In silico Binding Predictions for Identification of HLA-DR-Promiscuous Regions and Epitopes of *Mycobacterium tuberculosis* Protein MPT64 (Rv1980c) and Their Recognition by Human Th1 Cells

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Key Words

Mycobacterium tuberculosis • Rv1980c • In silico predictions • HLA-DR-promiscuous regions and epitopes • Th1 cell reactivity

Abstract

Objective: To identify HLA-promiscuous regions and epitopes of MPT64 (Rv1980c), a major secreted antigen of *Mycobacterium tuberculosis*, by in silico analysis for binding to HLA-DR molecules. **Materials and Methods:** The sequence of mature MPT64 protein (aa 1–205) was analyzed in silico for HLA-DR binding regions and T cell epitopes using ProPred, a web-based prediction server. The prediction results were experimentally verified by testing 20-mer synthetic peptides corresponding to the predicted HLA-DR binding regions and epitopes with T cell lines established from peripheral blood mononuclear cells of PPD-positive and HLA-heterogeneous healthy subjects in Th1 cell assays (antigen-induced proliferation and IFN- γ secretion). **Results:** The in silico analysis for binding of the mature MPT64 sequence to HLA-DR molecules suggested that it could bind to molecules expressed from all HLA-DR alleles (n = 51) included in ProPred. Furthermore, ProPred identified 26 epitopes and 8 nonoverlapping HLA-DR binding regions (9–35 aa in length) in the Rv1980c

sequence, with 5 regions (aa 20–44, aa 68–102, aa 132–146, aa 164–186 and aa 194–202) being HLA-DR-promiscuous. By using synthetic peptides and T cell lines in Th1 cell assays, 4 peptides of MPT64 (aa 21–40, aa 81–100, aa 171–190 and aa 191–20), from 4 of the 5 HLA-DR-promiscuous regions predicted by ProPred, were experimentally verified to be HLA-DR-promiscuous and to have immunodominant epitopes. **Conclusion:** The in silico method (ProPred) suggested promiscuous HLA-DR-binding of mature MPT64 and identified HLA-promiscuous and immunodominant regions and epitopes of this protein.

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Introduction

Tuberculosis (TB) is a major infectious disease of worldwide prevalence. About one third of the world population is infected with *Mycobacterium tuberculosis*, 9.3 million people develop active TB, and 1.8 million people die of TB each year [1]. The current efforts to reduce the global problem of TB have been focused on improved diagnosis and effective chemotherapy; however, these efforts have not been effective in reducing the worldwide problem of TB [1]. Therefore, improved diagnostic and

therapeutic efforts need to be combined with additional preventive efforts. Hence, the development of effective vaccines to prevent the development of active disease is considered a major goal to control the worldwide epidemic of TB [2].

A primary criterion to identify antigens/peptides of *M. tuberculosis* as potential vaccine candidates against TB is their recognition by Th1 cells, which are the major players in protective immunity against TB [3–5]. It has previously been shown that MPT64 (Rv1980c), a major secreted antigen of *M. tuberculosis*, is recognized by Th1 cells from *M. bovis* BCG-vaccinated and *M. tuberculosis*-infected healthy subjects [6, 7], and thus could be useful as a candidate vaccine against TB. However, human Th1 cells recognize mycobacterial protein/peptide antigens in association with human leukocyte antigen (HLA) class II molecules and, in particular, in association with highly polymorphic HLA-DR molecules [8–12]. To be useful in HLA-heterogeneous human populations, it is imperative that a candidate protein/peptide vaccine or diagnostic antigen is able to bind HLA-DR molecules promiscuously, i.e. to several commonly expressed HLA-DR molecules [13–15]. Several antigenic proteins proposed as candidate vaccines against TB and/or diagnostic antigens, e.g. ESAT-6, CFP10, Ag85B, MPT70 or MPT63, have been characterized for HLA-DR binding by *in silico* prediction using the ProPred server [16], and found promiscuous HLA-DR binders both with respect to full-length proteins and immunodominant epitopes [17–19]. To our knowledge, the MPT64 sequence has not been characterized for HLA-DR binding prediction and identification of epitope regions using ProPred. Furthermore, the analysis of such data in relation to actual presentation of Rv1980c and the predicted regions and epitopes to Th1 cells from healthy subjects is also lacking.

