**Epitope and affinity determination of recombinant *Mycobacterium tuberculosis* Ag85B antigen towards anti-Ag85 antibodies using proteolytic-affinity mass spectrometry and biosensor analysis**

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**Abstract**

Tuberculosis (TB) is the first cause of death from infectious diseases worldwide. Only a single anti-TB vaccine is currently available for clinical use, but its efficacy is not achieved with certainty. The aim of this work is to provide a basis for the rational design of a *neo*-glycoconjugate vaccine against TB. Structural characterization of recombinant antigenic proteins from *Mycobacterium tuberculosis* (MTB) Ag85B (rAg85B, variants and semi-synthetic glycoconjugates) was initially carried out. Identification of antibody epitope analyses by proteolytic affinity-mass spectrometry and surface plasmon resonance (SPR) biosensor analyses were performed in order to qualitatively identify and quantitatively characterize interaction structures of the antigens with antibodies from different sources. A commercial monoclonal antibody, and polyclonal antibodies from different sources (patients with active TB, vaccinated individuals and a healthy control) were employed to analyze antigen-antibody interactions. These combined approaches provided the identification of different assembled epitope regions on the recombinant MTB antigens, their affinity binding constants in the interactions with specific antibodies and revealed the importance of protection from excessive glycosylation. The identified epitope peptides should constitute a suitable basis for the design of new specific target vaccines.

**Keywords:** antibody epitope identification; affinity mass spectrometry; surface plasmon resonance; Ag85B; tuberculosis; glycoconjugate vaccine.

## 1. Introduction

Tuberculosis (TB) still constitutes one of the main causes of death in the world and, in 2016, it ranked above HIV/AIDS as the most lethal infectious disease [1]. TB is caused by the bacillus *Mycobacterium tuberculosis* (MTB), from which various drug-resistant strains emerged and spread. The only antitubercular vaccine currently in clinical use is Bacillus Calmette-Guérin (BCG), developed in 1921 from the attenuation of a laboratory strain of *Mycobacterium bovis*. However, the variable protective efficacy observed for BCG and the growing public health concern for drug-resistant forms of TB pointed out the need for new effective agents against this disease [1].

The development of a new vaccine requires a well-defined correlation between design and protection. Two key prerequisites for the rational design of effective prophylactic agents capable to elicit a long-term protection are the comprehension of epitope/antibody interactions and the quantitative evaluation of vaccine-induced immune responses [2, 3]. To identify antigenic regions of molecules coming from microorganisms, which might be valuable vaccine candidates, B cell epitope determination is a promising approach, especially for the discovery of assembled discontinuous epitopes [2]. Mass spectrometry (MS)-based epitope identification approaches are among the most powerful; a strategy widely used is proteolytic epitope extraction (PROTEX). This approach includes antigen digestion by a proteolytic enzyme; incubation with the binder antibody (typically in the immobilized form); washing steps to remove unbound peptides; and MS analysis of the elution fractions to identify the epitope peptides involved in the binding [4-12]. Surface plasmon resonance (SPR) biosensor technology has proven to be a useful technique for vaccine characterization, as it quantifies binding affinities and provides reliable kinetic data to evaluate specificity and mechanism of action [13]. Noteworthy, affinity-MS epitope determination and SPR approaches can be used as a combination to investigate immune responses directly in serum samples, representative of the total human antibody set [4, 13].

In this study, several analytical techniques were employed for the characterization of a new potential glycoconjugate vaccine against TB. This kind of glycovaccine can be obtained by conjugation of antigenic proteins, which are expressed by MTB and are known to induce a strong immune reaction, with carbohydrate moieties capable to mimic lipoarabinomannan (LAM), one of the most important saccharide superficial antigen of MTB [14-16]. The *neo*-glycoconjugate might induce both humoral and T cell-mediated immune responses, resulting in an improved antigenicity compared to the single constituents [17], but at the same time glycosylation might hamper the recognition of selected protein epitopes. Ag85B was selected as carrier protein since it is one of the most potent antigen species expressed by MTB; in addition, different studies proved its anti-TB protective efficacy in mice, guinea pigs and other animal models, and showed good antibody and T cell responses to this antigen in patients with active TB as well as BCG-vaccinated individuals [18-21]. Therefore, a recombinant form of Ag85B (rAg85B) with an additional pre-sequence of 7 amino acids at the *N*-terminus was produced in *E. coli* [18] and conjugated with simplified models of saccharide moieties (saccharides of mannose [17, 21-26]).

