The indistinguishability of epitopes from protein surface is explained by the distinct binding preferences of each of the six antigen-binding loops

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General protein–protein interfaces are known to be enriched, compared with other surface patches, with amino acids that can form stabilizing interactions. However, several studies reported that there are hardly any differences between the amino acid composition of B-cell epitopes and that of antigen surface residues. If the amino acid composition of epitopes is indistinguishable from other surface patches, how do antibodies (Abs) identify epitopes? Here, we analyze the antigen binding regions (ABRs, roughly corresponding to the complementarity determining regions) and the epitopes in a non-redundant set of all known Ab–antigen complexes. We find that the ABRs differ significantly from each other in their amino acid composition and length. Analysis of the energetic contribution of each ABR to antigen binding reveals that, while H3 often plays a key role in antigen binding, in many antibodies other ABRs are more important. Moreover, each ABR has a distinct propensity to bind different amino acids on the antigen. The combined binding preferences of the ABRs yield a total preference to amino acids with a composition that is virtually identical to that of surface residues. These results suggest that antibodies evolved to recognize protein surfaces. They may help in improving Ab engineering and B-cell epitope prediction.

Keywords: antibody–antigen interactions/antigen binding regions/complementarity determining regions/epitopes

Introduction

Paratopes identify patches that are indistinct from protein surface

Antigen (Ag) binding sites dictate what epitopes will be recognized by the antibody (Ab). It has been shown that protein–protein interfaces differ in their characteristics and amino acid composition from the rest of the protein surface (Jones and Thornton, 1997a,b; Jones and Thornton, 1997a,b; Lo Conte et al., 1999; Chakrabarti and Janin, 2002; Ofran and Rost, 2003; Neuviirth et al., 2004; Keskin et al., 2008). This is, of course, not surprising given that some amino acids (e.g. hydrophobic or charged) are more likely than others to form non-covalent interactions that could stabilize protein–protein complexes. Ab–Ag interfaces, however, are unlike other interfaces (Lo Conte et al., 1999; Jones and Thornton, 1996; Ofran et al., 2008). While B-cell epitopes differ significantly in their characteristics (e.g. amino acid composition) from other types of protein–protein interfaces (Jones and Thornton, 1996; Ofran et al., 2008; Soga et al., 2010), their characteristics are similar to those of protein surfaces in general (Janin and Chothia, 1990; Lollier et al., 2011; Kringleum et al., 2012). This poses an interesting question in molecular recognition: how can paratopes reach high affinity without showing a clear preference to bind residues that can form stabilizing interactions? Exploring the characteristics of paratopes and epitopes may help address this question.

Properties of paratopes

Length of complementarity determining regions. Early systematic characterization of Abs (Wu and Kabat, 1970; Kabat et al., 1983) revealed that complementarity determining regions (CDRs) vary in length and sequence. Later studies found that the length of the loops is a primary factor in determining the topography of the Ag-binding site (Vargas-Madrazo et al., 1995; Collis et al., 2003). The length diversity of CDRs has been investigated by several groups that reached different, and sometimes contradicting, results (Wu et al., 1993; Collis et al., 2003; Zemlin et al., 2003; North et al., 2011; Raghunathan et al., 2012). For example, North et al. (2011) found H3 to be the longest CDR, while Collis et al. (2003) found that H2 may be as long as or even longer than H3. These differences may stem from the fact that each study used a different set of Abs and from the fact there is more than one way to define CDRs (e.g. Kabat (Wu and Kabat, 1970), Chothia (Chothia and Lesk, 1987), immunogenetics information (IMGT) (Lefranc et al., 2003)).

The composition and relative importance of each CDR. It has been shown that the number of residues that interact with the Ag differ in Abs recognizing Ags of different type and size (MacCallum et al., 1996; Almagro, 2004; Raghunathan et al., 2012), and that there is a wide variation in the contribution of each CDR to Ag binding (Padlan et al., 1995). The amino acid composition of CDRs differs from that of other loops in proteins (Collis et al., 2003) and from that of homo- and heterodimer interfaces (Zhao and Li, 2010) and also from each other (Zhao and Li, 2010; Raghunathan et al., 2012). The amino acid composition of Ag-binding residues has been shown to be distinct and highly enriched in some residues such as Try, Ser, Trp and Asn (Collis et al., 2003; Almagro, 2004; Ofran et al., 2008; Kringelum et al., 2012; Raghunathan et al., 2012). Since different types of protein–protein interfaces were shown to have different amino acid compositions and different contact preferences (Ofran and Rost, 2003; Soga et al., 2010), one might expect that this will hold true also for Ab–Ag
interfaces. However, to the best of our knowledge, the contact preferences of each CDR were not systematically analyzed so far.

