Humanization of Antibodies Using Heavy Chain Complementarity-determining Region 3 Grafting Coupled with in Vitro Somatic Hypermutation*

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Background: Humanization of murine monoclonal antibodies by CDR grafting is a widely used technique.

Results: In vitro somatic hypermutation was coupled with minimal CDR grafting to produce potent, pM affinity antibodies.

Conclusion: This methodology can rapidly generate potent, humanized antibodies containing a minimum of donor sequence.

Significance: Antibodies produced using this approach contain reduced rodent antibody donor content and possess potential advantages in manufacturability and immunogenicity.

A method for simultaneous humanization and affinity maturation of monoclonal antibodies has been developed using heavy chain complementarity-determining region (CDR) 3 grafting combined with somatic hypermutation in vitro. To minimize the amount of murine antibody-derived antibody sequence used during humanization, only the CDR3 region from a murine antibody that recognizes the cytokine hNGF was grafted into a nonhomologous human germline V region. The resulting CDR3-grafted HC was paired with a CDR-grafted light chain, displayed on the surface of HEK293 cells, and matured using in vitro somatic hypermutation. A high affinity humanized antibody was derived that was considerably more potent than the parental antibody, possessed a low pM dissociation constant, and demonstrated potent inhibition of hNGF activity in vitro. The resulting antibody contained half the heavy chain murine donor sequence compared with the same antibody humanized using traditional methods.

The majority of therapeutic monoclonal antibodies approved by the Food and Drug Administration for human use are derived from mouse immunization, with 21 of the 30 marketed molecules originating from murine hybridoma sources (1). Hybridoma-derived monoclonal antibodies possessing desired functional properties are typically humanized by replacing those regions not required for antigen binding specificity with the corresponding human sequence. The goal of this process is to produce a molecule with reduced nonhuman sequence content that possesses the same or improved activity and affinity relative to the parent antibody but with reduced immunogenicity. The first methods developed replaced the constant domains of the mouse donor antibody to produce a chimeric molecule that was ~70% human sequence (2). Subsequently, both the constant domains and variable domain framework regions were replaced with human sequence, leading to complementarity-determining region (CDR)-grafted antibodies with ~90% human antibody sequence (3, 4).

CDR-grafted antibodies often exhibit reduced binding affinity, and additional engineering is generally required to recover the binding properties of the original clone. Structure-based (5–7) and library-based methods (8) have been demonstrated for antibody humanization that regain or may improve upon the binding affinity of the originating molecule. Affinity maturation of antibodies can be accomplished by a number of methods including random mutagenesis (9, 10), random mutagenesis of CDR sequences (11), directed mutagenesis of residues (12, 13), and approaches that reproduce SHM in vitro (14). Random methods for humanization and affinity maturation rely on multiple rounds of labor-intensive trial and error mutagenesis, and may result in an additional non-germ line sequence being incorporated into the final antibody.

During in vivo adaptive immunity, affinity maturation in B-cells is effected by Ig SHM combined with clonal selection. Activation-induced cytidine deaminase (AID) is the enzyme that initiates SHM, and its action, in concert with additional, ancillary factors, introduces mutations into the DNA of antibody V regions, preferentially targeting amino acids important for antigen binding such as those capable of direct contact with antigen. The position and identity of SHM mutations have been explored in detail by a number of groups and have led to the identification of specific hotspot motifs (e.g., WRCH) (15). Expression of AID in either a B-cell or non-B-cell context in vitro has been shown to be sufficient to initiate SHM and results

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2 The abbreviations used are: CDR, complementarity-determining region; SHM, somatic hypermutation; AID, activation-induced cytidine deaminase; HC, heavy chain; LC, light chain; IGHV, immunoglobulin heavy chain variable region; IGLV, immunoglobulin A variable region; IGGK, immunoglobulin k variable region; SPR, surface plasmon resonance; HTRF, homogeneous time-resolved fluorescence; hNGF, human β NGF; H1, HC CDR1; H2, HC CDR2; H3, HC CDR3; L1, LC CDR1; L2, LC CDR2; L3, LC CDR3.
in replication of the amino acid diversity generated by SHM in vivo (16–18).