In a previous part of this project [21], different experimental and computational methods suggested that K30 and K282 residues (the two most reactive amino acids of rAg85B involved in the glycosylation) are antigenic. Therefore, substitutions of these two lysines with arginine residues were designed and rAg85B variants were produced to direct the glycosylation towards non-antigenic amino acids, thus preserving immunogenicity of the constructs [25]. In the present work, the areas of rAg85B protein involved in the interaction with antibodies from different sources (commercial monoclonal antibody from mouse, total antibodies from sera of active TB-patients, BCG-vaccinated subjects and a healthy control) were investigated using the PROTEX-MS epitope identification method. In addition, affinities of rAg85B protein, its variants and its glycoconjugates for the commercial monoclonal antibody (mAb) were compared by SPR analysis to support the mutagenesis approach.

## 2. Materials and methods

### *2.1. Materials, reagents and chemicals*

*Mycobacterium tuberculosis* Ag85 monoclonal antibody was purchased from Thermo Fisher Scientific (Darmstadt, Germany). Total antibodies from sera of patients with active TB, individuals vaccinated against TB (BCG vaccination) and a healthy control were provided from the University of Rome "Tor Vergata" (Department of Biology and Animal Technology Station, Rome, Italy). Ammonium sulfate precipitation was used to isolate total immunoglobulins from sera, and antibodies were then transferred in a phosphate buffered saline (PBS) solution. All sera were tested for antibody reactivity to the protein antigens by the enzyme-linked immunosorbent assay (ELISA), following the same procedure as described in [18].

rAg85B recombinant protein was produced as described in [18]. K30R and K282R variants of rAg85B protein were similarly also prepared [25]. For SPR measurements, rAg85B was conjugated with 2-iminomethoxyethyl activated α-D-arabinofuranosyl-(1→6)-α-D-mannopyranoside (AraMan-IME) synthesized as in [22]. Glycosylation was performed as previously described [17, 25].

16-Mercaptohexadecanoic acid, α-chymotrypsin from bovine pancreas, calcium chloride, ethanolamine, iodoacetamide, *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC), sodium phosphate dibasic, sodium azide, *N*-hydroxysuccinimide (NHS), sodium acetate and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2,5-dihydroxybenzoic acid (2,5-DHB) and Super DHB (SDHB) were from Bruker Daltonics (Billerica, MA, USA), acetonitrile and sodium chloride from Carl Roth GmbH (Karlsruhe, Germany), ammonium bicarbonate from Merck KGaA (Darmstadt, Germany), dithiothreitol (DTT) from Roche (Mannheim, Germany), urea from VWR (Radnor, PA, USA). Water was obtained from a Direct-Q® 3 UV system (Merck KGaA, Darmstadt, Germany).

POROS™ A material and Zeba™ spin desalting columns were purchased from Thermo Fisher Scientific (Darmstadt, Germany). Amicon® Ultra-0.5 mL centrifugal filters and ZipTip® pipette tips were from Merck KGaA (Darmstadt, Germany).

*2.2. Proteolytic extraction-mass spectrometry*

For proteolytic extraction-MS experiments, rAg85B protein and its variants were digested with chymotrypsin prior to incubation with immobilized antibodies. Protein solutions (1 mg/mL in 100 mM ammonium bicarbonate buffer, pH 8.5) were incubated at 95 °C for 20 minutes. After cooling to room temperature, urea and DTT were added to a final concentration of 6 M and 5 mM, respectively. Solutions were incubated at 60 °C for 30 minutes to reduce disulfide bonds and cooled to room temperature. The addition of iodoacetamide to 14 mM final concentration was followed by incubation at room temperature in the dark for 30 minutes to alkylate cysteines. DTT was added to an additional 5 mM concentration and protein mixtures were incubated at room temperature in the dark for 15 minutes to quench unreacted iodoacetamide. Solutions were diluted 1:5 in 100 mM ammonium bicarbonate buffer, pH 8.5 to reduce the concentration of urea to less than 2 M, followed by the addition of calcium chloride to 1 mM concentration. Digestion was achieved by the addition of chymotrypsin at 1/50 enzyme/substrate ratio (w/w) and the incubation of the reaction mixture overnight at 37 °C. Reactions were stopped by separation of the peptide mixture from the enzyme using 10 kDa Amicon® Ultra centrifugal filters (centrifugation at 10410 rcf for 5 minutes, 2 steps). Peptides were transferred in 10 mM sodium phosphate, pH 7 in presence of 0.02% (w/v) sodium azide by Zeba™ spin desalting columns, and concentrated to 2 mg/mL.

A Protein A affinity column was prepared by loading POROS™ A medium on a 100 µL micro-column. The column was equilibrated with the running buffer (10 mM sodium phosphate, pH 7 added with 0.02% w/v sodium azide) for 10 minutes with a constant flow rate of 1 mL/min. Each Protein A-antibody column was obtained by immobilization of 50 µL of antibody solution (commercial mAb, total antibodies from sera of active-TB patients, vaccinated subjects or healthy control) in phosphate buffer, using a constant flow rate of 500 µL/min. The column was washed for 10 minutes at 500 µL/min to remove any species not captured from the Protein A.