In this study, the Rv1980c sequence was analyzed *in silico* for binding to molecules expressed from 51 HLA-DR alleles included in ProPred, and regions predicted to be HLA-DR binders were evaluated for HLA-promiscuous Th1 cell reactivity using synthetic peptides and T cell lines established from PPD-positive and HLA-DR-typed healthy subjects.

Materials and Methods

In silico Analysis of MPT64 for HLA-DR Binding Regions and Identification of T Cell Epitopes

The primary structure of the mature MPT64 sequence was first analyzed for T cell epitopes by the ProPred method for binding to HLA-DR molecules using the server ([\[res.in/raghava/propred/\]\(http://res.in/raghava/propred/\)\) \[16\]. The ProPred analysis was performed at the default setting, i.e. threshold value of 3.0. The peptides predicted to bind >50% \(\$\geq 5/9\$ \) serologically defined HLA-DR molecules included in ProPred were considered promiscuous for binding \[18\].](http://www.imtech.</p></div><div data-bbox=)

Synthetic Peptides

Synthetic peptides (20-mers; overlapping with neighboring peptides by 10 aa, if more than 1 peptide was required to cover the HLA-DR binding region) spanning the sequence of the ProPred-predicted HLA-DR binding regions of MPT64 were purchased from Thermo Hybaid GmbH, Ulm, Germany. These peptides were synthesized using fluorenylmethoxycarbonyl chemistry, as described previously [20, 21]. The stock concentrations (5 mg/ml) of the peptides were prepared in normal saline (0.9%) by vigorous pipetting, and the working concentrations were prepared by further dilution in tissue culture medium RPMI-1640 (Life Technologies, Paisley, UK) [22].

Experimental Verification of ProPred-Predicted Epitopes in Th1 cell Assays

The synthetic peptides were experimentally verified for Th1 cell reactivity by testing T cell lines, established from HLA-heterogeneous healthy humans, in peptide-induced proliferation and IFN- γ secretion assays using procedures described previously [23]. For proliferation assays, the results were calculated as stimulation index, which is defined as cpm in peptide-stimulated cultures/cpm in cultures without peptide, and stimulation indices of ≥ 2 were considered positive [24]. Secretion of IFN- γ in response to a given peptide was considered positive with E/C >2 (E/C = IFN- γ concentration in cultures stimulated with peptide/IFN- γ concentration in cultures without peptide) and IFN- γ concentration ≥ 1.5 IU/ml [24]. The Th1 cell responses were considered strong, moderate and weak with peptides showing positive responses with T cell lines from >60, 30–60 and <30% donors, respectively [25].

Informed consent was obtained from all the subjects included in the study, and the experimental protocol was approved by the Ethics Committee, Faculty of Medicine, Kuwait University, Kuwait.

Results

The ProPred analysis of the mature MPT64 sequence (aa 1–205) showed that this protein was predicted to bind all serologically defined HLA-DR molecules expressed from 51 (100%) HLA-DR alleles included in the ProPred graphical web tool (table 1), and it had a total of 26 epitopes (9-mers each), which were scattered throughout the protein sequence (table 2). In addition, a total of 8 nonoverlapping regions from 9 to 35 aa in length (aa 9–17, aa 20–44, aa 46–62, aa 68–102, aa 115–127, aa 132–146, aa 164–186 and aa 194–202) were predicted to bind HLA-DR alleles with binding frequencies ranging from 2/51 (4%) to 47/51 (92%) (table 1). Five of these regions, i.e. region 2 (aa 20–44), region 4 (aa 68–102), region 6 (aa 132–146), region 7

Table 1. ProPred analysis for HLA-DR binding regions of MPT64 to 51 alleles of 9 serologically defined HLA-DR molecules

Binding region		Binding to HLA-DR molecules									All alleles	All molecules ¹
No.	sequence	B1.1	B1.3	B1.4	B1.7	B1.8	B1.11	B1.13	B1.15	B5.1	P/T	P/T
1	aa 9–17	0/2	0/7	2/9	0/2	0/6	0/9	0/11	0/3	0/2	2/51 (4)	1/9 (11)
2	aa 20–44	2/2	7/7	9/9	0/2	6/6	9/9	11/11	3/3	0/2	47/51 (92)	7/9 (78)
3	aa 46–62	0/2	1/7	5/9	0/2	0/6	1/9	1/11	0/3	0/2	8/51 (16)	4/9 (44)
4	aa 68–102	1/2	1/7	9/9	0/2	5/6	3/9	2/11	2/3	2/2	25/51 (49)	8/9 (89)
5	aa 115–127	0/2	1/7	2/9	0/2	0/6	1/9	0/11	0/3	0/2	4/51 (8)	3/9 (33)
6	aa 132–146	0/2	5/7	3/9	0/2	2/6	4/9	2/11	0/3	0/2	16/51 (31)	5/9 (56)
7	aa 164–186	2/2	7/7	4/9	2/2	3/6	2/9	3/11	3/3	0/2	26/51 (51)	8/9 (89)
8	aa 194–202	0/2	4/7	1/9	0/2	6/6	9/9	11/11	2/3	0/2	33/51 (65)	6/9 (67)