**What do Abs like to bind?**

Some amino acids are more likely than others to be found in protein–protein interfaces (Jones and Thornton, 1997a,b; Lo Conte et al., 1999; Chakrabarti and Janin, 2002; Neuvirth et al., 2004). It has been suggested that B-cell epitopes are also enriched with certain types of amino acids (Rubinstein et al., 2008; Soga et al., 2010; Sun et al., 2011). However, several analyses did not find any significant differences between the amino acid composition of epitopes and that of Ag surface residues (Janin and Chothia, 1990; Kringleum et al., 2012) or suggested that epitopes do not necessarily possess clear intrinsic properties distinguishing them from other surface patches (Ponomarenko and Bourne, 2007). General protein–protein interfaces are typically composed of pairs of surface patches that have good shape and electrostatic complementarity with each other (Laver et al., 1990; Jones and Thornton, 1996; McCoy et al., 1997; Cohen et al., 2005). In this sense, the composition of epitopes is determined by the composition of paratopes, as they should complement each other. Thus, if the amino acid composition of epitopes is indistinguishable from the composition of protein surface, the composition of paratopes must reflect this indifference.

Identification of paratopes is often done through identification of CDRs. However, we have recently shown that CDRs, as identified by methods such as Kabat (Wu and Kabat, 1970), Chothia (Chothia and Lesk, 1987) and IMGT (Lefranc et al., 2003), may miss ~20% of the Ag-binding residues (Kunik et al., 2012a,b). Nonetheless, virtually all the residues that bind the Ag lie in regions of structural binding consensus among Abs, termed antigen binding regions (ABRs). ABRs roughly correspond to the CDRs (i.e. six ABRs correspond to the six CDRs but slightly differ in their boundaries) and can be computationally identified from sequence or structure (Kunik et al., 2012a,b).

We analyzed the ABRs of a non-redundant set of all Ab–Ag complexes available from the protein data bank (PDB) (Berman et al., 2000). In particular, we explored (i) the length of the ABRs, (ii) the number of Ag-binding residues in each of them, (iii) their amino acid composition, (iv) the contact preferences of each ABR and (v) their energetic contribution to Ag binding. In addition, we analyzed epitopes as defined by the ABRs with which they interact. We found that the six ABRs differ significantly from each other in their typical length, composition and binding preferences. The amino acid composition of the residues that a given ABR tends to bind is different than the amino acids preferred by the other ABRs, and from the composition of protein surfaces. However, the combined preference of all ABRs together, as reflected by the amino acid composition of epitopes, is virtually the same as the composition of Ag surfaces. These results suggest that ABRs evolved to recognize together protein Ag surfaces in general. Knowledge of the binding preferences of each ABR can improve Ab engineering, Ab–Ag docking and B-cell epitope predictions.

**Materials and methods**

**Dataset construction**

We used a representative, non-redundant set of experimentally determined 3D structures of Ab–Ag complexes from the PDB. The full details of data curation and redundancy removal procedure are described in detail elsewhere (Kunik et al., 2012a,b). This resulted in a non-redundant set of 200 Ab–Ag complexes, listed in Supplementary data S1.

**Identification of ABRs**

To identify the ABRs, we used the Paratome web server (Kunik et al., 2012a,b). The PDB ids of the Abs in our set were used as an input to the Paratome structure-based ABRs identification module. The full list of ABRs is in Supplementary data S2.

**Extraction of Ab–Ag interactions**

For each Ab–Ag complex in our set, an ABR Ab residue and an Ag residue were considered to be in contact if at least one of their respective atoms were ≤6 Å of each other. While such permissive cut-off may introduce false positives, it enables the curation of an unbiased dataset (Ofran and Rost, 2003; Ofran, 2009). The resulting dataset contained 14 286 pairs of Ab–Ag interacting residues, which originated from 5062 distinct Ab residues and 3589 distinct Ag residues. Using distance cut-offs of 5 and 4.5 Å did not change the trends reported (data not shown, see also Supplementary Table S14).

**Solvent accessibility analysis**

The solvent accessible surface area of each structure was calculated using NACCESS (Hubbard and Thornton, 1992–6), with solvent probe radius of 1.4 Å and Van der Waals radii according to Chothia (1976). A residue was considered exposed if its relative accessibility was ≥10%. The relative accessibility is computed as the percent of the computed accessibility of a residue out of the accessibility of that amino acid in an extended ALA-X-ALA tripeptide (where X is the type of amino acid).

