Universal Blood

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Towards the Production of Universal Blood by Structure-Guided Directed Evolution of Glycoside Hydrolases

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The ABO blood group system discovered by Landsteiner more than one hundred years ago and its modified form used today constitutes an essential guide when performing blood transfusions.[1] The ABO system describes antigens with specific structurally defined sugars which are exposed on the exterior of red blood cells (RBCs; erythrocytes), these being different according to blood type. The specificity of a given blood type is defined by the nature and linkage of the monosaccharides at the ends of the carbohydrate chains. The antigenic portion comprises one or more carbohydrate chains which are linked to a ceramide or a peptide backbone, these being embedded in the lipid bilayer of the cell membrane. If the wrong blood type is administered, transfusion triggers an immune response with adverse effects, sometimes with fatal consequences.[1b]

Scientists have long sought to generate a "universal blood" by enzyme-catalyzed selective cleavage of the antigenic components. In an early study, B-type erythrocytes were converted into universal O-type blood, catalyzed by an exoglycosidase.[2] Selective cleavage of αGalNAc and αGal residues of the A and B trisaccharide antigens was achieved with no impairment of the cells' ability to survive subsequent transfusion. Unfortunately, enzyme activity was far too low for practical clinical applications. Since then other attempts have been made to implement the original vision, but the lack of acceptable levels of activity persisted.[3a,b] The use of bacterial glycosidases led to improvements,[3c] but the problem of insufficient enzyme activity remained.

Now a team led by Steve Withers collaborating with other Canadian researchers and a French group, has taken a major step forward in this challenging endeavor.[4] In order to increase the activity of a selective glycoside hydrolase significantly, they applied structure-guided directed evolution in a proof-of-principle study. This protein engineering technique involves recursive cycles of gene mutagenesis, expression and screening (or selection), a "Darwinian" procedure for evolving essentially any protein property including enzyme stability, activity and stereoselectivity.[5,6] Several gene mutagenesis methods and strategies for applying them have been developed for maximizing efficiency in laboratory evolution, iterative saturation mutagenesis (ISM) at sites lining the binding pocket based on the combinatorial active-site saturation test (CAST) being one of the most reliable techniques for evolving activity and/or stereoselectivity.[6] Accordingly, the best hit in a randomization library at one site near the binding pocket is used as a template for saturation mutagenesis at another site, and the process is continued at the remaining sites until the desired degree of catalyst improvement has been reached.

In essence, Withers and co-workers applied several cycles of directed evolution based on ISM using a glycoside hydrolase from *Streptococcus pneumonia* SP3-BS71 (Sp3GH98), which cleaves the entire terminal trisaccharide antigenic determinants of both A- and B-antigens from some of the linkages on RBC surface glycans, although so slowly that applications remained impossible.[4] Clinically, these are the most important antigens which must be considered in blood transfusions and tissue or organ transplantations.[1] The goal was to enhance enzyme activity for the cleavage of the Galβ-1,3-GlcNAc linkage of type 1A antigens (Figure 1a–d). A microtiter high-throughput screening assay was first established in which a fluorogenic substrate was prepared comprising the type 1A blood group pentasaccharide linked by a β-glycosidic bond to a methylumbelliferyl moiety. In the presence of two "coupling" enzymes, the desired hydrolytic cleavage generates fluorescent methylumbelliferone, a technique used in other fluorescence-based screening systems.[7]

Guided by the crystal structure of Sp3GH98 harboring type 2A Lewis^Y pentasaccharide (PDB ID 2WMK),[8] seven first- and second-sphere CAST sites were chosen for saturation mutagenesis, Tyr530, Asn559, Tyr560, Trp561, Ile562, Asn592 and Lys624 (Figure 1E). These amino acid positions are not the only ones that can be considered, and indeed two more residues were added at a later stage. The seven initial libraries were screened by the on-plate fluorescence assay, leading to the discovery of three single-residue mutants, Asn559Ser, Asn592Val and Asn592Ser showing a three-fold improved hydrolytic activity. Then the mutations were combined with formation of double mutants Asn559Ser/Asn592Val and Asn559Ser/Asn592Ser, which displayed