For epitope extraction, 50 µL of the previously prepared rAg85B (or rAg85B variants) proteolytic peptide mixtures were loaded on the protein A-antibody column in the running buffer at a flow rate of 20 µL/min. The flow through was monitored at 220 and 280 nm using a UV-Vis detector (Dionex, Thermo Fisher Scientific, Darmstadt, Germany) and the column was washed until absorption reached again the baseline. Elution was performed by switching the running buffer to 150 mM sodium chloride, pH 2.5 with 0.02% (w/v) sodium azide and the flow rate to 500 µL/min. Elution fractions were collected and peptides were separated from antibodies using 10 kDa Amicon® Ultra centrifugal filters. After 2 centrifugation steps at 10410 rcf (13000 rpm) for 5 minutes, 0.1% TFA in water (v/v) was added to the filters, which were centrifuged for additional 10 minutes at 10410 rcf. The peptide solutions passed through the filters were concentrated, desalted using the ZipTip® procedure [27] and analyzed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS.

*2.3. Mass spectrometric analysis*

Mass spectrometric analyses were performed using an Autoflex III Smartbeam (Bruker Daltonics, Billerica, MA, USA) MALDI-TOF MS equipped with a Nd:YAG laser source.

For intact protein determinations, 0.5 µL of each sample solution were spotted on a Ground Steel MALDI Target Plate (Bruker Daltonics, Billerica, MA, USA), mixed with 0.5 µL of Super DHB matrix solution (50 mg/mL SDHB in 50/50 v/v acetonitrile / 0.1% TFA in water) and allowed to dry at room temperature. Spectra were acquired using the following instrumental conditions: linear mode; mass range 10000-35000 Da for proteins and glycoconjugates, 20000-100000 and 100000-200000 Da for antibodies; detector gain 10 (proteins and glycoconjugates) or 100 V (antibodies); sample rate 2.00 GS/s; electronic gain 50 mV.

For peptide mapping and epitope identification, 0.5 µL of 2,5-DHB matrix solution (10 mg/mL 2,5-DHB in 30/70 v/v acetonitrile / 0.1% TFA in water) were spotted on an AnchorChip MALDI Target Plate (Bruker Daltonics, Billerica, MA, USA). 1 µL of sample solution (after desalting by ZipTip®) was deposited onto each matrix spot and allowed to dry. Spectra were acquired using the following instrumental conditions: linear mode; mass range 240-8000 Da; detector gain 5.5 V; sample rate 2.00 GS/s; electronic gain 50 mV.

Data processing was performed using mMass open source software (version 5.5) [28]. Peptides were identified by comparing experimental data with the protein fragment list generated *in silico*; a mass tolerance of 1 Da was applied for data matching.

*2.4. SPR biosensor determinations*

Determination of affinity constants was carried out on a SR7500DC dual channel SPR biosensor (Ametek-Reichert Technologies, Depew, NY). MTB Ag85 monoclonal antibody (Thermo Fisher Scientific, Darmstadt, Germany) was immobilized on two different sensor slides: a carboxymethyl dextran hydrogel surface sensor chip and a gold surface sensor chip coated with a self-assembled monolayer (SAM) of 16-mercaptohexadecanoic acid.

Carboxyl groups on the chip surface were activated by injecting 250 µL of a freshly prepared solution of 200 mM EDC and 100 mM NHS at a constant flow rate of 25 µL/min. After washing the chip surface with 1 mL of phosphate buffer (10 mM sodium phosphate, 30 mM sodium chloride, pH 7.5), 250 µL of a 35 µg/mL antibody solution (in 10 mM sodium acetate buffer, pH 5.2) were injected at 25 µL/min for the immobilization. Unreacted NHS groups were blocked with 1 M ethanolamine, pH 8.5. The antibody was immobilized on left channel and right channel was used as a reference.

Affinity determinations were performed at 22 °C (SAM-coated chip) or 27 °C (dextran chip) in 10 mM sodium phosphate, 30 mM sodium chloride binding buffer, pH 7.5 at a flow rate of 25 µL/min, by injecting 100 µL of each sample. A dilution series from 0.2 to 6.4 µM (6.25-200 µg/mL) was analyzed for each protein. Each

sample injection was followed by a buffer injection (blank). Affinity $K_D$ constants and associated errors were calculated using the TraceDrawer software (Ridgeview Instruments AB, Vange, Sweden) according to a 1:1 binding model. The injection of a 0.2-6.4 μM dilution series of a negative control protein (myoglobin) was performed to confirm the specificity of the interaction.