**ABRs analysis**

For each Ab within the dataset and for each of the six types of ABRs we recorded:

(i) The length of the ABR,
(ii) The number of residues within the ABR that contact the Ag.

We then assessed the distributions of these values in each of the six ABRs across all Abs.

**Computing amino acid composition**

For each type of ABR (i.e. L1–L3 and H1–H3) we computed its amino acid composition, adding a prior of one to each amino acid type in each type of ABR.

**Measuring Ab–Ag contacts preferences**

We used the six lists of ABRs and the corresponding Ag residues with which they interact to compute the log odds ratio of the observed frequency of each pair over its expected frequency using the following equation:

$$L_x(Ab_i, Ag_j) = \log_2(P(Ab_i, Ag_j)/P(Ab_i)P(Ag_j))$$  \hspace{1cm} (1)

where the subscript \(x\) is one of the six ABRs (i.e. L1, L2, L3, H1, H2 or H3), \(i\) is the Ab amino acid from ABR of type \(x\) and \(j\) is the Ag amino acid, \(P(Ab_i, Ag_j)\) is the observed probability of a contact between amino acid of type \(i\) within ABR of type
Assessing significance of the differences

To determine whether the amino acid compositions and Ab–Ag residue–residue preferences differ significantly from each other, we implemented a bootstrap-like procedure based on Jensen–Shannon (JS) divergence (Lin, 1991), which is often used for measuring divergence between samples (Grosse et al., 2002; Azad and Lawrence, 2007; Capra and Singh, 2007; Ravishankar et al., 2009). Briefly, this procedure works as follows:

Let \( p_1 \) and \( p_2 \) denote two probability distributions satisfying the constraints \( p_1 + p_2 = 1 \) and \( 0 \leq p_i \leq 1 \), \( i = 1, 2 \). Let \( \pi_1, \pi_2 \) denote the respective weights of the probability distributions, so that \( 0 \leq \pi_i \leq 1 \), \( i = 1, 2 \) and \( \pi_1 + \pi_2 = 1 \). The JS divergence between the probability distributions \( p_1 \) and \( p_2 \) with weights \( \pi_1 \) and \( \pi_2 \) is:

\[
\text{JSD}(p_1, p_2) = H[\pi_1 p_1 + \pi_2 p_2] - \pi_1 H[p_1] - \pi_2 H[p_2] \tag{2}
\]

where \( H[X] = -\sum_{x=1}^{n} p(x_i) \log_2 p(x_i) \), and \( X = (x_1, \ldots, x_n) \) is the Shannon entropy (Shannon, 1948).

The following procedure measured how often ABRs of a certain type were most similar in their amino acid composition to ABRs of the same type and how often they were most similar to any of the other ABR types:

(i) Draw a random sample, \( S_i \), with 30% of the ABR sequences of one of the ABRs (e.g. 30% of all L1 sequences).

(ii) Draw a random sample, \( S_j \), with 30% of the ABR sequences of each of the six ABRs, such that \( S_i \cap S_j = \phi \) for \( i \neq j \).

(iii) Compute the six values of JS divergence between \( S_i \) and \( S_j, i = 1, 6 \).

(iv) Record which ABR had the smallest divergence from \( S_i \) and also the value of this smallest JS divergence.

We repeated this procedure 6000 times (1000 for each of the six ABRs). If the amino acid compositions of the various types of ABRs differ significantly, we expect \( S_i \) and the least divergent sample to be drawn from the same type of ABR more often than other types of ABRs. In other words, we expect \( S_i \) and the sample most similar to \( S_i \) to be drawn from the same type of ABR in the majority of cases.

To establish that the Ab–Ag contact preferences of the various types of ABRs differ significantly, we implemented the procedure described above after deriving the 400-dimensional Ab–Ag contacts preferences vector of each ABR type. Next, we computed and recorded the JS divergence between the Ab–Ag contacts preferences vector of each ABR type and all other ABR types. A result is considered statistically significant if it is unlikely to have occurred randomly. Therefore, we examined how often we obtained JS divergence values equal or greater than those obtained by comparing Ab–Ag contacts preferences of different types of ABRs. This was done as follows:

For each pair of ABR types, \( S_i \) and \( S_j, i \neq j \), we performed the following procedure:

(i) Draw a random sample \( S_{ic} \) with the same number of Ab–Ag contacts as in \( S_i \).

(ii) Draw a random sample \( S_{jc} \) with the same number of Ab–Ag contacts as in \( S_j \).