Binding = Number of alleles predicted to bind/number of alleles included; P/T = number positive/number tested; figures in parentheses are percentages.

¹ The values showing promiscuous binding to HLA-DR molecules (as defined in 'Materials and Methods') are given in italics.

Table 2. Comparison of the number of predicted epitopes and HLA-DR binding frequency with the responses of T cell lines to peptides corresponding to HLA-DR binding regions in Th1 cell assays

Region No.	Sequence of peptide(s) covering the region ¹	Epitopes	Binding to HLA-DR molecules ²		Responders in Th1 cell assays ³	
			P/T	%	P/T	%
1	1-APKTYCEEL K GTD T GQACQ I -20	1	1/9	11	0/14	0
2	11-GTDTGQACQ I QMSDPAYNINISLPSYYPDQ K SLENYIAQT-50	4	7/9	78	11/14	79
3	41-KSL E NYIAQTRDKFLSAAT S STPREAPYEL-70	2	4/9	44	3/14	21
4	61-STPREAPYELN I TSATYQSAIPPRGTQAVVLKVYQNAGG T HP T TYKA F D-110	7	8/9	89	11/14	79
5	111-WDQAYRK P ITYDTLWQAD T D-130	2	3/9	33	2/14	14
6	131-PLP V VPFPIVQ G ELSKQ T GQ Q -150	3	5/9	56	4/14	29
7	161-PVNYQ N FAVTNDG V IFFFN P GELLPEAAG P -190	6	8/9	89	9/14	64
8	191-TQVLVPRSAID S MLA-205	1	6/9	67	9/14	64

P/T = Number positive/number tested.

¹ The sequences predicted to bind HLA-DR molecules are underlined and the obligatory anchor (starting) residues are marked in bold. ² The values showing promiscuous binding to HLA-DR molecules (as defined in 'Materials and Methods') are given in italics. ³ The values showing strong responses (as defined in 'Materials and Methods') in Th1 cell assays (antigen-induced proliferation and/or IFN- γ secretion) are given in italics.

(aa 164–186) and region 8 (aa 194–202), were predicted to be promiscuous HLA-DR binders (table 1). Furthermore, 21 of 26 epitopes were present in these HLA-DR-promiscuous regions with 4 regions having more than 1 overlapping epitope of 9 aa each, i.e. 4 epitopes in region 2 (aa 20–29, aa 27–35, aa 29–37 and aa 36–44), 7 epitopes in region 4 (aa 68–76, aa 70–78, aa 77–85, aa 81–89, aa 89–97, aa 91–99 and aa 94–102), 3 epitopes in region 6 (aa 132–140, aa 134–142 and aa 138–156) and 6 epitopes in region 7 (aa 164–172, aa 169–177, aa 174–182, aa 175–183, aa 176–184 and aa 178–186), whereas HLA-DR-promiscuous region 8 had only 1 epitope (aa 194–202) (table 2).

Testing of synthetic peptides with T cell lines showed that 4 of the 5 HLA-DR-promiscuous regions, i.e. regions 2, 4, 7 and 8, induced strong Th1 cell responses, whereas peptides belonging to all of the 3 non-HLA-DR-promiscuous regions, i.e. regions 1, 3 and 5, induced nil to weak responses in Th1 cell assays (table 2).