## 3. Results and discussion

### *3.1. Characterization of rAg85B antigens and anti-Ag85B antibodies*

All proteins (rAg85B, rAg85B-K30R, rAg85B-K282R), rAg85B glycoconjugates and antibodies used in this study were characterized by MALDI-TOF MS. Following determination of intact proteins providing a first identity characterization, peptide mapping analyses were carried out on rAg85B protein and its variants to confirm protein sequences. These analyses were performed by MALDI-TOF MS following chymotryptic digestion. Unmodified and mutated rAg85B proteins were completely digested and provided high sequence coverage (>90%) to ascertain the identity of all proteins. Data quality is supported by the specificity of chymotryptic cleavages and the match between most of the identified peptides and the preliminary peptide mapping of rAg85B and its variants by RPLC-UV-MS/MS [25]. As an example, Fig. S1, Table S1 and Fig. S2 (Supplementary Material) report the results of peptide mapping for the chymotryptic digest of rAg85B-K30R variant.

rAg85B glycoconjugates were also prepared and characterized. AraMan saccharide was activated with the 2-iminomethoxyethyl (IME) functional group [22], which selectively reacts with the ε-amino group of lysine residues [17]. This conjugation involves all lysine residues (8 in rAg85B, all located on the surface) yielding a mixture of glycoproteins with variable saccharide numbers and positioning. Intact protein determinations allowed to confirm the glycosylation structures, showing that rAg85B-AraMan glycoconjugates investigated in this work presented up to 4 glycan moieties *per* protein (Fig. 1). The glycosylation profile was also characterized by a previously developed hydrophilic interaction liquid chromatography (HILIC)-UV method [23], which allowed to assess yield and glycoform composition of the coupling product (Table S2).

MS analyses of all antibodies used in this study were also performed. The monoclonal antibody (IgG1, Thermo Fisher Scientific) was produced by immunization of mice with bacterial press extract from MTB H37Rv (the most studied TB strain) and detects the MTB complex of mycolytransferases composed of Ag85A, B and C proteins. MS analysis of the intact antibody provided a single main species with a molecular weight of 146 kDa (Fig. 2a), confirming its monoclonality. As expected, mass spectra of antibodies obtained from sera samples showed the presence of different populations (Fig. 2b), being representative for the total immunoglobulin content (comprising non-specific and specific polyclonal antibodies) of human sera.

*3.2. Mass spectrometric identification of epitope peptides*

To support the rational design of an effective vaccine against TB, the antibody epitope of rAg85B antigen was determined using proteolytic extraction-mass spectrometry. The use of clinical samples, and in particular of sera, for epitope determination is representative of the overall human antibody set [4]. Therefore, antibodies from sera of patients with active TB, BCG-vaccinated subjects and a healthy control were employed in this study. These samples consisted of the total immunoglobulins from human sera, including both specific and non-specific antibodies for Ag85B antigen. Anti-Ag85B specific antibodies are produced in patients and vaccinated subjects, but are not present in healthy controls. These specific immunoglobulins represent a minority component of the total antibody content, generally up to 5 % in patients with active TB infection. For this reason, a key point is the set-up of an appropriate immobilization procedure based on a suitable binding chemistry capable to orient the antibodies with the variable regions exposed towards the mobile phase and to maximize the specific interaction of the analyte.

In addition to clinical samples, a commercial monoclonal antibody against Ag85B was studied to experimentally determine the binding areas of the same antigens to the mAb and assess whether it might constitute a simplified model to study the interaction with recombinant proteins from MTB.

After testing different immobilization chemistries (cyanogen bromide method using CNBr-activated Sepharose 4B and amine coupling through EDC/NHS activation, data not shown), a POROS$^{TM}$ Protein A affinity column was selected. Protein A is naturally produced by *Staphylococcus aureus* and specifically binds the heavy chain constant region (Fc) of IgGs in the $CH_2$-$CH_3$ portion, thus allowing the interaction between the antibody variable region and its antigens.

For proteolytic extraction experiments, rAg85B was initially digested with trypsin. However, the use of this enzyme resulted in a poor sequence coverage (data not shown). Therefore, chymotryptic digests were used, yielding sequence coverage values >90% in all cases. The chymotryptic digests of rAg85B were incubated with the immobilized antibodies. After a washing step to remove unbound peptides, the elution fractions were collected and analyzed by MALDI-TOF MS to identify the peptide(s) involved in the interaction. Table 1 summarizes the peptides identified in the elution fractions collected from each column (prepared with the mAb or with antibodies from individual serum samples). Noteworthy, peptide maps from the same areas of the antigen protein (in red; Fig. 3) were found in the elution fractions from all columns with antibodies from mouse (mAb), patients and vaccinated subjects. Areas in blue in Fig. 3 represent recurring peptides and were identified in at least two elution fractions. The results showed that the detected common peptides belong to the same side of the protein, suggesting the presence of an assembled epitope(s) in this section or to an exposed surface. One important result is that the mAb binds to the same regions of the protein, thus validating the identified epitope(s). Two types of controls were used to verify the specificity of the epitope peptides: (a) immobilized antibodies from sera of healthy controls (which do not possess anti-Ag85B antibodies) and (b) unmodified Protein A column (without any immobilized antibody). Three replicates were performed for the proteolytic

epitope extraction using the healthy control affinity column, in each case no peptides were found in the elution fractions. The specificity of epitope peptides was ascertained by the negative control (unmodified Protein A column, in two replicates).

