Stuber, G, Leder, GH, Storkus, WT, Lotze, MT, Modrow, S, Székely, L et al.. Identification of wild-type and mutant p53 peptides binding to HLA-A2 assessed by a peptide loading-defi...

ARTICLE *in* EUROPEAN JOURNAL OF IMMUNOLOGY · MARCH 1994

Impact Factor: 4.03 · DOI: 10.1002/eji.1830240341 · Source: PubMed

Short paper

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Identification of wild-type and mutant p53 peptides binding to HLA-A2 assessed by a peptide loading-deficient cell line assay and a novel major histocompatibility complex class I peptide binding assay*

Mutations of the p53 gene are the most frequently observed genetic changes in human cancers; often leading to an overexpression of the wild-type (wt) p53 protein. Demonstrable T cell reactivity against tumor cells overexpressing wt or mutant p53-derived peptides could support the application of such epitopes in cancer immunotherapies. As the binding of peptide to MHC class I molecules is a prerequisite for antigen-specific T cell recognition, we evaluated the ability of wt and mutant p53 peptides to bind to HLA-A2.1 using two independent flow cytometry-based assay systems, the T2 major histocompatibility complex (MHC) class I peptide stabilization assay (stabilization assay) and the peptide-induced MHC class I reconstitution assay (reconstitution assay). The twenty selected wt sequences each conformed to the previously reported HLA-A2.1 peptide binding motif. Seven of the wt p53 and 2/13 mutant p53 peptides derived from the previously chosen wt peptides bound to HLA-A2.1 in both the stabilization and the reconstitution assays. An additional six wt and six mutant p53 peptides, presumably exhibiting lower affinity for HLA-A2.1, were identified only in the reconstitution assay. Those p53 peptides binding HLA-A2.1 may provide useful immunogens for the generation of HLA-A2.1-restricted cytolytic T lymphocytes *in vitro* and *in vivo*.

1 Introduction

Mutations or deletions of the p53 gene are the most commonly observed changes associated with human cancers [1, 2]. Mutated p53 protein may complex with, and stabilize, wt p53 resulting in overexpression and a prolonged $t_{1/2}$ of the complex in tumor cells [3, 4]. This resultant overexpression of p53 protein can be visualized immunohistochemically [5]. Such p53 overexpression may also serve to provide peptide epitopes, presented by tumor cell MHC molecules, in sufficient quantities to elicit humoral and cellular (*i.e.* T cell) immune responses in cancer patients [6, 7]. Mutant or wt p53 peptides presented by MHC class I molecules could stimulate anti-tumor $CD8^+$ CTL responses, potentially beneficial in the design of cancer immunotherapy. In this report, we studied a panel of wt and mutant p53 peptides for their capacity to bind to the HLA-A2.1 molecule using two independent flow cytometry-based assay systems: the T2 MHC class I peptide stabilization assay (stabilization assay) [8] and the peptide-induced MHC class I reconstitution assay (reconstitution assay) [9].

[I 12231]

* Both authors contributed equally to the study.

* Supported by grants from the Swedish Cancer Society and NIH grant CA 15840. G. H. Leder is supported by a grant from the Deutsche Forschungsgemeinschaft (DFG).

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Abbreviations: **ATCC:** American Tissue Culture Collection **hβ$_2$-m:** Human β_2-microglobulin **FI:** Fluorescence intensity **MFC:** Median fluorescence channel **PCI:** Pittsburgh Cancer Institute **UVR:** University of Regensburg **wt:** Wild-type

Key words: p53 / Peptide / Tumor suppressor / T cell epitope / Major histocompatibility complex class I

2 Materials and methods

2.1 Cell lines

The peptide presenting defective T2 (T (CEM) × B (.174) hybrid) cell line [10] (kindly provided by Dr. P. Cresswell, Howard Hughes Medical Institute, Immunobiology Section, Yale University School of Medicine, New Haven, CT) and the human C1R.A2.1 B-cell line [11] were grown in RPMI1640 medium supplemented with 2 mM L-glutamine, 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Hepes (0.1 mM) was also added to T2 cultures. All culture reagents were from Gibco (Grand Island, NY).