We sought to develop a simple method for humanization that would minimize both the originating murine-derived antibody sequence and secondary mutations required for affinity maturation, while improving upon the affinity and activity of the originating antibody. The CDR H3 of a murine antibody, directed against the neurotrophic growth factor hβNGF was grafted into a nonhomologous human V region and affinity-matured in vitro utilizing a combination of AID-directed SHM activity in HEK293 cells and also libraries exploring common SHM events observed in vivo. This approach resulted in the rapid generation of a humanized antibody with pxi affinity for hβNGF that exhibited potent anti-hβNGF in vitro and possessed half the number of non-germ line HC mutations and donor antibody sequence compared with the same antibody humanized using traditional methods.

**Experimental Procedures**

**Analysis of in Vivo Somatic Hypermutation**—The NCBI archive of antibody sequences was downloaded from NCBI and mined for sequences annotated as human IgG or IgM in origin. Germ line human IGHV, IGKV, and IGLV sequences and their allelic forms were assembled from three online antibody sequence sources, IMGT, NCBI Entrez, and VBASE, yielding a total of 232 IGHV, 56 IGKV, and 66 IGLV germ line alleles. The single germ line sequence that provided the best unique alignment to each of the matured antibody sequences was identified using an ungapped BLAST alignment with an expectation score of <1.0 × 10⁻50 and a minimum 93% sequence identity over the entire length of the antibody variable region. Mutations identified at the 5' and 3' portions (three residues) of the alignment were not considered further in this analysis. In this way, a total of 106909 IGHV, 24378 IGKV, and 24965 IGLV mutations were identified in 12956, 4165, and 3811 alignments to germ line sequences, respectively. Each DNA base in the germ line sequences was mapped to a unique codon and Kabat number-106909 IGHV, 24378 IGKV, and 24965 IGLV mutations were mined for sequences annotated as human IgG or IgM in origin. The DNA sequence for the closest human V gene homolog IGKV1–27 (leader peptide and FW1, 5'-ATGGAGATGAGGTTCCCTGTCACGGGTTCTCCCTGCTGTTTGGGCTGAG-GAGGAGCACGAGTCCAGCAAGGAGTTAAGCTACT-3') was taken from the closest human J-region IGJ6 (5'-TGGGGCGAAGGGACCGTGTCACCGTCTC-CTCA-3'). Kabat CDR definitions were used, and germ line sequences were based on IMGT database annotations (20, 21). Amino acid positions selected for diversification and the amino acid diversity at each position (Fig. 1) in this HC library were based on the bioinformatics analysis described above. The amino acids encoded in the library at each position were: H28, TAI; H30, STGNT; H31, SNDRT; H33, ATSVD; H35, NGTIS; H50, AGTSLV; H52a, GDVANT; H53, SRNTG; H55, AVRTDS; and H56, SRTGN. The germ line residue Gly-55 was not present in the library. The codons used to encode amino acid diversity at each position (Ser, AGC; Thr, ACT, Ala, GCT, Asn, AAC; Val, GTC; Arg, AGG; Ile, ATC; Asp, GAC; and Leu, CTG) were selected based on two criteria: observed codon usage at antigen contacting positions across all IGV genes and preservation of AID hot spots (WRC). The HC CDR3-grafted, CDR1.2 diversified library was assembled with IgG1 constant domains with a C-terminal transmembrane segment supporting cell surface display and cloned into in-house episomal vectors for stable selection in HEK293 cells.