**Table 1** Identified peptides in the elution fractions of rAg85B proteolytic mixtures loaded on affinity columns with immobilized antibodies from mouse (commercial mAb), TB-patients and BCG-vaccinated people. Peptides included in the protein areas identified in at least two fractions are listed

| Slice | Mis | m/z | z | Sequence | Commercial mAb | Patient 1 | Patient 2 | Patient 3 | Vaccinated 1 | Vaccinated 2 |
|---|---|---|---|---|---|---|---|---|---|---|
| [9-17] | 0 | 1017.5364 | 1 | f.SRPGLPVEY.l | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| [45-58] | 4 | 1635.7762 | 1 | y.LLDGLRAQDDYNGW.d | | ✓ | ✓ | ✓ | | ✓ |
| [46-55] | 2 | 1165.5484 | 1 | l.LDGLRAQDDY.n | | | ✓ | | | |
| [46-58] | 3 | 1522.6921 | 1 | l.LDGLRAQDDYNGW.d | ✓ | ✓ | | | | |
| [47-55] | 1 | 1052.4643 | 1 | l.DGLRAQDDY.n | ✓ | | | | ✓ | |
| [47-58] | 2 | 1409.6080 | 1 | l.DGLRAQDDYNGW.d | ✓ | ✓ | | | ✓ | ✓ |
| [50-58] | 1 | 1124.4756 | 1 | l.RAQDDYNGW.d | ✓ | | | | | |
| [133-150] | 2 | 1909.8935 | 1 | l.SMAGSSAMILAAYHPQQF.i | | | ✓ | | | |
| [143-150] | 1 | 961.4526 | 1 | l.AAYHPQQF.i | | | | | | ✓ |
| [236-245] | 2 | 1178.6164 | 1 | f.LENFVRSSNL.k | | ✓ | | ✓ | | ✓ |
| [237-245] | 1 | 1065.5323 | 1 | l.ENFVRSSNL.k | | | | ✓ | | |
| [240-251] | 2 | 1427.7278 | 1 | f.VRSSNLKFQDAY.n | | | ✓ | | | |
| [246-261] | 2 | 1709.8030 | 1 | l.KFQDAYNAAGGHNAVF.n | | | ✓ | ✓ | | ✓ |
| [252-261] | 0 | 957.4537 | 1 | y.NAAGGHNAVF.n | ✓ | ✓ | | | ✓ | ✓ |
| [262-271] | 0 | 1156.5170 | 1 | f.NFPPNGTHSW.e | ✓ | ✓ | | ✓ | | ✓ |
| [262-273] | 1 | 1448.6230 | 1 | f.NFPPNGTHSWEY.w | ✓ | | ✓ | | ✓ | ✓ |
| [272-289] | 4 | 2010.9590 | 1 | w.EYWGAQLNAMKGDLQSSL.g | | | ✓ | | | |
| [274-289] | 3 | 1718.8530 | 1 | y.WGAQLNAMKGDLQSSL.g | | | | ✓ | | ✓ |
| [279-292] | 2 | 1348.6525 | 1 | l.NAMKGDLQSSLGAG. | ✓ | | | ✓ | | ✓ |

These findings suggest a common assembled epitope or group of epitopes comprising different sequences recognized by anti-Ag85B antibodies from different sources. This aspect is of utmost importance also for the validation of the commercial mAb as a simplified model to screen new potential antigens.

Encouraged by the promising results, we decided to use the PROTEX-MS method on rAg85B variants. In a previous study [21], the assessment of rAg85B glycosylation sites, the *ex vivo* evaluation of *neo*-glycoproteins immunogenic activity and the prediction of protein B and T cell epitopes by a combination of *in silico* systems suggested that the two main glycosylated sites (K30 and K282) are antigenic. Thus, rAg85B variants at 30 and 282 residues were designed and produced in order to prevent the glycosylation in these positions and to preserve the immunogenicity of the glycoconjugates [25]. After proving that the introduced conservative mutations (K30R and K282R) do not affect the T-cell antigenicity of the protein [25], in the present work we examined the effect of the amino acid substitutions also on protein-antibody binding. Antibodies from a vaccinated individual (Vaccinated 2) were immobilized and an equimolar mixture of rAg85B-K30R and

rAg85B-K282R chymotryptic digests was incubated with the affinity column. In the elution, peptides including both unmodified (K) and mutated (R) 282 residue were identified (Table 2), suggesting that this substitution does not alter the binding. Antibodies from Patient 3 were also incubated with rAg85B-K282R chymotryptic digest (Table 3). Peptides comprising R282 were found in the elution fraction, supporting the evidence that replacement of this residue does not prevent antigen-antibody interactions. Peptides including K30 did not bind the tested antibodies, and the conservative mutation in R30 did not alter the immunogenicity of this site.

