National Institute for Public Health
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Ministry of Health, Welfare and Sport

Identification of a B Cell epitope of Diphtheria Toxin Utilizing Epitope Excision and Differential Stable Isotope Tagging

Introduction

B cells play a central role in the humoral immune pathway after an infection or vaccination. These immune cells bind to an antigen with high specificity and subsequently secret antibodies. Binding of the antigen to the paratope of the antibody is determined by the amino acid sequence and the spatial arrangement of the antigen. Identification and characterization of these epitopes on antigens is essential in improving vaccine concepts. Current MS-based methods to allocate the epitope often rely on H/D exchange analysis, epitope excision/extraction or probing the surface-exposed lysine residues of the antigen-antibody complex (*i.e.* immune complex).
Here, we further explore on these basic concepts by adapting dedicated stable isotope labeling strategies to unambiguously locate the antigenic determinant of the diphtheria toxin.

Methods

Diphtheria toxin was bound to the Dim9 monoclonal antibody under native conditions. Epitope excision of the complex was performed using trypsin as protease (Hager-Braun *et al.*, 2004). The resulting peptide mixture was split into two equimolar portions to differentially methylate peptides with native (**L**ight) formaldehyde/cyanoborohydride reagents ($CH_2O/NaBH_3CN$) and with **H**eavy stable isotopically labeled reagents ($^{13}CD_2O/NaBD_3CN$) (figure 1). In parallel, separate controls of antigen and antibody were digested under similar conditions and methylated with the **I**ntermediate stable isotopically labeled reagents ($CD_2O/NaBH_3CN$). All reaction mixtures were pooled in equimolar amounts and analyzed on a *nano*scale LC-ESI/MS system. Unique mass spectral doublets with an exact mass difference of 8.0444 Da, or multiples thereof, were allocated using MsXelerator® (MsMetrix, The Netherlands) and subsequently identified by their MS/MS spectral information against the amino acid sequence of the Diphtheria toxin (UniProt/SwissProt Accession number P00588).

Results

The differential methylation of the tryptic peptides results in the formation of many mass spectral triplets (figure 2, *top*). These triplets represent peptides from the digestion mixtures of both the controls as well as of the immune complex, since potential cleavage sites within the antigen are not (fully) shielded by the antibody. By contrast, putative cleavage site(s) within the antigenic determinant are hardly or not accessible upon proteolytic digestion of the immune complex, due to (partial) shielding by the antibody. This will result in unique miss cleavages upon proteolytic digestion and thus in the formation of peptides solely present in the digestion mixture of the immune complex. These peptides can unambiguously be recognized by their unique mass spectral doublets (figure 2, *bottom*). Table 1 lists all peptides recognized by their mass spectral doublets, covering 3 surface-exposed protein regions located on both the A and B fragment of the toxin (figure 3). Since inhibition studies of Dim9 only showed specificity for the A fragment (Metz, 2005), the shielded protein regions on the B fragment were interpreted as being residues in close contact to the antibody but not contributing to the binding, a feature distinguished by the terms *structural* and *functional* epitopes.
Although proteolytic digestion of immune complexes may affect the interaction of the constituents to a certain extent, it did not hamper the recognition of the epitope region using this strategy.

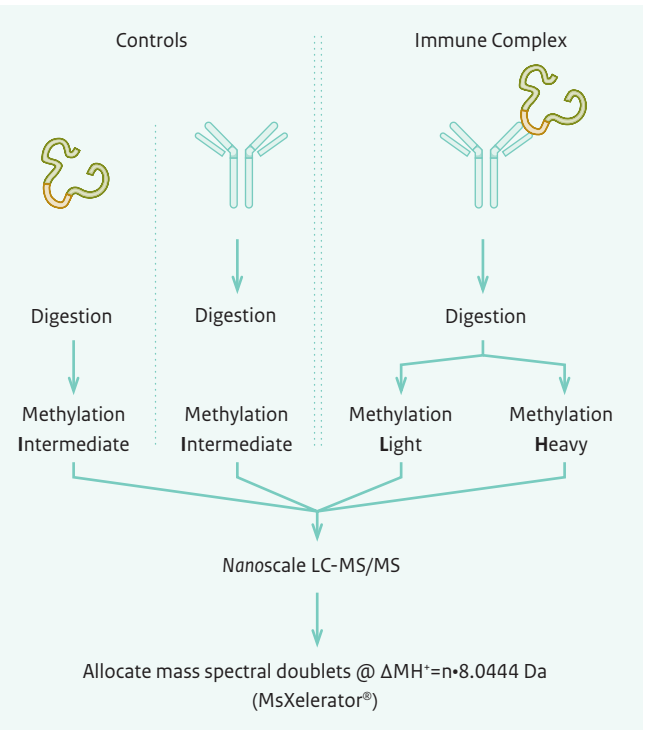

Figure 1 Schematic outline of the epitope excision strategy in combination with multiplexed methylation after proteolytic digestion. All resulting tryptic peptides are methylated and their origin can be deduced from their mass spectral information:
Singlet: peptide tagged with the **I**ntermediate label, solely present in the Controls digestion mixtures.
Doublet: peptide tagged with the **L**ight and **H**eavy labels, solely present in the Immune Complex digestion mixture.
Triplet: peptide tagged with de **L**ight , **I**ntermediate and **H**eavy labels, present in all digestion mixtures.