(iii) Compute and record the JS divergence between \( S_{ic} \) and \( S_{jc} \).

The procedure was repeated 6000 times (1000 times for each ABR type). The random samples were drawn from the set of Ab–Ag contacts of all types of ABRs. Furthermore, to eliminate the effect of group size, the samples included the same number of Ab–Ag contacts as the number of contacts in the compared ABRs. If the Ab–Ag contacts preferences differ significantly, we expect the random samples to be, in most cases, less divergent. Thus, we expect the random samples to yield smaller JS divergence values than those obtained by the real amino acid contacts preferences of the different ABRs.

Computational alanine-scanning mutagenesis

We used the FoldX algorithm (Guerois et al., 2002; Schymkowitz et al., 2005), to assess \( \Delta \Delta G \) effects of mutations to alanine. FoldX is based on empirical energy terms that were compared and correlated with experimental data of a large number of mutations in monomeric proteins and protein–protein complexes (Guerois et al., 2002). The predicted energetic effect of \( >1000 \) mutations were found to have a correlation of 0.83 with the experimental results (Guerois et al., 2002). Thus, given the speed and ease of using FoldX, it can provide a good evaluation of trends in large datasets. FoldX (version 3.0b4) was applied to all Ag-binding residues in the following manner: (i) 3D structures were taken from the PDB (Supplementary data S1) and optimized using the FoldX repairPDB function, (ii) structures corresponding to each of the single-point mutants were generated using the BuildModel protein mutagenesis function, (iii) the interaction energy of the wild-type structure and the mutated structure were calculated using the Complex Analysis energy calculation function and (iv) \( \Delta \Delta G \) values were obtained using the following equation:

\[
\Delta \Delta G = \Delta G_{\text{mutant}} - \Delta G_{\text{wild-type}} \tag{3}
\]

The binding energy of mutated structures was classified as follows: destabilizing, if \( \Delta \Delta G > 1 \) kcal/mol; neutral, if \( 0 \leq \Delta \Delta G \leq 1 \) kcal/mol and stabilizing, if \( \Delta \Delta G < 0 \) kcal/mol. We divided our dataset of 200 Ab–Ag complexes into seven random mutually exclusive sets, analyzed each set separately and recorded the average and standard error (Supplementary data S4 and S5).

Epitopes analysis

For each Ab–Ag complex in the dataset, we compiled the list of residues within the Ag that are in contact with the ABRs as described above, marked the chains and ABRs with which it interacts and computed the amino acid composition of the following: (i) all epitopes, (ii) Ag residues contacting the light chain, (iii) Ag residues contacting the heavy chain, (iv) Ag
residues contacting L1, (v) Ag residues contacting L2, (vi) Ag residues contacting L3, (vii) Ag residues contacting H1, (viii) Ag residues contacting H2 and (ix) Ag residues contacting H3.

To identify interfaces of hetero complexes, we used the advanced search option in the RCSB website (on 17 December 2011) with the following search criteria: (i) number of chains (asymmetric unit): 2–10, (ii) macromolecule type: does not contain DNA and/or RNA, (iii) has no modified residues, (iv) minimum chain length: 50, (v) resolution, 3Å and (vi) maximum sequence similarity: 30%. The search resulted in a list of 2889 complexes. We then removed: (i) structures with only one chain in their biological unit, (ii) structures of Abs or Ab-like sequences (i.e. BLAST e-value <0.001 against a sample of either major histocompatibility complex-I (MHC-I), MHC-II, T-cell receptor-A (TCR-A), TCR-B, Ab light chain and Ab heavy chain), (iii) structures in which there was at least one chain with <50 residues and (iv) structures with homo-oligomers (i.e. a chain in the biological unit with >90% sequence identity to another chain). The final list contained 210 hetero complexes (Supplementary data S6). For all proteins in this set we extracted the amino acid composition of interface residues as described above.

**Results**

**ABRs differ significantly**

**ABRs differ in their length.** The length and number of Ag-binding residues in each ABR are presented in Fig. 1. As seen in Fig. 1A, ABR H2 has the longest median length (14 residues long), followed by H3 and L2 (11 residues). While H3 shows the highest length diversity, L2, L3 and H1 show almost no length diversity. L1 and L3 have the shortest median length (eight for both).