The LCV region sequence was assembled using the germ line sequence for the closest human V gene homolog IGHV1–27 (leader peptide and FW1, 5'-ATGGAGATGAGGTTCCCTGTCACGGGTTCTCCCTGCTGTTTGGGCTGAG-GAGGAGCACGAGTCCAGCAAGGAGTTAAGCTACT-3') and FWF1 at position 53, and HAEQ at position 55. As defined by Kabat CDR definitions. The entire sequence was synthesized (DNA2.0, Menlo Park, CA) and joined with a human IGK constant domain and assembled in in-house episomal vectors.

Two light chain V region libraries, termed ML28 and ML30, were also prepared during affinity maturation. They were based on the most frequently observed SHM events in antibody light chains as described for heavy chains. Both contained variations at three positions. ML28 contained ERHQGK at position 27, DGANSTPR at position 28, and NGDAS at position 50, whereas ML30 contained ANFSYTD at position 32, TNRS at position 53, and HAEQ at position 55.

**Transformation, Stable Expression, and Selection of HEK293 c18 Cells**—HEK293 c18 cell lines stably expressing the CDR3-grafted, IgG heavy chains modified with a C-terminal transmembrane domain (22) and CDR1,2,3-grafted light chain, together with AID were generated using individual episomal vectors under independent selection for expression of HC, LC, and AID as described (14). T75 culture flasks were seeded with 3 × 10⁶ HEK293 c18 cells in DMEM containing 10% FBS (Invitrogen). Plasmids were transfected using OptiMEM (Invitrogen) and HD-FuGENE (Roche Applied Science). Three days post-transfection, cell growth medium was exchanged with DMEM containing 10% FBS, 50 μg/ml geneticin, 10 μl/ml antibiotic-antimycotic solution, 1.5 μg/ml puromycin, 15 μg/ml blasticidin, and/or 350 μg/ml hygromycin (all from Invitrogen), and...
the cells were incubated for approximately 4 weeks with periodic reseeding and exchange of the cell culture medium.

**Antigen and Antibody Expression and Purification**—Antibody variants were expressed transiently in HEK293 c18 cells as full-length IgG1 kappa molecules. Supernatants from transfected cells were loaded on a protein A/G-agarose resin (Thermo Scientific), washed with 6 column volumes of PBS, pH 7.4, and eluted with 100 mM glycine, pH 3.0, with the resulting purified IgGs buffer exchanged into PBS. Human hH9252 NGF was expressed transiently in HEK293 c18 cells and purified using standard His tag affinity purification methodologies. Fluorescently labeled antigens utilized for FACS were prepared using standard amine coupling chemistry. In addition, a fusion protein of hH9252 NGF linked to wasabi fluorescent protein was also expressed and purified using standard His tag affinity purification methods.

**FACS Selection**—To assess initial antigen binding and optimal conditions for FACS, HEK293 c18 cells displaying cell-surface antibody (5 × 10^5 cells in 0.5 ml PBS, 0.1% BSA) were incubated with various concentrations of fluorescently tagged hβNGF and FITC-AffiniPure Fab fragment goat anti-human IgG (H+L) (Jackson ImmunoResearch) for 0.5 h at 4 °C. The cells were then pelleted and resuspended in 0.3 ml of 0.2 μg/ml DAPI in PBS, 0.1% BSA (Sigma-Aldrich) and analyzed for fluorescence on a BD Influx cell sorter (BD Biosciences). For FACS selection, stably transfected HEK293 c18 cells were incubated with fluorescently tagged hβNGF (at concentrations determined empirically from FACS analysis discussed above). In each instance, ~20–40 million cells were sorted, typically selecting 0.1–0.5% of the most fluorescent cells normalized against cell surface IgG expression. The amino acid composition of the HC and LC in the final populations was assessed via

**FIGURE 1. SHM in vivo.** Analysis of human affinity-matured antibodies compared with their germ line antecedents highlights the features of SHM in vivo. The 20 most common codon mutations are shown for the IGHV 3–23 V region (A). The arrow widths are proportional to the frequency with which a mutation of a codon was observed (e.g., AGC to AAC corresponds to 3529 events). Common in vivo SHM events form an interconnected network of differentiation seeded from SHM AID hot spots that correspond to antigen contacting positions (B). Specific IGHV positions account for a majority of the diversity created during in vivo affinity maturation. Mutations at 10 CDR1 and CDR2 positions, denoted by arrows, were selected for a combinatorial library containing 6E6 members, accounting for ~30% of all in vivo SHM events observed in this V region.
Sanger sequencing from cell pellets collected following each FACS selection.