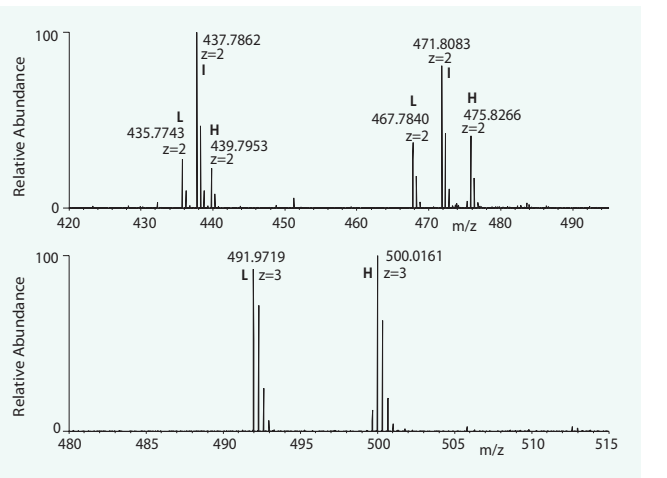

Figure 2 Mass spectral triplets (*top*) and doublet (*bottom*) illustrating the different isotope patterns for peptides that are not shielded and shielded, respectively, during digestion of the immune complex.

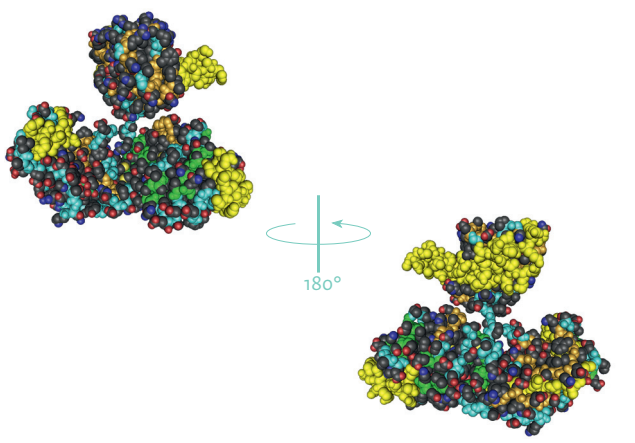

Figure 3 Spatial arrangement of the amino acids of the Diphtheria toxin (front and rear view). Yellow-coloured spheres indicate the surface-exposed protein regions shielded by the Dim9 antibody.

References

Christine Hager-Braun and Kenneth B. Tomer (2004). *Determination of Epitopes by Mass Spectrometry.* Methods Mol Med **94**:109-120.
Bernard Metz (2005). *Structrural Characterisation of Diphtheria Toxoid.* Dissertation **Ch 8**:157-173. Http://igitur-archive.library.uu.nl/dissertations/2005-0303-105230/.

Table 1 Diphtheria toxin-originating tryptic peptides exclusively present in the digestion mixture of the Immune Complex

Sequence	Location on DT125		# Methylgroups	MH^+ (Light)	MH^+ (Heavy)	ΔMH^+
AGGVV**K**VTYPGLTK	[77-90]	A fragment	6	1473.9026	1498.0347	24.1321
VLAL**K**VDNAETI**K**K	[91-104]	A fragment	8	1654.0510	1686.2267	32.1757
T**K**IESL**K**EHGPI**K**NK	[215-229]	B fragment	10	1862.1463	1902.3637	40.2174
MSESPN**K**TVSEEK	[230-242]	B fragment	6	1549.7811	1573.9143	24.1332
MSESPN**K**TVSEEKAK	[230-244]	B fragment	8	1776.9427	1809.1173	32.1746
TVSEEKAK	[237-244]	B fragment	6	975.5711	999.7034	24.1323
SSSE**K**IHSNEISSDSIGVLGYQK	[494-516]	B fragment	6	2549.3105	2573.4420	24.1315
TVDHT**K**VNSK	[517-526]	B fragment	6	1212.6940	1236.8260	24.1320
LSLFFEI**K**S	[527-535]	B fragment	4	1139.6714	1155.7597	16.0883

Conclusions

- The differential stable isotope labeling strategy in combination with proteolytic digestion enhances the allocation of shielded protein sites in noncovalently bound (immune) complexes.
- In addition to trypsin as primary protease, different shielded cleavage sites can be targeted using a panel of proteases to define the minimum epitope more accurately.
- The relative large Fc fragment of the antibody may lead to the recovery of nonfunctionally shielded protein regions (*structural* epitopes). Hence, in further studies only the Fab fragment of the antibody will be used to specifically target the *functional* epitope.

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