and IL-23 M7 Alanine shave mutant with equal potency yet neutralizes M9 and M10 IL-23 with reduced potency. Further, BMS-986010 does not neutralize the biological activity of M5 (no inhibition). The EC50 and IC50 data taken together demonstrate that the IL-23 M5 mutant is functionally similar to WT except for its lack of blockade by BMS-986010 (Table 3). We tested two antibodies from the literature to characterize further the functionality of the mutants. Ustekinumab⁴⁸, which binds the other subunit, the IL-12 p40 domain neutralizes the biological activity of WT IL-23 and all the IL-23 Alanine shave mutants with equal potency in the pSTAT3 assay. The 7G10 (an IL-23 p19 mAb)⁴⁷ neutralizes the biological activity of WT IL-23, M7, M9 and M10 Alanine shave mutants with equal potency and does not neutralize the biological activity of M5. Results with single alanine mutants are consistent with these results and provide further details on the dominant residues of interaction. BMS-986010 binding site found in mutants M5, M9, and M10 has been localized to residues M35, D36, and L97 as shown by the IC50 shift in Table 4 relative to wild type IL-23 in Table 3. A slight shift in IC50 is observed for E93A though the potency effect is not convincing without the Biacore result in Table 2 to confirm it. The IC50 of Ustekinumab does not shift for any of the single mutants. The 7G10 mAb does shift in IC50 for E93A, but not for M35A, D36A, and L97A. This indicates that, although both BMS-986010 and 7G10 lose binding to the M5 mutant, their binding interaction with IL-23 is contributed mostly by different residues in the M5 pentamutant.

Biophysical Characterization of Mutants

SEC-MALS data demonstrate that all mutants are mainly monomeric, similar to the wild-type as shown in Figures S5 and Table 5. Further, a SEC-MALS analysis of the mutant complexes with the 7B7 Fab shows the M5 mutant does not shift significantly after pre-incubation with the Fab, demonstrating that little if any complex is formed in the 10 μ M concentration range of this experiment. The retention time for the M5 mixture is consistent with the two species eluting when not mixed. M7 complexes elute similarly to the wild-type. The M9 and M10 mutants form complexes at these concentrations, but the mass is somewhat less than that of the wild-type, and the retention time is slightly later than those of the wild-type and M7, suggesting that their affinity for BMS-986010 is weaker than that of the WT and M7. These results are consistent with the affinity and functional results obtained for the mutants by Biacore and pSTAT3 assay.

We also measured the thermal stability profile for the wild type and mutant IL-23 heterodimers by differential scanning calorimetry (DSC); denaturation of each molecule was characterized by two unfolding transitions (fitted transition midpoint (T_m) values are shown in Table 5). The results show that none of the mutants show significant thermal destabilization relative to WT. Secondary structure comparison of mutants and wild type of IL-23 by FTIR spectroscopy shows that secondary structure content, as calculated using the structure-sensitive Amide I peak, is similar. Approximately an equal quantity of α -helix and β -sheet are in IL-23 samples as indicated by peaks at 1637 cm^{-1} for α -helix and at 1637 cm^{-1} and the shoulder at 1687 cm^{-1} for β -sheet. No significant difference in the FTIR spectrum and the calculated secondary structure pertains to the mutants compared to the WT, as shown in Figure S6. We also compared the secondary structure of mutants and WT using CD spectroscopy and found no significant difference in secondary structure of mutants compared to the WT (Figure S7).

One-dimensional ^1H NMR spectra for the multi-alanine shave proteins reveals that each mutant protein is properly folded, as evidenced by the well-dispersed resonances in both the high- ($< 0.5\text{ ppm}$) and low-field ($> 6.5\text{ ppm}$) regions of the spectra. The high-field methyl resonances indicate the presence of an intact hydrophobic core; the downfield amide protons (See Figure S8) reflect the existence of well-formed secondary structure. Comparison of the spectra with that of WT indicates a close match, precluding the existence of large conformational changes in the protein structure induced by the amino-acid substitutions. In addition, the NMR results indicate that extra loss in activity in M5 is unlikely due to an extra-large disruption in structural integrity at the mutation sites in M5. The fact that M5 is considerably closer to the wild-type protein in the PCA-plot (see SI) than M9 suggests that the H34A, M35A and E93A M5-mutations, which are missing in M9, do not cause much disruption in the M5-structure.