**HTRF Antibody Competition Assay**—The binding affinity rank order of anti-NGF antibodies was determined in an HTRF assay. A reference antibody to hβNGF (tanezumab) was biotinylated, and purified hβNGF was labeled with N-hydroxysuccinimide activated cryptate using a HTRF® cryptate labeling kit following the manufacturer’s protocol (Cisbio Bioassays). The biotinylated reference antibody was mixed with Streptavidin-XL665 (Cisbio), with the test antibody added at varied concentrations, followed by incubation with labeled hβNGF at room temperature for 2.5 min in PBS, pH 7.2, 0.1% BSA. The reaction was read in a ProxiPlate-384 Plus (PerkinElmer Life Sciences) using an EnVision plate reader (PerkinElmer Life Sciences) (320-nm excitation, dual emission at 620 and 665 nm). Binding of hβNGF to the reference antibody was represented as the ratio of emission at 665/620 nm. To determine the IC₅₀ values of the test antibodies, the 665/620-nm emission ratios were fitted by a three-parameter inhibitory curve using GraphPad Prism (GraphPad Software).

**NGF Signaling Assay**—The rat pheochromocytoma-derived cell line, PC12, that differentiates in response to hNGF (CRL-1721) and maintained in DMEM supplemented with 15% horse serum, 2.5% FBS. For signaling assays PC12 cells were plated at 0.8–1.0 × 10⁵ cells/well in collagen type IV coated 96-well microplates (BD BioCoatTM; BD Biosciences) and incubated overnight at 37 °C. Following a 4-h serum starvation in DMEM, 0.1% BSA PC12 cells were stimulated with human hβNGF (R&D Systems, 10 ng/ml) in the presence or absence of various concentrations of anti-βNGF antibodies for 15 min at 37 °C. Cell lysates were made in freshly prepared 1 × lysis buffer for AlphaScreen® (PerkinElmer Life Sciences) according to the manufacturer’s instructions for adherent cells. Activated ERK1/2 in cell lysates was quantified in an AlphaScreen® SureFire® p-ERK1/2 (Thr-202/Tyr-204) assay (PerkinElmer Life Sciences) according to the manufacturer’s instructions for adherent cells. The plates were read on an EnVision® 2103 multilabel plate reader (PerkinElmer Life Sciences). The data were graphed and analyzed using GraphPad Prism (GraphPad Software, Inc.) using three-parameter, nonlinear regression curve fits.

**Affinity Screening of Antibody/βNGF Binding**—Secreted antibodies from 96-well plate array transfections were screened and ranked using a Biacore 4000 (GE Healthcare) surface plasmon resonance (SPR) instrument. Four of five spots in four flow cells on a Series S CM5 chip (GE Healthcare) were coupled with ~10,000 resonance units of anti-human IgG (Fc) (human antibody capture kit; GE Healthcare). The fifth flow cell was kept blank to serve as a reference. Culture medium from antibody transfectant HEK293 cells was diluted 1:1 with HBS-EP+ buffer (10 mM Hepes, 150 mM sodium chloride, 3 mM EDTA, 0.05% Polysorbate 20) with 0.1% BSA, pH 8.0, and centrifuged. Secreted antibody was captured on the CM5 chip by flowing diluted culture medium over the outer spots for 120 s at 10 ml/min. hβNGF at 500 and 50 nM was then passed over all flow cells for 120 s at 30 ml/min and then allowed to dissociate for 600 s. The capture surface was regenerated using glycine, pH 1.5, for 120 s. Resulting sensorgrams were double reference subtracted, and the kinetics were analyzed using Biacore 4000 evaluation software, version 1.0.