Antibodies from Vaccinated 2 and Patient 3 were employed also to assess the repeatability of the epitope determination. Duplicate experiments of the entire epitope extraction procedure were performed, starting from the immobilization of a new aliquot of antibodies from the same source. The results (Tables 2 and 3, Fig. S3) show that the majority of identified protein areas are found in both analyses, despite the use of different proteolytic mixtures and the preparation of different Protein A-antibody affinity columns.

**Table 2** List of the peptides identified from the epitope determination of rAg85B and variants peptide mixtures incubated with antibodies from Vaccinated 2 (two replicates). Peptides including K282 are underlined in light blue, those with R282 are in orange

| Slice | Mis | m/z | z | Sequence | Replicate 1 | Replicate 2 |
|---|---|---|---|---|---|---|
| [1-17] | 1 | 1849.9153 | 1 | .AMAISDPFSRPGLPVEY.l | | ✓ |
| [9-17] | 0 | 1017.5364 | 1 | f.SRPGLPVEY.l | ✓ | ✓ |
| [34-44] | 0 | 1093.4909 | 1 | f.QSGGNNSPAVY.l | | ✓ |
| [45-58] | 4 | 1635.7762 | 1 | y.LLDGLRAQDDYNGW.d | ✓ | ✓ |
| [47-58] | 2 | 1409.6080 | 1 | l.DGLRAQDDYNGW.d | ✓ | |
| [74-86] | 1 | 1371.6613 | 1 | l.SIVMPVGGQSSFY.s | ✓ | |
| [105-116] | 3 | 1463.7417 | 1 | w.ETFLTSELPQWL.s | | ✓ |
| [143-150] | 1 | 961.4526 | 1 | l.AAYHPQQF.i | ✓ | ✓ |
| [236-245] | 2 | 1178.6164 | 1 | f.LENFVRSSNL.k | ✓ | ✓ |
| [246-261] | 2 | 1709.8030 | 1 | l.KFQDAYNAAGGHNAVF.n | ✓ | ✓ |
| [252-261] | 0 | 957.4537 | 1 | y.NAAGGHNAVF.n | ✓ | ✓ |
| [262-271] | 0 | 1156.5170 | 1 | f.NFPPNGTHSW.e | ✓ | ✓ |
| [262-273] | 1 | 1448.6230 | 1 | f.NFPPNGTHSWEY.w | ✓ | |
| [274-289] | 3 | 1746.8592 | 1 | y.WGAQLNAMRGDLQSSL.g | | ✓ |
| [279-292] | 2 | 1376.6587 | 1 | l.NAMRGDLQSSLGAG. | ✓ | ✓ |
| [279-292] | 2 | 1348.6525 | 1 | l.NAMKGDLQSSLGAG. | ✓ | |

**Table 3** List of the peptides identified from the epitope determination of rAg85B and variants peptide mixtures incubated with antibodies from Patient 3 (two replicates). Peptides including R282 are in orange

| Slice | Mis | m/z | z | Sequence | Replicate 1 | Replicate 2 |
|---|---|---|---|---|---|---|
| [9-17] | 0 | 1017.5364 | 1 | f.SRPGLPVEY.l | ✓ | ✓ |
| [45-58] | 4 | 1635.7762 | 1 | y.LLDGLRAQDDYNGW.d | ✓ | ✓ |
| [236-245] | 2 | 1178.6164 | 1 | f.LENFVRSSNL.k | ✓ | |
| [237-245] | 1 | 1065.5323 | 1 | l.ENFVRSSNL.k | | ✓ |

| [246-261] | 2 | 1709.8030 | 1 | l.KFQDAYNAAGGHNAVF.n | ✓ | ✓ |
|---|---|---|---|---|---|---|
| [262-271] | 0 | 1156.5170 | 1 | f.NFPPNGTHSW.e | ✓ | ✓ |
| [274-289] | 3 | 1746.8592 | 1 | y.WGAQLNAMRGDLQSSL.g | ✓ | |
| [279-292] | 2 | 1376.6587 | 1 | l.NAMRGDLQSSLGAG. | ✓ | |