Among the 4 HLA-DR-promiscuous and strong Th1 cell-stimulating regions, only region 8 was represented by a single peptide, whereas the other 3 regions required more than 1 peptide to cover the sequences, i.e. 3 peptides for region 2, 4 peptides for region 4 and 2 peptides for region 7 (table 3). These peptides were further analyzed for

Table 3. Comparison of the number of predicted epitopes and HLA-DR binding frequency of peptides of regions 2, 4 and 6 with Th1 cell responses of T cell lines

Region No.	Peptide sequence ¹	Epitopes	Binding to HLA-DR molecules ²		Responders in Th1 cell assays ³	
			P/T	%	P/T	%
2	11-GTDTGQACQIQMSDPAYNIN-30	1	4/9	44	2/14	14
	21-QMSDPAYNINISLPSYYPDQ-40	2	7/9	78	8/14	57
	31-ISLPSYYPDQKSLENYIAQT-50	1	2/9	22	3/14	21
4	61-STPREAPYELNITSATYQSA-80	2	2/9	22	3/14	21
	71-NITSATYQSAIPPRGTQAVV-90	2	5/9	56	2/14	14
	81-IPPRGTQAVVLKVYQNAGGT-100	3	6/9	67	8/14	57
	90-LKVYQNAGGTHPTTTYKAFD-110	2	4/9	44	3/14	21
7	161-PVNYQNFAVTNDGVIFFFNP-180	2	5/9	56	3/14	21
	171-NDGVIFFFNPGELLPEAAGP-190	4	6/9	67	9/14	64

P/T = Number positive/number tested.

¹ The sequences predicted to bind HLA-DR molecules are underlined and the obligatory anchor (starting) residues are marked in bold. ² The values showing promiscuous binding to HLA-DR molecules (as defined in 'Materials and Methods') are given in italics.

³ The values showing moderate to strong responders (as defined in 'Materials and Methods') in Th1 cell assays (antigen-induced proliferation and/or IFN- γ secretion) are given in italics.

HLA-DR binding prediction and number of epitopes, which were correlated with the responses of T cell lines in Th1 cell assays (table 3). The results showed that the peptides aa 21–40, aa 81–100 and aa 171–190, of regions 2, 4 and 7, respectively, showed the highest binding predictions to HLA-DR molecules, had the highest number of epitopes, and were the best stimulators of Th1 cells (table 3).

A further analysis of the predicted HLA-DR binding regions and peptides of MPT64 for Th1 cell responses in relation to expression of HLA-DR molecules by the donors of T cell lines confirmed the promiscuous presentation of the peptides aa 21–40, aa 81–100, aa 171–190 and aa 191–205 because these peptides were recognized by T cell lines from donors with varied HLA-DR types, i.e. HLA-DR 3, 4, 5, 6, 7, 13, 52 and 53 (table 4).

Discussion

MPT64 (Rv1980c) is among the major secreted protein antigens of *M. tuberculosis*, and like other secreted proteins of the pathogen, which are recognized by protective Th1 cells in humans, e.g. ESAT-6, CFP10, Ag85B, MPT70 or MPT63 [17–19], this protein is also recognized by human Th1 cells [6, 7] and thus could be useful in the diagnosis of or included among the new candidate vaccines

against TB. However, any protein as a vaccine or diagnostic antigen for use in humans must be able to bind several HLA-DR molecules so that it is presented to Th1 cells in a HLA-promiscuous manner, a requirement for any new vaccine or antigen to be useful in HLA-heterogeneous human populations [13–15]. All of the other secreted proteins of *M. tuberculosis* mentioned above have previously been analyzed for HLA binding prediction using the ProPred server [19–21]; however, to our knowledge, such an analysis for MPT64 has been performed for the first time in this study.

The HLA-DR binding analysis of the MPT64 sequence by ProPred suggests that there are 8 nonoverlapping regions scattered throughout the protein sequence, which could bind to HLA-DR alleles with frequencies ranging from 4 to 92%, and the complete mature protein could bind to all (51/51, 100%) HLA-DR alleles of 9 frequently expressed and serologically defined HLA-DR molecules included in the ProPred [16]. Although none of the HLA-DR binding regions were predicted to bind all the alleles by themselves, the complete sequence of MPT64 was predicted to bind all the molecules expressed from 51 HLA-DR alleles included in the ProPred, because of differential binding of the regions to various HLA-DR alleles. Furthermore, 5 of the 9 HLA-DR binding regions are predicted to be promiscuous HLA-DR binders, because these regions were predicted to bind >50%, i.e. >5/9, serologi-

Table 4. Comparison of HLA-DR binding prediction of peptides of MPT64 using ProPred with responders in Th1 cell assays and HLA-DR types of responding donors