**ABRs differ significantly in their number of Ag-binding residues.** Residues within ABRs can contribute to Ag binding either directly, by contacting the Ag, or indirectly by shaping the ABR in a way that allows other residues to contact the Ag. Figure 1B presents the number of residues in each ABR that actually contact the Ag. ABRs H3 and H2 have the largest median number of Ag-binding residues (six residues). However, H3 is more diverse than H2 in the number of Ag-binding residues it contains, and may include up to 14 residues that contact the Ag. ABRs H1 and L1 have a median of four and three Ag-binding residues, respectively, yet L1 is slightly more diverse than H1. While the median length of L2 is the second longest and identical to that of H3 (11 residues), it has a median of only one Ag-binding residue. Hence, usually it does not contribute much to Ag binding. Nevertheless, in some Abs L2 may hold up to 10 Ag-binding residues thus becoming a major factor in Ag binding. It is important to note that for all ABRs there are cases in which they form no contacts with the Ag at all (i.e. zero residues that contact the Ag).

**ABRs differ significantly in their amino acid compositions.** Figure 2 shows the similarity and differences between ABRs using the average JS divergence values between their amino acid compositions. JS divergence can be used to assess the similarity between distributions (very similar distributions have JS values that are close to zero). Each cell in the figure represents the average of 1000 comparisons of ABRs from 60 randomly selected Abs. For example, the top left cell indicates that the average divergence between 1000 comparisons of the amino acid composition of L1 from 60 Abs and the amino acid composition of L1 from other 60 Abs was 0.01. Note that this matrix is based on comparing random samples of the data, which introduce some level of...
Distinct binding preferences of loops explain epitopes

Ab–Ag contacts preferences differ significantly between ABRs. Figure 4 shows Ab–Ag amino acids contact preferences of each ABR. Interaction between charged residues are observed in heavy chain ABRs (especially H2) more often than expected at random, while interactions between polar residues are observed more or less as expected. On the other hand, some of the interactions between polar residues are observed more frequently than expected in light chain ABRs (specifically in L1 and L3), whereas these ABRs are rather depleted in contacts between charged residues with respect to ABRs on the heavy chain. While interactions between Glu in the Ag and Arg in the Ab are the only interactions observed more frequently than expected in L2, a higher than expected frequency of quite a few types of interactions, such as charged, aromatic, hydrophobic and cation–π interactions, are observed in heavy chain ABRs and in L3. Thus, each ABR tends to bind different types of amino acids. The differences between all ABRs were found to be significant (P ≤ 0.006, see Supplementary data S12).

ABRs differ significantly in their energetic contribution to Ag binding. To assess the energetic contribution of each ABR to Ag binding, we performed a computational alanine-scanning analysis using the FoldX algorithm (Guerois et al., 2002; Schymkowitz et al., 2005). The energetic contribution of each ABRs to Ag binding is presented in Fig. 5. The relative distribution of destabilizing mutations (i.e. mutation to alanine that resulted in ΔΔG > 1 kcal/mol, suggesting that the original amino acid in this position is energetically important for Ag binding) is depicted in Fig. 5A. For each ABR, the graph shows the ratio of residues that were found to have a destabilizing effect on Ag binding, out of all destabilizing residues. H3 and H2 have the most prominent effect on Ag binding, comprising ~31 and 23%, respectively, of the energetically important residues. On the other hand, L2 contributes only 6.19% of the energetically important Ag-binding residues. The average energetic contribution of H1 (13.56%), L1 (13.15%) and L3 (13.58%) is essentially the same, consistent with the number of Ag-binding residues in these ABRs (Fig. 1B). Figure 5B shows, for each ABR, the percentage of Ag-binding residues that are energetically important for Ag binding out of all Ag-binding residues in that ABR. H3 has the highest percentage (~29%) of Ag-binding residues that are energetically important for Ag binding. This may explain why H3 has the highest energetic contribution to Ag binding (Fig. 5A), despite the fact that H2 is usually longer (Fig. 1B). L1 and H2 have a similar proportion (~24 and ~22%, respectively) of energetically important Ag-binding residues. H1 and L2 have essentially the same percentage (~19%) of energetically important Ag-binding residues. L3 has the lowest percentage (~17%) of Ag-binding residues that are energetically important for Ag binding.