DISCUSSION

The integrated approach to epitope mapping presented for the 7B7 epitope on IL-23 illustrates the need for complementary methods to identify regions and to determine the key energetic determinants of an antibody binding its antigen. We identified the hot spot residues on IL-23 for the antibody epitope by a combination of HDX-MS, FPOP, computational surface residue analysis, alanine shave mutagenesis, and binding analytics. HDX found five IL-23 (p19 subunit) epitopes that are important for binding of the 7B7 antibody. These epitopes, found by FPOP, when mapped to the linear sequence of IL23p19 (Figure 3B), show an overlap with four epitope regions detected by HDX, specifically, in the N-terminal and middle regions of the p19 subunit. One discrepancy is the C-terminal region of the antigen, for which HDX identified a peptide covering the middle of helix D (residues 145–153) yet for which there are no detectable oxidation products of the corresponding peptide (residues 143–153) because this region is nearly unreactive to $\cdot\text{OH}$. In addition, FPOP identified a site located in the N-terminal half of helix D, represented by peptide 124–139, that shows minor reactivity change upon binding. Protection of peptide 124–139 is not observed with HDX. One explanation is that HDX monitors the exchange level of the local protein sequence over various time points. Therefore, HDX rates not only reflect protein high order structure, but also structural stability in the time course study. On the other hand,

FPOP monitors side-chain oxidation level on the sub millisecond timescale and can therefore report more rapid changes in solvent accessibility in the side chains. Some minor remote conformational changes that occur upon antibody binding to IL-23 may not involve changes in the hydrogen-bonding pattern of the protein and would not be reported by HDX but possibly by FPOP. Nevertheless, taken together, these results demonstrate FPOP as a complementary method to HDX for epitope mapping at the peptide level. One advantage of FPOP is the ability to obtain residue-specific information without using electron-based fragmentation techniques to minimize H/D scrambling accompanying the usual collision-induced fragmentation of HDX.

Alanine shave mutagenesis provides the ability to probe energetically the residues identified by the two MS methods. The energetic determinants for 7B7 binding to IL-23 are found in the M5 mutant. The M5 mutant shows a dramatic loss of binding to BMS-986010 whereas M9 and M10, which each contain two of the same mutated residues as M5, show partial loss of binding to BMS-986010. The affinity of the M5 IL-23 mutant is 5 μ M for BMS-986010, which is approximately 27,000-fold weaker than that of WT IL-23, demonstrating that the residues in the M5 mutant define the dominant energetic epitope for the 7B7 Fab in BMS-986010 and BMS-986113 binding to IL-23.

We also confirmed the regional alanine shave mutants (M5, M9, M10), both biophysically and functionally, to be comparable to WT IL-23 except for binding affinity to the 7B7 Fab contained in both BMS-986113 and BMS-986010. Single alanine mutants of IL-23 containing residues found in the M5, M9, and M10 mutants show the specific residues contributing to the binding affinity. M35, D36, and L97 each contribute over 2 kcal/mole in binding energy, and E93 contributes about 1 kcal/mole. These values are consistent with the epitope of the 7B7 Fab on IL-23 as a conformational epitope comprised of two separate linear epitopes (Figure 2). Regions A & B identified by HDX and FPOP are involved in the M5 epitope, whereas region C was not identified in the energetic epitope, suggesting that conformational changes upon antibody binding lead to changes in region C that are not directly involved in the binding epitope. A comparison between individual residues identified by FPOP, HDX and the Alanine Shave mutagenesis energetic analysis is shown in Figure 3B.

In conclusion, we are able to locate the dominant binding residues at the interface between an anti-IL-23 antibody and its antigen by a combination methods. Mapping the energetic epitope of an antibody of interest onto its target distinguishes the specific residues that dominate the antibody interaction from the total residues buried in the complex. Knowledge of the specific residues on the target antigen that interact with the antibody support the mechanistic interpretation of the antibody function, allow antibody optimization to improve affinity or remove liability residues, and assess epitope novelty for intellectual property purposes. Identification and characterization of the binding sites of antibodies can aid in the discovery and development of new therapeutics, vaccines, and diagnostic molecules. The abilities to map efficiently and accurately antibody epitopes using such an integrated approach and to pinpoint the critical residues responsible for the energetics of binding constitutes a major analytical advance for the discovery and development of antibody therapeutics. The combination of MS-based methods, computational modeling, and alanine-

shave mutagenesis allows this critical information to be readily obtained for important therapeutic targets in the absence of an X-ray structure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The abbreviations used

SPR	surface plasmon resonance
IL-23	interleukin-23
mAb	monoclonal antibody
HDX-MS	hydrogen/deuterium exchange mass spectrometry
FPOP	fast photochemical oxidation of proteins
Fab	fragment antigen-binding region of an antibody
ODA	optimal docking area
SASA	solvent accessible surface area
SEC-MALS	size exclusion chromatography with inline multi-angle light scattering
DSC	differential scanning calorimetry
T_m	thermal transition midpoint

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Figure 1.

Linear regions on the mature sequence of IL-23 identified by HDX-MS and FPOP for targeted alanine shave mutagenesis. HDX peptides are identified with yellow arrows and FPOP arrows are identified by blue arrows. Summary regions are colored red (region A), blue (region B), and purple (region C). Residues selected for inclusion in alanine shave mutants are underlined.

Figure 2. Mapping of regional alanine shave mutants onto 3 linear regions. The p40 domain is shown in purple. The p19 domain is shown in grey. Region A is shown in red, region B is shown in blue, region C is shown in green. M5 residues are shown in orange. M6 residues are shown in dark grey. M7 residues are shown in yellow.