A literature search was performed to compare our data with previously published results. Although different T cell epitope determinations (reviewed in [29]) were carried out for MTB Ag85B, few experimental data on B cell epitopes are available for this antigen. To our knowledge, this is the first report describing the extraction of epitope peptides from proteolytic mixtures of Ag85B incubated with immobilized antibodies. All previous work about the interactions between antibodies and Ag85B peptides [20, 30-32] was performed with synthetic peptides, either selected on the basis of *in silico* predictions of B epitopes [20, 30] or synthesized to cover the entire Ag85B protein sequence [31, 32]. An intriguing previous result is that Kadir et al. [20] recently ascertained the involvement of Ag85B T cell epitopes $L_{101}$SELPQWLSANRAV$_{115}$ (corresponding to 108-122 in rAg85B), $S_{126}$MAGSSAMILAAYHP$_{140}$ and $T_{261}$HSWEYWGAQLNAMK$_{275}$ (identified in [33]) also in the humoral response. The sequences $A_{136}$AYHP$_{140}$ (part of the second epitope peptide in [20]) and $T_{261}$HSWEYWGAQLNAMK$_{275}$ (third epitope) were in agreement with the epitope determination in our present work. Furthermore, peptides based on the sequences $P_{7}$VEYLQVPS$_{15}$ and $G_{249}$HNAVFN$_{255}$ of Ag85 proteins were demonstrated by ELISA to be immunoreactive [30]. Both sequences (except for $L_{11}$QVPS$_{15}$ amino acids) were identified in all elution fractions examined in this study. Some epitope regions in our work ($L_{38}$-$G_{50}$ and $Q_{141}$-$F_{143}$ in MTB Ag85B) were also recognized by antibodies from TB patients in [31]. The peptide $P_{258}$NGTHSWEYWGAQ$_{270}$, included in the areas of Ag85B protein found in our epitope determination, showed a significantly higher humoral response in TB patients than in healthy individuals in a previous microarray experiment on human sera [32]. Finally, many epitope regions identified in the present study were found in the previous B cell epitope predictions of Ag85B [34] and Ag85A [35], a protein belonging to the complex of Ag85B that shows high sequence homology with this antigen (about 77% shared amino acids).

### *3.3. Determination of affinity binding constants*

The validation of the monoclonal MTB Ag85 antibody by the epitope determinations prompted us to use this simplified model to assess the affinity binding constants of rAg85B, its variants and its glycosylated forms by SPR analysis. This study was not possible using antibodies from sera due to the low content of specific antibodies in the samples, which did not allow to analyze specific interactions (data not shown).

First, the effect of protein glycosylation was evaluated. The use of a carboxymethyl dextran chip that provides a stable, flexible surface favouring easy coupling and accessibility of binding sites was not suitable for determination of glycoprotein equilibrium dissociation constants ($K_D$). The glycosylated antigen showed a similar binding to the channels with and without (reference) the immobilized antibody, suggesting the

occurrence of non-specific interactions between the carbohydrate moieties and the dextran surface. Therefore, a SAM-coated chip was employed as an alternative immobilization support. The two supports gave comparable maximal responses upon analyte binding ($R_{max}$), 15.3 ± 2.5 µRIU for the dextran chip and 18.0 ± 3.6 µRIU for the SAM-coated chip (mean values of three independent determinations at 1.6 µM protein concentration). The response depends on the number of immobilized ligand molecules and on the size of ligand and analyte (which were identical for the two chips), providing a measure of the maximum analyte binding capacity of the surface. The obtained $R_{max}$ values suggest that the two surfaces gave similar yields in terms of amount and proper orientation for the interaction of the immobilized antibodies. An additional confirmation of the comparable antibody immobilization on the two chips was obtained from the rAg85B $K_D$ values, which were found to be consistent with each other (Table 4 and ~~Figure~~ Fig. S4). The 1:1 binding model, indicating that one immobilized antibody binds one analyte from the injected solution, was chosen to fit the obtained sensorgrams.

Using the SAM-coated chip (~~Figure~~ Fig. S4, panel b), a considerably slow dissociation rate between protein and antibody was observed. A regeneration step of the chip within consecutive injections was exploited, as suggested by the supplier. However, both basic and acidic regeneration solutions resulted unsuccessful. For these reasons, increasing concentration levels of the protein antigen were flowed, running a buffer sample between injections. In addition, replicate injections of the lower concentration level were analyzed for each protein sample to assess repeatability and a negative control protein (myoglobin) was used to prove the specificity of the measured interactions.

In order to provide a proof of principle of the mutagenesis approach to prevent the glycosylation of antigenic amino acids, the affinity of single point variants (K30R and K282R) of rAg85B protein was quantified. The analysis of rAg85B variants on the SAM-coated chip yielded~~The~~ $K_D$ values ~~obtained from the SAM-coated chip were~~ comparable with those calculated on the dextran chip (Table 4, Fig. S5 and S6), supporting the reliability of the results. ~~Replicate injections of the lower concentration level were analyzed for each protein sample (Figure S4, panel b) to assess repeatability. In addition, a negative control protein (myoglobin) was used to prove the specificity of the measured interactions. Thus~~Furthermore, the use of the SAM-coated chip allowed to define the $K_D$ for rAg85B glycosylated with AraMan-IME, resulting in a 9-fold decrease of the binding affinity relative to the unmodified protein (Table 4 and Fig. S6). This finding is in agreement with the general view of the masking of protein epitopes by glycosylation.