2.2 Peptides

Twenty wt p53 peptides conforming to the HLA-A2.1 binding motif [12] were selected using a peptide mapping program ([13], Table 1). Peptides were synthesized as

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Table 1. p53 wild-type and mutant peptides

Name (amino acids)	Source	Sequence (position) 1 2 3 4 5 6 7 8 9
p53 24-32	UVR	K L L P E N N V L
p53 42-50	PCI	D L M L S P D D I
p53 43-52	UVR	L M L S P D D I E Q
p53 65-73	UVR/PCI	R M P E A A P P V
p53 92-100	PCI	P L S S S V P S Q
p53 110-118	PCI	R L G F L H S G T
p53 129-137	UVR/PCI	A L N K M F C Q L
p53 132-140	UVR	K M F C Q L A K T
p53 132 M1	UVR	**Q** M F C Q L A K T
p53 136-144	UVR	Q L A K T C P V Q
p53 159-167	UVR	A M A I Y K Q S Q
p53 159 M1	UVR	A M A I **H** K Q S Q
p53 186-196	PCI	D G L A P P Q H L I R
p53 187-195	UVR	G L A P P Q H L I
p53 187 M1	UVR	G L A P P Q H **E** I
p53 193-201	UVR/PCI	H L I R V E G N L
p53 193 M1	UVR	**R** L I R V E G N L
p53 193 M2	UVR	H L I **K** V E G N L
p53 193 M3	UVR	**E** L I R V E G N L
p53 193 M4	UVR	H **E** I R V E G N L
p53 245-253	UVR	G M N R R P I L T
p53 245 M1	UVR	G M N **C** R P I L T
p53 245 M2	UVR	G M N **K** R P I L T
p53 245 M3	UVR	G M N **E** R P I L T
p53 245 M4	UVR	G M N R **H** P I L T
p53 250-258	UVR	P I L T I I T L E
p53 256-265	UVR/PCI	T L E D S S G N L L
p53 263-272	UVR	N L L G R N S F E V
p53 264-272	PCI	L L G R N S F E V
p53 264 M1	UVR	L L G R N S F E **M**
p53 264 M2	UVR	L L G R N S **E** E **M**
p53 322-330	UVR/PCI	P L D G E Y F T L
p53 331-339	UVR	Q I R G R E R F E

9-mers or, for increased solubility as 10- or 11-mers, by the Peptide Synthesis Facility (Shared Resource, Pittsburgh Cancer Institute) or as previously described [14]. Thirteen additional peptides containing natural point mutations observed in the p53 gene were also synthesized. HPLC-purified peptides were dissolved in PBS (1 mM) just prior to use. Human β_2-microglobulin (hβ_2-m; Sigma Chemical Co., St. Louis, MO) was diluted in PBS (100 μM) and aliquots stored at − 20 °C prior to use.

2.3 Stabilization assay

The assay was performed as previously described [8]. Briefly, T2 cells (2.5×10^5) were incubated with 100 μM peptides for 24 h at 37 °C, or for increased sensitivity at 26 °C in AIM V medium. Cells were then stained for HLA-A2 by indirect immunofluorescence using mAB BB7.2 (anti-HLA-A2, American Type Culture Collection, Rockville, MD) and FITC-labeled (1:20) rabbit anti-mouse antibodies (Sigma). Results were determined on a FACS sorter (FACS IV, Becton Dickinson, Mountain View, CA) and expressed as x-fold increase in fluorescence intensity (FI): FI sample/FI control (cells without peptide).

2.4 Reconstitution assay

The assay was performed as previously described [9]. Briefly, C1R.A2.1 cells were treated for 1 min with pH 3.2 citrate-phosphate buffer to denature class I complexes. After neutralization with excess AIM-V medium (Gibco), cells (> 92 % viable) were incubated in 100 nM hβ_2-m, 2 μg mAb BB7.2, and either no peptide (negative control) or 10 μM peptide for 4 h at room temperature (total sample volume = 200 μl). After washing with AIM-V media, 2 μl of FITC-conjugated goat anti-mouse IgG F(ab')$_2$ was added to cell pellets for 30 min at 4 °C. Results were determined as noted above for the stabilization assay.

3 Results

3.1 wt (7/19) and mutant (2/13) p53 peptides bind HLA-A2.1 in the stabilization assay

As depicted in Fig. 1, 7/19 wt and 2/13 mutant p53 peptides induced a greater than 1.4-fold increase in HLA-A2.1