Frequency of amino acids not always reveals their importance in binding. Figure 5C shows the five most frequent amino acids among energetically important Ag-binding amino acids in each ABR. In all cases, Tyr is the most frequent one. Arg and Trp are both highly frequent in all ABRs except H1 and L1, respectively. Phe is common to L1, L2, L3 and H1. Asp is common to all heavy chain ABRs, while Asn appears only in L1 and L3. Interestingly, Gly is highly abundant in all heavy chain ABRs. Note that positive ΔΔG for the mutation Gly-to-Ala does not necessarily reflect direct binding of the Gly. Rather, the increased volume of the Ala might destabilize the complex through clashes/steric hindrances. Despite the fact that Phe, Arg and Trp are not frequent among Ag-binding amino acids, Phe is frequent among energetically important amino acids in all light chain ABRs and Arg is frequent among energetically important amino acids in all ABRs, except for H1. Trp is not a frequent Ag-binding amino acid, nevertheless, it is among the five most frequent energetically contributing amino acids in all of them. Thus, the abundance of an amino acid among the Ag-binding residues does not necessarily indicate its energetic contribution to Ag binding. The amino acid composition of all energetically important Ag-binding residues is available in Supplementary data S13. The observed trend does not change when we use different cut-offs (e.g. ΔΔG > 2 kcal/mol and contacting residues are within 4.5 Å of each other. See Supplementary Table S14).

The Ag side of the interface

Considering that the six ABRs have significantly different amino acid compositions and contact preferences, one would expect epitopes to be a distinguishable set of residues on the presence of quite a few types of interactions, such as charged, aromatic, hydrophobic and cation–π interactions, are observed in heavy chain ABRs and in L3. Thus, each ABR tends to bind different types of amino acids. The differences between all ABRs were found to be significant (P ≤ 0.006, see Supplementary data S12).
surface of the Ag, complementing the distinct composition of the paratopes. Figure 6 presents the amino acid composition of Ag surface residues, epitopes, in general, and residues contacted by each type of ABR. Comparing the amino acid composition of epitopes and that of Ag surface residues does not reveal any noticeable differences (Fig. 6A). Apart from Glu and Thr, which are slightly (1–2%) more frequent in Ag surface residues and Trp and Gly, which are marginally (~1%) more abundant in epitope residues, all other amino acids show virtually identical frequencies in both sets. When the set of epitope residues was mapped into groups according to the ABR they contact (e.g. Ag residues contacting L1), differences between the amino acid compositions became noticeable. Figure 6B depicts the amino acid composition of Ag surface residues and epitopes of each ABR.

Fig. 3. The five most frequent Ag-binding amino acids in each ABR. For each ABR, we compiled the list of residues that contact the Ag and measured the frequency of each amino acid in this set. L1–L3, H1–H3 denotes the light and heavy chain ABRs, respectively.

Fig. 4. Ab–Ag amino acids contact preferences of the six ABRs. The y-axis denotes antigenic residues and the x-axis denotes the Ab residues. Each panel represents one ABR. The cells are color-coded according to the preference of the amino acid pairs to interact. A red square indicates that the interaction is favoured, and a blue square indicates that it occurs less frequently than expected at random. The black open rectangles indicate (left-to-right, bottom-to-top) contacts between charged, aromatic, aliphatic hydrophobic and polar residues. Amino acids that appear <15 times either in the ABR or in the Ag were ignored for that ABR (e.g. Cys in H1 and Met in H2).
surface residues and Ag residues contacted by light chain ABRs. L2 tends to bind more Lys and His, and L1 prefers Leu. Figure 6C depicts the amino acid composition of Ag surface residues and Ag residues contacting heavy chain ABRs. H3 shows preferences to bind Ile and disfavors Thr. Lys is, more or less, as frequent in Ag residues contacting heavy chain ABRs as in Ag surface residues.

The residues contacting each ABR are not a random sample of surface residues. To investigate the difference between the amino acid compositions of Ag surface residues and residues that contact different types of ABRs, we used the JS divergence. The results of the analysis are presented in Fig. 7. Two sets of residues that have identical compositions will have a JS divergence of zero. Larger JS divergence values indicate...
greater ‘dissimilarity’ between the sets. Epitope residues are most similar in their amino acid composition to Ag surface residues. As a reference, we also present the divergence between Ag surface residues and protein–protein interfaces (excluding Ab–Ag complexes), which are the most divergent from Ag surface residues. In between, are the divergences of Ag residues contacting each type of ABR. Their divergence from the general composition of Ag surface residues, suggests that they are not random samples of Ag surface residues. That is, each ABR prefers to bind a distinct set of amino acids. However, the combination of all these preferences yields a composition that is highly similar to protein surface in general. This is further illustrated by the fact that the combined preference of all ABRs on the light chain is closer to the general composition of Ag surface residues than any of the light chain ABRs. Similarly, the combined preference of all ABRs on the heavy chain is closer to the general composition of Ag surface residues than any of the heavy chain ABRs.
ABRs. Finally, the combined preference of both the light and heavy chains altogether is closer to the general composition of the surface than any of them. The preference of all ABRs on the light chain is more divergent from the general composition of Ag surface residues than the preference of all ABRs on the heavy chain. Specifically, the amino acid compositions of Ag residues contacting ABR L1 and Ag residues contacting ABR L2 are the most divergent from the general composition of Ag surface residues.