~~In order to provide a proof of principle of the mutagenesis approach to prevent the glycosylation of antigenic amino acids, the affinity of single point variants (K30R and K282R) of rAg85B protein was quantified. The measurements were performed on both SPR chips, giving comparable results (Table 4).~~ In summary, these data appear to support the validity of the mutagenesis approach, since the introduced mutations do not considerably alter the binding affinity, while glycosylation of rAg85B reduces affinity compared to the non-glycosylated antigen.

**Table 4** Equilibrium dissociation constants ($K_D$) and estimated errors calculated for rAg85B protein, its variants (rAg85B-K30R and rAg85B-K282R) and its glycosylated form (rAg85B-AraMan) interacting with MTB Ag85 mAb

| Sample | $K_D$ ± estimated error (µM) Dextran chip | $K_D$ ± estimated error (µM) SAM-coated chip |
|---|---|---|
| rAg85B | 0.6397 ± 0.0091 | 0.9838 ± 0.0580 |
| rAg85B-K30R | 1.5451 ± 0.1420 | 1.8352 ± 0.0045 |
| rAg85B-K282R | 1.1697 ± 0.0356 | 1.3633 ± 0.0108 |
| rAg85B-AraMan | not determined | 9.1652 ± 0.0162 |

## 4. Conclusions

In this work, the mass spectrometric epitope determination and SPR biosensor analysis allowed the identification and characterization of the interactions between a known antigenic protein from MTB (Ag85B) and antibodies from different sources, both qualitatively and quantitatively.

It was possible to use human clinical samples to determine the Ag85B epitope. Noteworthy, it was not necessary to isolate Ag85B-specific antibodies (which have very low abundance in serum, typically ≤5% of total antibodies), since a simple ammonium sulfate precipitation of the total immunoglobulin content of sera samples followed by antibody immobilization on a Protein A column was sufficient to isolate epitope peptides from rAg85B digestion mixtures.

Proteolytic mixtures of rAg85B and its single point variants were injected on several affinity columns, prepared by immobilizing a commercial MTB Ag85 monoclonal antibody and antibodies from sera of TB-patients and BCG-vaccinated individuals. Despite the use of different samples and Protein A-antibody columns, epitope peptides covering common protein areas were identified in the elution fractions collected from each column. The recognition of redundant chymotryptic peptides mapping in the same protein regions provided an additional confirmation of their involvement in the binding. The protein regions found to contribute to the antibody binding include the sequences [9-17], [45-58], [143-150] and [236-292] of rAg85B. The localization of these areas on the same side of protein surface suggests the possible contribution of protein conformation in antibody binding and the consequent presence of an assembled epitope or group of epitopes. The repeatability of the method was assessed by performing two replicates of the whole epitope extraction procedure on antibodies, both from active TB and vaccinated subjects. The specificity of the epitope peptides was confirmed by performing the same PROTEX-MS experiments on immobilized antibodies from a healthy control serum, as well as on the unmodified Protein A column.

The identification of identical binding areas of the protein interacting with a monoclonal antibody and with antibodies from human sera allowed to validate the simplified monoclonal model, confirming its applicability

in SPR experiments. Therefore, an alternative screening method for the immunological evaluation of antigenic proteins from MTB has been developed. With this *in vitro* approach, it was possible to study the effect of the introduction of conservative mutations (K30R and K282R) in the rAg85B protein designed to prevent excessive chemical glycosylation. SPR analyses allowed to quantitatively compare the affinity binding constants of intact rAg85B, its single point variants and its glycosylated form, by immobilizing MTB Ag85 mAb on two different sensor slides.

Both the SPR and epitope identification results indicate that the introduced mutations did not alter the binding properties of rAg85B, while glycosylation of the unmodified protein reduced the binding affinity due to the masking of protein epitopes (especially in the case of K282 glycosylation).

These results provide a molecular basis for an improved rational design of a new potential glycoconjugate vaccine against TB.

## Figure captions

**Fig. 1** MALDI-TOF mass spectra of rAg85B protein conjugated with AraMan-IME. Mono-, bi- and tri-charged ions of the glycoprotein are shown

**Fig. 2** MALDI-TOF mass spectra of commercial MTB Ag85 monoclonal antibody (**a**) and polyclonal antibodies from serum of a BCG-vaccinated individual (**b**)

**Fig. 3** Summary results of the PROTEX-MS epitope identification. Amino acids in red were identified in all the elution fractions collected from the different Protein A-antibody affinity columns, amino acids in blue in at least two elution fractions. Upper panel: epitope areas in rAg85B sequence. Lower panel: areas of rAg85B protein interacting with commercial mAb (**a**), TB-patients polyclonal antibodies (**b**) and vaccinated people polyclonal antibodies (**c**)

## Conflict of interest

The authors declare that they have no conflict of interest.

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