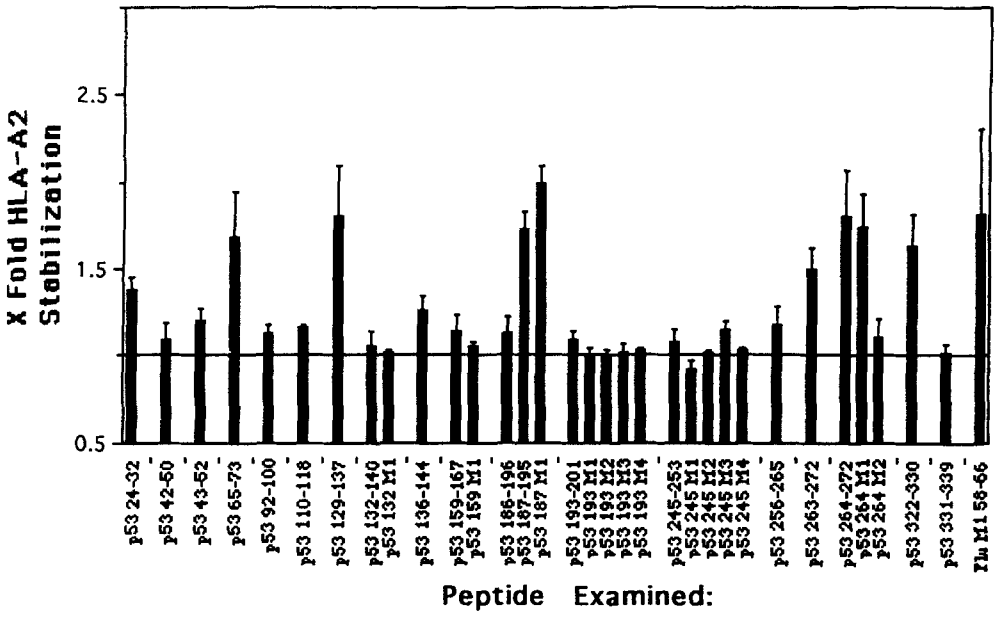

Figure 1. HLA-A2 binding capacity of wt and mutant p53 peptides assessed by the MHC class I stabilization assay at 26 °C. The standard deviations of the individual tests are calculated from the FI values of ten control samples. The Fig. summarizes the cumulative result of five assays. Flu M1 58–66 is an HLA-A2.1 binding peptide used as control.

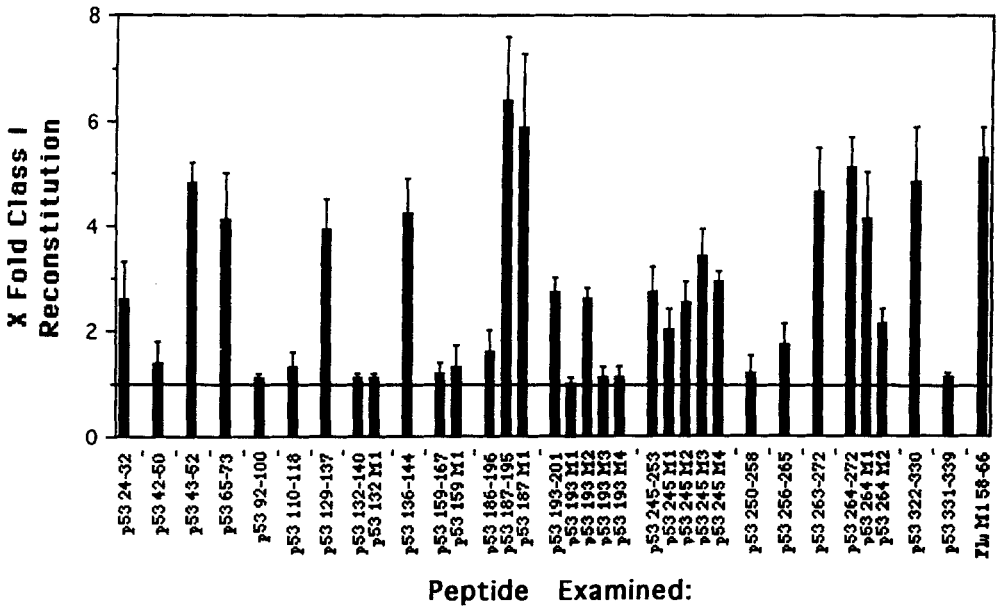

Figure 2. HLA-A2 binding capacity of wt p53 peptides assessed by the reconstitution of acid denatured MHC complexes. Experimental results are comprehensive for wt peptides from ten, and for the mutant peptides from four assays.

expression by T2 after 24 h of co-incubation at 26 °C. One of the wt (p53 322–330) and both of the mutant p53 peptides did not stabilize HLA-A2.1 on T2 when incubations were carried out at 37 °C, suggesting that these peptide-class I interactions are of somewhat lower affinity than those involving p53 peptides stabilizing HLA-A2.1 at both temperatures [15]. The sensitivity of the stabilization assay was enhanced at the reduced (26%) temperature, a phenomenon similarly noted in the binding of peptides to RMA-S cells [15]. A minimum of 1–10 nM peptide was required for stabilization; lower doses were insufficient, as also seen with the known CTL epitope influenza matrix 58–66 (Flu M1 58–66) [16].