Region No.	Peptide	HLA-DR binding prediction ¹		Responders in Th1 cell assays ²		HLA-DR types of responding donors ³
		P/T	%	P/T	%	
1	aa 1–20	1/9	11	0/14	0	not applicable
2	aa 11–30	4/9	44	2/14	14	4, 53
	aa 21–40	7/9	78	8/14	57	3, 4, 5, 6, 7, 13, 52, 53
	aa 31–50	2/9	22	3/14	21	4, 13, 52, 53
3	aa 41–70	4/9	44	3/14	21	4, 6, 7, 52, 53
4	aa 61–80	2/9	22	3/14	21	3, 6, 7, 52, 53
	aa 71–90	5/9	56	2/14	14	4, 5, 6, 7, 52, 53
	aa 81–100	6/9	67	8/14	57	3, 4, 5, 6, 7, 13, 52, 53
	aa 90–110	4/9	44	3/14	21	3, 4, 6, 7, 52, 53
5	aa 111–130	3/9	33	2/14	14	6, 7, 52, 53
6	aa 131–150	5/9	56	4/14	29	4, 6, 7, 13, 52, 53
7	aa 161–180	5/9	56	3/14	21	4, 5, 6, 7, 52, 53
	aa 171–190	6/9	67	9/14	64	3, 4, 5, 6, 7, 13, 52, 53
8	aa 191–205	6/9	67	9/14	64	3, 4, 5, 6, 7, 13, 52, 53

P/T = Number positive/number tested.

¹ The values showing promiscuous binding to HLA-DR molecules (as defined in ‘Materials and Methods’) are given in *italics*. ² The values showing moderate to strong responders (as defined in ‘Materials and Methods’) in Th1 cell assays (antigen-induced proliferation and/or IFN- γ secretion) are given in *italics*. ³ The donors of T cell lines used for Th1 cell assays were positive for HLA-DR 3, 4, 5, 6, 7, 13, 52 and 53 molecules.

cally defined HLA-DR molecules included in the ProPred [18, 19]. In addition to being promiscuous for HLA-DR binding, the ProPred analysis also predicted that MPT64 has a large number of Th1 cell epitopes scattered throughout its sequence. These results are compatible with previous findings of the recognition of MPT64 and a large number of its peptides by peripheral blood mononuclear cells and T cell lines from HLA-heterogeneous donors in Th1 cell assays [6, 7]. Thus, the present results reinforce the highly promiscuous nature of MPT64 for presentation to Th1 cells and further support the use of this protein in vaccine or diagnostic applications.

In addition to prediction of HLA-DR binding regions by ProPred, we have also evaluated the Th1 cell reactivity of these regions using 20-mer synthetic peptides. Because these regions were of varying length, i.e. 9–35 aa, 4 regions required more than 1 peptide to cover their sequence, i.e. region 2 (3 peptides), region 3 (2 peptides), region 4 (4 peptides) and region 7 (2 peptides). The evaluation of all regions by using synthetic peptides in Th1 cell

assays showed that 4 of the 5 regions predicted as HLA-DR promiscuous by ProPred were also strong stimulators of Th1 cell reactivity among T cell lines from donors with varied HLA-DR types. The reason for 1 of the 5 predicted HLA-DR-promiscuous regions, i.e. region 6, not showing strong Th1 cell reactivity could be due to its relatively lower level of binding to HLA-DR alleles (16/51, 31%) compared to other regions (from 25/51 to 47/51, 49–92%), which showed strong Th1 cell reactivity. Alternatively, Th1 cells capable of recognizing the epitopes of region 6 may have been lacking in the tested individuals, as has been reported previously for some other mycobacterial proteins [11, 17–19]. Further analyses of HLA-DR-promiscuous regions with strong Th1 cell reactivity, which required more than 1 peptide to cover their sequence, was performed with individual peptides to identify the HLA-DR-promiscuous and immunodominant peptides. The results show that for each region, the immunodominant peptides had the highest numbers of T cell epitopes and were HLA-DR-promiscuous for binding by ProPred.

Thus, except for one region and its peptide showing discordant results, all other regions and peptides showed concordance in terms of HLA-DR binding prediction by ProPred and response in Th1 cell assays with T cell lines from donors of varied HLA-DR types.

Conclusion

The in silico analysis suggests that MPT64 has several HLA-DR-promiscuous regions and a large number of T cell epitopes scattered throughout the sequence of this

protein. Furthermore, strong recognition of HLA-DR-promiscuous regions by Th1 cells supports the notion that MPT64 deserves consideration for inclusion in diagnostic applications or as a candidate vaccine against human TB.

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