Figure 3.

(A) Epitope regions determined by FPOP mapped on the crystal structure of IL-23. Color code: no significant difference (gray), minor epitope region (cyan), and major epitope region (blue). The p40 subunit is colored in purple. (B) Epitope regions determined by FPOP, HDX, and Ala Shave Energetics as mapped on the linear sequence of the IL-23 p19.

Figure 4.

(A) Extent of FPOP modification of free (blue bars) and Fab-bound IL-23 p19 (red bars) at the residue level. (B) Changes in FPOP labeling of five key residues of free IL-23 relative to the IL-23/Fab complex as mapped on the crystal structure of IL-23. Differences are discussed in text.

Figure 5.

(A) Changes in FPOP labeling of five key residues of free IL-23 relative to the IL-23/Fab complex as mapped on the crystal structure of IL-23. Color code: no significant difference (gray) and major FPOP protection (blue); the p40 subunit is not involved in binding and is colored in purple in the back of the structure. (B) Three major residues (green) identified by Alanine Scanning Mutagenesis as the energetic hot spot of IL-21 for binding to the Fab.

A.

B.

Figure 6.

Biacore binding analysis of alanine shave mutants & single mutants binding antibodies and receptor-Fc fusions captured on protein A sensor surface, error bars are shown for the normalized standard deviation of replicates (A) Normalized Biacore response of purified regional alanine shave mutants (B) Normalized Biacore response of purified single point mutants

Table 1

Alanine Shave IL-23 Mutant Residues

Name	Region Mutated	IL-23p19 Residues Mutated to Alanine
M5 *	A and B	H34A, M35A, E93A, L97A and D99A
M6	B and C	T23A, W26A, H29A, F144A, Q151A
M7 *	C	W123A, E124A, T125A, Q126A and Q127A
M8	A, B, and C	H34A, E93A, Q135A, W137A
M9 *	B	L97A, D99A and Q104A
M10 *	A	H34A, M35A, D36A and F144A
M11	C	Q123A, T125A, Q127A

* Mutants selected for scale-up and purification.

Table 2

Biacore kinetic analysis of IL-23 mutants binding 7B7

Variant	Kd (nM)	Kd-shift (fold weaker than WT)	G (kcal/mole)
WT	0.2	–	–
M5	5000	27,000	6.0
M7	0.4	2	0.5
M9	25	140	2.9
M10	43	230	3.2
H34A (region A)	0.2	1.5	0.2
M35A (region A)	6.8	48	2.3
D36A (region A)	9.6	68	2.5
E93A (region B)	0.7	5	0.9
L97A (region B)	46	330	3.4
D99A (region B)	0.1	0.5	–0.4

Region A: H34, M35, D36

Region B: E93, L97, D99

EC50 of mutants and IC50 Values for BMS-986010, Ustekinumab IL-12 p40 mAb, and 7G10 IL-23 p19 mAb inhibition of IL-23 Alanine shave mutants induced pSTAT3 in BaF3/huIL-23R α /huIL-12R β 1 transfectants

Table 3

Antibody	WT IL-23	IL-23 M5	IL-23 M7	IL-23 M9	IL-23 M10
EC50	21 pM	26 pM	21 pM	33 pM	19 pM
BMS-986010	19 pM	–	17 pM	2400 pM	5300 pM
Ustekinumab IL-12 p40	79 pM	62 pM	59 pM	67 pM	71 pM
7G10 IL-23 p19	380 pM	–	310 pM	260 pM	350 pM

EC50 of mutants and IC50 Values for BMS-986010, Ustekinumab IL-12 p40 mAb, and 7G10 IL-23 p19 mAb inhibition of IL-23 Alanine single mutants induced pSTAT3 in BaF3/huIL-23R α /huIL-12R β 1 transfectants

Table 4

Antibody	H34A	M35A	D36A	E93A	L97A	D99A
EC50	27 pM	17 pM	22pM	18 pM	17 pM	25 pM
BMS-986010	15 pM	710 pM	1200 pM	25 pM	>3000 pM	12 pM
Ustekinumab IL-12 p40	78 pM	81 pM	77 pM	33 pM	54 pM	52 pM
7G10 IL-23 p19	190 pM	270 pM	180 pM	>3000 pM	240 pM	220 pM

Table 5

SEC-MALS analysis of IL-23 mutants and complex formation with 7B7 Fab

IL-23	Average solution mass (kDa)	Main peak retention time (min)	Complex average solution mass (kDa)	Complex peak retention time (min)	T _m 1(°C)	T _m 2(°C)
wild-type	55.3	20.1	105	17.7	63	67
M5	56.5	20.8	54.2, 61	20.7, 20.2	60	66
M7	57.2	19.7	106	17.6	62	68
M9	53	20.3	99.2	17.9	61	67
M10	55.6	19.9	95.4	17.9	63	68

^aMW of 7B7 Fab determined by this method is 50.0 kDa, and its retention time is 20.7 min.