3.2 wt (13/20) and mutant (8/13) p53 peptides bind HLA-A2.1 in the reconstitution assay

As shown in Fig. 2, 13/20 wt and 8/13 mutant p53 peptides significantly (1.8–6.4-fold control values) reconstituted HLA-A2.1 complexes on the C1R.A2.1 cell line in the presence of $h\beta_2$-m. Each of the p53 peptides determined to bind HLA-A2.1 in the stabilization assay were shown to bind HLA-A2.1 in the reconstitution assay. Six additional wt and six additional mutant p53 peptides were deduced to bind HLA-A2.1 only in the reconstitution assay.

4 Discussion

The high frequency of p53 mutations and wt p53 protein overexpression in human tumors *vs.* normal tissue makes p53 a potential candidate tumor rejection antigen. The documentation of humoral IgG immune responses directed against p53 in cancer patients supports the idea of cellular (*i.e.* $CD4^+$ T_h-cell) immune recognition of p53 peptide epitopes [6, 7]. We and others hypothesize that $CD8^+$ T cell reactivity to p53 sequences might also be naturally generated in cancer patients, and that such T cell reactivity would be directed against short eight to ten-amino acid peptides presented by MHC class I molecules [17, 18]. We have identified a series of p53 peptides, conforming to the previously reported HLA-A2.1 binding motif [12], that bind to the HLA-A2.1 class I molecule using two independent flow cytometry-based assays. Recently, each of these assay systems has been demonstrated in titration experiments to have a detection limit of approximately 1–10 nM peptide for the binding of the CTL epitope influenza matrix 58–66 to HLA-A2.1 [16]. The effective concentration of peptide and catalytic mechanisms at the site of peptide loading are not known, and it is therefore difficult to speculate about the physiological implications of the peptide concentrations used in the assays. We note, however, that the lower detection limit for several of the positive peptides in this study is in the same range as the one observed for several known CTL epitopes in similar assays ([8, 15, 16], unpublished observations). Each assay system is capable of clearly discerning low *vs.* high affinity class I-binding peptides [16], with the stabilization (26 °C) assay displaying a higher degree of sensitivity than the stabilization (37 °C) assay [15]. Both assay systems support the strong binding of six wt p53 peptides (p53 65–73, p53 129–137, p53 187–195, p53 263–272, p53 264–272, p53 322–330) and somewhat lower affinity binding of wt p53 24–32 to HLA-A2.1. In contrast to the stabilization assay, the reconstitution assay suggested the additional strong binding of wt p53 43–52 and p53 136–144 and the intermediate/low affinity binding of p53 186–196, p53 193–201, p53 245–253, and p53 256–265 to HLA-A2.1. The binding analysis of mutant p53 peptides suggests: (1) no mutant p53 peptide bound to HLA-A2.1 with an apparent higher affinity than its wt p53 analog, (2) the 9-mer p53 187–195 bound far better than the 11-mer p53 186–196, emphasizing the critical importance of peptide length in binding to class I molecules [19, 20], and (3) several mutations appeared to inhibit p53 peptide binding to HLA-A2.1. In particular, the substitution of position 1 H-> R or H-> E in p53 193M1 and p53 193M3, respectively, ablates peptide binding to HLA-A2.1. In addition, as expected, substitution of the position 2 "anchor" L-> E in p53 193M4 also ablated binding. Further, while a V-> M mutation (p53 264M1) at position 9 was tolerated by the HLA-A2.1 binder p53 264–272, the additional F-> E mutation at position 7 (p53 264M2) substantially reduced the binding

capacity of the peptide. Minor changes in HLA-A2.1 binding capacity resulted from amino acid substitutions at wt p53 peptide positions 4/5 (p53 186M1, p53 193M2, p53 245M1-M4), which according to the Rammensee motif [12] are predicted to be less involved in MHC class I-peptide interactions. These positions are predicted to influence T cell receptor contact with class I-peptide complexes, and would be anticipated to result in potential neo p53 T-cell epitopes.

In conclusion, this study has identified a series of p53-derived peptides capable of binding to HLA-A2.1 using two independent assay systems. We do not know whether any of these sequences is naturally processed and presented by HLA-A2.1 on HLA-A2.1$^+$ tumor cells, and if so, whether they can be recognized as T cell epitopes. Nevertheless, these results provide a panel of potential T cell epitopes that may now be evaluated for their immunogenicity *in vitro*.

We thank Petter Höglund, Lars Franksson and Hans Gustav Ljunggren for stimulating discussions, Markus Maeurer and Chiara Castelli for critical review of the manuscript and Ed Scheid for technical assistance.

Received August 25; in revised form December 3, 1993; accepted December 9, 1993.

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