Discussion

Early analyses of Ab–Ag recognition were based on a small number of complexes. The comprehensive analysis of antigenic interfaces we present here reveals some basic characteristics of epitopes and paratopes. Obviously, like any large-scale analysis, our reliance on data from the PDB may introduce biases towards proteins that are more widely studied or those that are more easily expressed, purified or crystallized. These biases notwithstanding, we are able to suggest some general principles of Ab–Ag recognition.

The Ag-binding site

Diversity is a key factor in the adaptive immune system. Most of the noticeable sequence and structural diversity of Abs is confined to the six hypervariable loops (Wu and Kabat, 1970; Kabat et al., 1983). Collis et al. (2003) demonstrated that the length diversity of these loops plays a major role in defining shape and electrostatic complementarity between the epitope and the paratope. In agreement with their findings, our results show that L1 has the highest length diversity among the light chain ABRs, H1 has the lowest length diversity among the heavy chain ABRs and H3 the highest length diversity among ABRs. Our results show that light chain ABRs form fewer interactions with the Ag in comparison with heavy chain ABRs and are in line with previous reports (MacCallum et al., 1996; Almagro, 2004). As for the amino acid composition of paratopes, it has been suggested that Ag-binding residues are generally enriched with Tyr, Trp, Ser and Asn and are depleted in Cys, Glu, Lys and Pro, relative to loops in proteins (Collis et al., 2003) and to proteins in general (Ofran et al., 2008). In a series of studies, Fellouse et al. (2004, 2006) showed that Tyr contributes over 70% of the interfacial area of the Ab. Using a diversity of only four amino acids (Tyr, Ala, Asp and Ser) in display libraries, they were able to generate high specificity and affinity in engineered Abs. Their choice of these amino acids is based on their abundance in CDRs. Our in silico alanine-scanning analysis (Fig. 5) suggests that Tyr is also the amino acid that contributes most energetically to Ag binding. This is probably due to its exceptional versatility in mediating contacts (Van der Waals, aromatic interactions and hydrogen bonds). However, despite the high abundance of Ser among Ag-binding amino acids in all ABRs, it does not seem to contribute much energetically in any of the ABRs. Gly, which does not come up as unusually abundant in the paratopes, is a frequent and an important energy contributor in both H2 and H3 (Fig. 3). Birtalan et al. (2008) engineered Abs with Tyr, Ser, Gly and Arg, and suggested that the most specific Abs were those with the highest Tyr content. They also suggested that Ser and Gly appear frequently in the binding site due to their size and the fact that they allow flexibility of the chain and avoid steric hindrances, which is in line with the fact that H2 and H3 are the longest ABRs and may be subject to conformational constraints. Gly is an important energetic contributor in H1 as well, which may also be subject to structural constraints that require flexibility due to its location between the longer ABRs, H2 and H3.

Abs recognize indistinct patches on Ag surface

Unlike protein–protein complexes that co-evolve and optimize their interfaces to best fit each other while maintaining their other molecular and biological functions (Keskin et al., 2008), in Ab–Ag complexes only the Ab goes through rapid development to fit the epitope. Electrostatic, hydrophobic and aromatic interactions between protein surfaces are the main forces driving protein association (Jones and Thornton, 1995; Xu et al., 1997a,b; Xu et al., 1997a,b; Lo Conte et al., 1999; Sheinerman et al., 2000; Ofran and Rost, 2003; Crowley and Golovin, 2005). However, our results, which are consistent with previous studies (Janin and Chothia, 1990; Kringleum et al., 2012), found no substantial enrichment of residues that mediate these types of interactions in epitopes compared with their propensities on the surface of Ags. This result is also in agreement with studies that showed that the entire accessible surface of a protein is a continuum of potential antigenic sites, detectable by Abs (Berzofsky, 1985; Novotny et al., 1986), and with the work of Blythe and Flower (2005) that could not significantly associate 484 amino acid propensity scales from the AIndex database (Kawashima and Kanehisa, 2000) with known linear epitopes locations. From an evolutionary standpoint, it is clear that it would be advantageous if Abs could bind any surface patch, without being limited to rarely occurring patches that are ‘stickier’. From a physicochemical perspective, however, it is not clear how this can be realized. Our results may suggest a mechanism that can resolve this ostensible contradiction. We find that each ABR has a different set of amino acid contact preferences. That is, each ABR tends to bind different types of amino acids. For example, Ab Thr and Ag Asn are slightly disfavored in all ABRs, but L2 and L3. H2 shows a strong preference to form charged interactions, while the other ABRs have a much weaker inclination to do so. Interactions of light chain ABRs with Ag aromatic amino acids are completely absent, yet all heavy chain ABRs interact with Ag aromatic amino acids. Thus, Abs may have evolved so that the intricate combination of the preferences of each ABR results in an overall binding preference to bind epitopes that are indistinguishable from the rest of the surface of the protein.