Antibody variants exhibiting the highest affinity binding by Biacore 4000 analysis were chosen for scale-up, purification, and additional characterization of kinetic constants using a Biacore T200 (GE Healthcare). Each of four flow cells on a Series S CM5 chip was immobilized with ~1,000 resonance units of anti-human IgG (Fc). Antibodies (~1 mg/ml) were captured for 60 s at a flow rate of 10 ml/min. hβNGF was diluted in running buffer (HBS-EP+, 0.1% BSA, pH 7.4) starting ~10-fold higher concentration than each antibody’s Kᵯ. Each hβNGF concentration was passed over all flow cells for 120 s at 30 ml/min, then allowed to dissociate for 600 s. Surfaces were regenerated with
3 m \(\text{MgCl}_2\) for 180 s. Association and dissociation kinetic constants (\(k_{\text{on}}\) and \(k_{\text{off}}\)) and steady-state affinity (\(K_D\)) were derived from the resulting sensorgrams using Biacore T200 evaluation software, version 1.0.

**RESULTS**

**Analysis of SHM in Vivo**—Humanization was initiated by grafting the CDR H3 from a rat monoclonal antibody to h\(\beta\)NGF (24) into a nonhomologous V region (Fig. 2). A combinational HC V region library with amino acid variation at ten CDR1 and two positions was used to initiate grafting prior to in vitro SHM and affinity maturation. The V gene IGHV3–23 was chosen for CDR3 grafting based on its good biophysical characteristics, its usage in existing therapeutics, and its high degree of usage in human in vivo antibody repertoires. Positions were varied to encompass the most frequent in vivo SHM events observed in the IGHV3–23 gene. Mutations resulting from in vivo SHM were obtained by comparing matured human antibody sequences with their corresponding germ line V gene sequences. A total of 106,909 IGHV, 24,378 IGKV, and 24,965 IGLV mutations were identified in 12956, 4165, and 3811 alignments to germ line V gene sequences, respectively. These data can be viewed as a fate map of likely mutations observed during in vivo SHM (Fig. 1A), revealing that particular SHM hot spots (e.g., AGC) propagate the majority of amino acid diversity. Hot spot motifs are often localized to antigen contacting residues, contained primarily within CDR1, CDR2, and FW3, as shown for the V gene IGHV3–23 in Fig. 1B. This analysis was applied to create the V gene combinatorial library with variability at ten positions within CDR1 and CDR2 as described under “Experimental Procedures” (Fig. 3). The library encoded four to six possible codons at each varied position and had a total complexity of \(1.6E+07\) members, reflecting the most common V gene, position-specific amino acid mutations as described above.

**CDR H3 Grafting in V Region SHM Diversified Libraries for Humanization**—To initiate humanization, the combinatorial IGHV3–23 library described above was paired with a LC containing the originating antibody L1, L2, and L3 CDRs grafted into its closest human \(\kappa\) V region ortholog (IGKV1–27). The library was assembled in episomes in a full-length IgG1 format that supports simultaneous secretion and cell surface display in mammalian cells (14).

Following transfection and stable episomal selection in HEK293 cells, the cell population was expanded and subjected to iterative rounds of FACS (Fig. 2). To apply selective pressure, the cells were stained with diminishing concentrations of fluorescently labeled antigen. Single cells were sorted into 96-well plates, and kinetics of antibody-antigen binding was characterized using Biacore T200 evaluation software version 1.0 (B). The dissociation rate for APE424 is 1.7 s\(^{-1}\), which was subsequently improved \(-1000\)-fold by four AID-derived mutations identified during affinity maturation (APE894, APE896, and APE897).