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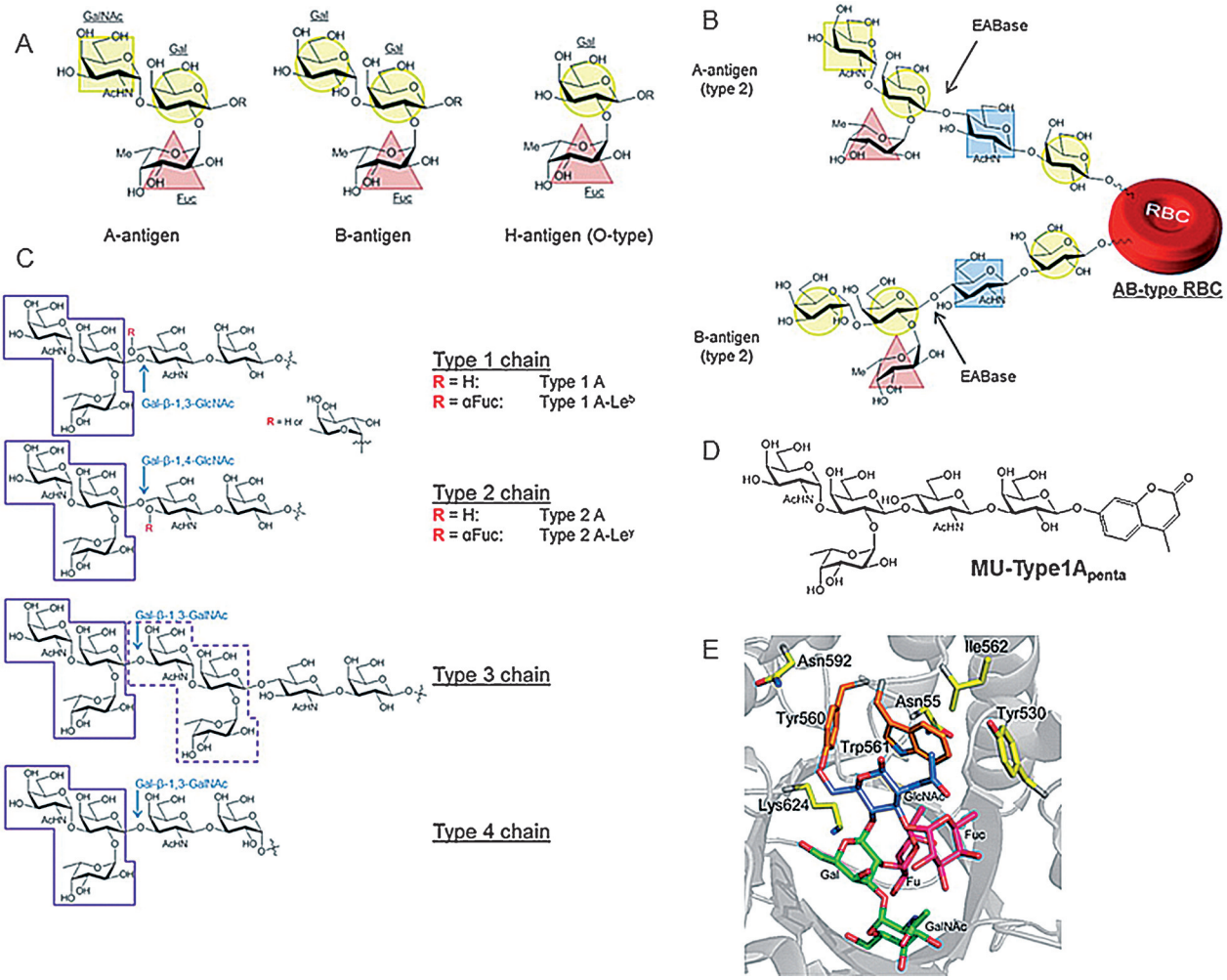

Figure 1. A) Carbohydrate antigenic determinants of A-, B-, and H-antigens.[4] The H-antigen is present on glycans of the O blood-group, and typically nonantigenic except in rare cases. B) Site of cleavage of A- and B-antigens by GH98 EABase enzymes from type 2 chains of erythrocytes. C) Various chain types to which A-antigens are present on erythrocytes and other cell types. D) Structure of the fluorogenic substrate MUType1A$_{penta}$. E) First- and second-sphere randomization sites chosen for iterative saturation mutagenesis (ISM),[4] guided by the X-ray structure of Sp3GH98.[8] First sphere: Tyr560 and Trp561; second sphere: Tyr530, Asn559, Ile562, Asn592 and Lys624. Reproduced with permission from the American Chemical Society.

a roughly six-fold activity enhancement. Subsequently, ISM[6] was applied by using these double mutants as templates for individual randomization at amino acid positions Tyr530, Tyr560, Trp561, Ile562 and Lys624. This was followed by further ISM involving various 2-residue sites formed from the above individual amino acid positions and spatially close residues Glu630, Glu663 and Lys677. The researchers anticipated cooperative effects operating between individual point mutations within a multi-residue site and between sets of mutations in an ISM process as shown in other studies.[6] This approach provided the best variant at this stage of the evolutionary optimization: Tyr530His/Asn559Ser/Asn592Val/Glu630Leu/Lys677Leu with a 120-fold enhancement in k_{cat}/K_M. One round of error-prone polymerase chain reaction raised activity only to a small extent by a sixth mutation Leu692Ile.[4] The strategy proved to be successful. Perhaps an even better ISM approach would have been the grouping of the individual amino acid positions into multi-residue sites from the very beginning, followed by ISM using reduced amino acid alphabets.[6]

Thermostability of the mutant ($T_m = 44\,°C$) suffices for practical applications.[4] The improvements evolved for the type 1A oligosaccharide do not cause significant activity loss towards type 2A oligosaccharide. Therefore, it was suggested that the activities of the enzyme towards the two linkages are balanced, which makes the complete removal of antigens possible. Finally, antibody-based immunofluorescence experiments were performed with the real substrates, demonstrating highly improved removal of type 1A antigens from the surface of red blood cells.

This study has shown for the first time that directed evolution can be used to broaden the substrate specificity of Sp3GH98 in the cleavage of the Galβ-1,3-GlcNAc linkage of type 1 chains without drastically compromising activity towards the Galβ-1,4-GlcNAc linkage of type 2 chains. The results set the stage for future directed evolution for broad-

ening the substrate specificity towards Galβ-1,3-GalNAc linkage of type 3 and 4 chains. This involves A antigens (but not B-type) occurring in some individuals belonging to A_1 phenotype (80% of blood group A). More work is necessary before the production of truly universal blood can be implemented, but the present study has opened a new door in this exciting endeavor.

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