This is also consistent with what we see when we review the divergence between the residues that are favored by each ABR (Fig. 7). Taken together, epitope residues are the least divergent from Ag surface residues. Divergence increases when epitope residues are grouped according to the Ab chain they contact and increases further when they are divided according to the specific ABR they contact. Consistent with previous results (Jones and Thornton, 1995; Lo Conte et al., 1999), we found that protein–protein interfaces have significantly different amino acid composition than that of non-interfaces. Abs and Ags may be subjects to opposing pressures: while the function of Abs is to bind the Ag, a pathogen will be evolutionary successful if its Ags escape recognition by the immune system. The ability of Abs to recognize indistinct patches can be crucial in such red queen’s race.
H2 takes on a key role in Ag binding

Of the six CDRs, H3 has been known to have the highest variability in length, sequence and structure (Chothia et al., 1989; Al-Lazikani et al., 1997; Kuroda et al., 2008). It is generally known to form the largest number of contacts with the Ag (Zhao et al., 2011) and to be the most important CDR for Ag recognition and binding (Al-Lazikani et al., 1997; Barrios et al., 2004; Kuroda et al., 2008; Sela-Culang et al., 2012). Our results indicate that indeed, H3 has the highest length variability and that it contributes more to Ag-binding energy than any other ABR. Nevertheless, we found that the median length of H2 is substantially longer than that of H3 and that it typically forms the same number of interactions with the Ag as H3 does. H2 contributes ~23% of the residues that are energetically important for Ag binding (compared with 31% that are contribute by H3). When inspecting the residue–residue preferences of H2, it can be seen that it is enriched with residues that can readily form salt bridges and aromatic interactions. In fact, the most frequently occurring energetically important amino acids in H2 are Tyr, Asp, Trp, Arg and Gly. This enrichment may explain, to some extent, the relatively high energetic contribution of H2 to Ag binding. In some cases, H2 is more important energetically to Ag binding than H3. For example, the structure of HyHEL-10 complexed with HEL (pdb id: 2DQJ) (Burkovitz et al., 2012). While the energetic contribution of L2 is in most cases negligible (6%), L1, L3 and H1 contribute equally to the energetics of Ag binding (~13%). It is important to note that these results come from lumping all Abs together. However, there are cases in which most of the energetically important residues are from L1 (PDB ids: 1V7M and 1H0D). Thus, each of the six ABRs may be the key to binding a given epitope and each of them can sometimes be inconsequential for Ag binding. Kuroda et al. (2012) reviewed several studies in which computational methods were utilized to identify favorable positions for experimental mutagenesis (Barderas et al., 2008). They concluded that not only H3 but also the other five loops are good candidates for affinity-enhancing mutations. In addition, a recent analysis showed that the length of L3 accounts for the increased neutralization potency of a class of Abs targeting the CD4-binding site of HIV-1 gp120 (West et al., 2012), further emphasizing that each ABR may be a major contributor to Ag binding.

In summary, we have shown that ABRs differ significantly from each other in length, number of Ag residues they bind, amino acid composition, amino acid contact preferences and the energetic contribution to Ag binding. H2, and not only H3, takes on a key role in Ag binding. Moreover, by combining the distinct binding preferences of the ABRs, Abs are capable of recognizing indistinct patches on the surface of Ags.

A set of antigenic residues cannot become an epitope in the absence of a specific Ab capable of binding it (Greenbaum et al., 2007; Van Regenmortel, 2009). While most methods for identifying B-cell epitopes focus on continuous epitopes, most real Ab binding sites in native proteins are complex conformational discontinuous epitopes. Identifying paratope–epitope binding preferences and better understanding of the way paratopes identify epitopes could improve epitope prediction. In addition, detailed binding preferences of each CDR may also assist attempts to engineer Abs.

Supplementary data

Supplementary data are available at PEDS online.

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References

Distinct binding preferences of loops explain epitopes