**TABLE 1**

| Antibody | \(k_{\text{on}}\) | \(k_{\text{off}}\) | \(K_D\) |
|----------|----------------|----------------|--------|
| APE424   | 7.3E + 06      | 1.7E - 02      | 2.3E - 09 |
| APE520   | 9.3E + 06      | 1.9E - 04      | 2.1E - 11 |
| APE521   | 1.6E + 07      | 7.2E - 05      | 4.5E - 12 (<1E-11) |
| APE890   | 9.4E + 06      | 3.9E - 05*     | 4.2E - 12 (<1E-11) |
| APE894   | 8.7E + 06      | 1.9E - 05*     | 2.2E - 12 (<1E-11) |
| APE896   | 6.5E + 06      | 4.6E - 08      | 9.9E - 15 (<1E-11) |
| APE897   | 9.1E + 06      | 1.7E - 05*     | 1.8E - 12 (<1E-11) |
| PG110    | 4.7E + 06      | 1.9E - 07*     | 4.0E - 14 (<1E-11) |

* These values are considered to be at or below the level of detection for this instrument.
Humanization Using CDR3 Grafting with Somatic Hypermutation

Affinity Maturation—APE424 was affinity-matured using two parallel strategies: in vitro SHM initiated by transfection of AID and small combinatorial libraries incorporating the most frequent in vivo SHM events in the LC CDR1 and 2. In the first approach, the HC and LC were stably transfected with AID into HEK293 cells as described (14). In the second approach, positions within the LC CDR1 and CDR2 were varied in combinatorial libraries to identify beneficial mutations. The two LC libraries (ML28 and ML30) encompassed a total of 6 L1 and L2 antigen-contacting positions with mutations frequently observed during SHM in vivo. As with the AID-mediated SHM approach, these libraries were paired with the APE424 HC, transfected with AID in HEK293 cells, stably selected, and subjected to 4–6 rounds of cell sorting in the presence of diminishing concentrations of fluorescently labeled antigen.

Affinity maturation was observed within two rounds of cell sorting from the in vitro SHM strategy, as evidenced by enriched mutations observed in sequences recovered from cells binding antigen with higher affinity (Table 2). Mutations identified from this cell population were recombined by oligonucleotide-directed mutagenesis, expressed, and characterized by SPR. A construct incorporating LC mutations G66E, H90Q, and G93D (APE520) showed a 100-fold improvement in binding affinity for hNGF relative to APE424, and the addition of LC mutations D17N and F71L improved affinity by an additional 4-fold (AP571) (Tables 1 and 2 and Fig. 4). An additional mutation, D61Y, was observed to be enriched in the HC and was included in the final library used to recombine the SHM-derived mutations described below.

Mutations were enriched from both LC SHM-diversified libraries, including CDR1 mutations E27K and N28D, and CDR2 mutations T53R and H55Q, each providing only modest (<2-fold) improvements in affinity. Transfection of AID into the library cells resulted in additional mutations generated, and the AID-mediated mutation H90Q was also enriched by round 2 of cell sorting. To identify the optimal HC and LC from the enriched mutations identified, mutations from the in vitro SHM and SHM-diversified library strategies were recombined by overlap extension PCR in a final combinatorial library, which was transfected and stably selected in HEK293 cells. This population was subjected to iterative rounds of fluorescence-activated cell sorting using diminishing concentrations of antigen.

In FACS rounds 4 and 5, the cells were incubated with 1 nM Dylight-labeled antigen for 30 min, followed by washing and incubation with unlabeled hNGF (25). Following round 5, the cells were isolated by FACS, HC/LC pairs were isolated, the corresponding antibodies were expressed and purified, and binding kinetics were characterized by SPR.

Characterization of Affinity-matured Antibody Variants—The binding kinetics and $K_D$ of affinity-matured antibodies were determined to be in the mid to low pM range as determined by SPR experiments (Table 1 and Fig. 4), with $k_{off}$ values

### Table 2

Mutations accompanying affinity maturation

| Origin | Kabat Chain | Amino acid | Mutation | Source | Position | APE424 | APE520 | APE571 | APE890 | APE896 | APE897 |
|--------|-------------|------------|----------|--------|----------|--------|--------|--------|--------|--------|--------|
| Library 30 | HC | Ser | Thr | M2 | CDR1 | × | × | × | × | × |
| Library 31 | HC | Ser | Thr | M2 | CDR1 | × | × | × | × | × |
| Library 33 | HC | Ala | Asn | M2 | CDR1 | × | × | × | × | × |
| Library 50 | HC | Ala | Val | M2 | CDR2 | × | × | × | × | × |
| Library 52A | HC | Gly | Thr | M2 | CDR2 | × | × | × | × | × |
| Library 53 | HC | Ser | Gly | M2 | CDR2 | × | × | × | × | × |
| Library 55 | HC | Gly | Ser | M2 | CDR2 | × | × | × | × | × |
| Library 56 | HC | Ser | Asn | M2 | CDR2 | × | × | × | × | × |
| AID 61 | HC | Asp | Tyr | AID | CDR2 | × | × | × | × |
| AID 17 | LC | Asp | Asn | AID | FW1 | × | × | × | × |
| AID 66 | LC | Gly | Gln | AID | CDR2 | × | × | × | × | × |
| AID 71 | LC | Phe | Leu | AID | FW3 | × | × | × | × | × |
| AID 90 | LC | His | Gln | AID | CDR3 | × | × | × | × | × |
| AID 93 | LC | Gly | Asp | AID | CDR3 | × | × | × | × | × |
| AID 53 | LC | Thr | Arg-Thr | ML30 | CDR2 | × | × | × | × | × |

FIGURE 4. Analysis of antibody binding kinetics. SPR sensograms are shown for the initial clone isolated from the SHM-diversified, H3-grafted library, APE424 (A) and for affinity-matured variants APE520 (B), APE571 (C), and APE896 (D). Association for all antibodies is at or near the diffusion-limited rate for proteins of this size (−1E7 M$^{-1}$ s$^{-1}$). RU, resonance units.
at or below the limit of detection (\(1 \times 10^{-5} \text{s}^{-1}\)). Parental and affinity-matured variants were analyzed for their ability to block h\(\beta\)NGF binding to and activation of its cognate receptor TrkA in two assays: competition for binding to h\(\beta\)NGF with a known receptor blocking anti-\(\beta\)NGF antibody (tanezumab) and inhibition of h\(\beta\)NGF-dependent TrkA signaling in a rat pheochromocytoma-derived cell line (PC12). Antibody APE424 inhibited binding of tanezumab to h\(\beta\)NGF in a HTRF assay with an IC\(\text{So}_{50}\) of \(\sim 1 \mu\text{M}\) (Fig. 5A). The affinity improvement observed among the recombined antibody variants was well matched to their improved potency, with antibody APE897 possessing an IC\(\text{So}_{50}\) value of 4 nM (250-fold improvement) in the HTRF assay. Parental and affinity-matured variants were also analyzed for their ability to demonstrate dose-dependent inhibition of h\(\beta\)NGF-induced TrkA signaling and activation of ERK1/2 in PC12 cells (Fig. 5B). Again, the final affinity-matured variants (e.g., APE890; IC\(\text{So}_{50} = 300\) pM) showed significant improvement relative to the starting construct (APE424; IC\(\text{So}_{50} = 10\) nM) in their ability to inhibit PC12 signaling, which mirrors the improvement in affinity as measured by SPR.

**Amino Acid Sequences of the Final Antibody Constructs**

The amino acid changes in the highest affinity recombined antibody HC and LCs are shown in Fig. 6 on a model of the Fab structure. In Fig. 6A, sequences originating from mouse hybridoma and human germ line sources are highlighted in a ribbon diagram of the modeled Fab. In Fig. 6B and C, the origin of mutations is illustrated in a spacing filling model, illustrated by different colors, with contributions from CDR grafting, SHM-diversified libraries, and in vitro AID-mediated SHM. Only one of the library-derived LC mutations was included in
the final construct, with a majority of the affinity maturation events (five of six) derived from AID-mediated mutations in the LC, particularly within FW2 and FW3 regions. These sites do not correspond to previously identified mutations shown to facilitate humanization (5).

**DISCUSSION**

A novel method has been developed for the humanization of a rat antibody was grafted into a nonhomologous human V region containing CDR H1 and H2 residues varied to mimic in vivo SHM diversity. The HC library was paired with a CDR-grafted LC and presented as a full-length IgG on the surface of HEK293 cells. Cells expressing low affinity antibodies were isolated by FACS and affinity-matured rapidly using SHM in vitro, resulting in high potency antibodies with pm affinity. From the perspective of therapeutic antibody development, this approach has the advantage of enabling the rapid humanization of functional high potency antibodies containing a minimum of nonhuman donor antibody-derived sequence. The adaptive nature of this methodology should also facilitate the use of human V genes possessing lower homology to the murine antecedent, and the successful humanization of CDR-grafted constructs possessing lower starting affinities for the antigen.

The method of pairing mammalian display with in vitro SHM to affinity-matured antibodies could likely be applied in other venues. Recent studies have demonstrated the potential of computational methods to design grafted or de novo libraries of proteins with the desired binding properties (26–28). One or more loops of a donor antigen have been grafted onto a soluble surrogate scaffold protein for application in immunization and directed evolution. Likewise, novel binding proteins have been designed de novo utilizing only computational methods. In each instance, library ensembles of related molecules are first screened to resolve minority populations possessing desired functionality from nonbinding or nonfunctional members. As with the humanization example presented here, pairing in vitro SHM with mammalian display should provide a powerful tool for the rapid interrogation and subsequent affinity maturation of computationally derived binding proteins.

The originating rat hybridoma antibody has been independently humanized and affinity-matured (PG110) and has completed phase I clinical trials for the indication of pain associated with osteoarthritis of the knee (29). Antibody APE896, derived from the same original monoclonal antibody, possessed half the number of IGHV non-germ line mutations when compared with PG110 (9 mutations localized to CDR1 and CDR2 for APE896 versus 20 mutations found in FW1, CDR1, FW2, CDR2, and FW3 for PG110), possessed sequence identity of 89% with the closest human germ line sequence throughout the HC and LC V regions (versus 80% for PG110), and possessed an affinity equivalent to that of PG110 (Table 1). In addition, because this method minimizes the number of mutations needed to humanize and affinity-mature an antibody, the APE896 HC possessed greater sequence identity to its parental V gene segment (IGHV3–23) (89%) than did the original hybridoma rat V region sequence to either its originating rat germ line segment (86%) or to the closest human V gene IGHV4–59 (60%). The adaptive nature of the methodology and its ability to mature antibodies to high potency facilitates the use of human V regions with distant homology to the originating murine sequence, providing more flexibility in gene selection based on additional criteria such as manufacturability and known expression characteristics (30).

A HC V region library informed by likely SHM events observed in vivo was utilized to initiate affinity maturation of the CDR-grafted antibody, and similar libraries have been employed to effect modest affinity maturation when paired with phage and other display technologies (31, 32). In this instance, significant additional maturation was required, and AID-mediated in vitro SHM was successful in the identification of a small number of mutations (Table 2) that when combined improved the affinity of APE424 to low pm. Mutations selected in vitro SHM to enhance binding kinetics were at positions unanticipated by bioinformatics analysis and not associated with canonical antigen contacting positions or hot spots (e.g., HC mutations D17N, G66E, and F71L and LC mutation D61Y). These results demonstrate that in vitro SHM, coupled with minimal CDR grafting, can be sufficient for rapid humanization and affinity maturation to generate potent antibodies containing a minimum of sequence from the nonhuman donor antibody.

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