Direct Binding of Thymopentin to Surface Class II Major Histocompatibility Complex in Living Cells

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The molecular analysis of thymopentin (TP5)/class II major histocompatibility complex (MHC II) complexes has been basically understood; however, the mechanism by which TP5-MHC II complexes are formed is largely unexplored. Compared with Epstein−Barr virus (EBV)-transformed B cells expressing human leucocyte antigen DR (HLA-DR), no fluorescent signal was observed on the DR-deficient cell line. This indicates that FITC labeled TP5 (FITC-TP5) is genuinely bound to HLA-DR. The binding specificity was confirmed by incubating FITC-TP5 with unlabeled TP5 and HA peptide as well as mAb for DR molecules. In addition, the binding appeared to be rapid in living antigen-presenting cell (APC), which implies that TP5 is demonstrated on APC surface and does not require processing before associating with DR. Additional support for this surface binding arises from the observation that pretreatment of cells with a variety of metabolic inhibitors failed to decrease the level of TP5/DR complexes. However, temperature has an effect on the rate of binding between TP5 and DR molecules, which is well consistent with the qualitative predication of transition state theory. The formation of antigenic complexes is accelerated at acidic pH, which shows that the formation of TP5/DR complexes is a pH-dependent process.

1. Introduction

Class II major histocompatibility complex (MHC II) proteins have been reported to be the principal proteins anchored in antigen-presenting cell (APC) membrane, where they present antigenic peptides to CD4 positive T helper cells for triggering an immune response.[1,2] Recent advances[3−11] have greatly provided insight into how MHC II proteins interacted with peptides and the general characteristics of antigens processing pathway that prepared antigenic peptides for recognition by MHC II proteins in living APC (Figure 1). In addition, the differences of antigens presentation pathway have prompted the studies on the loading mechanisms.[12,13]

Thymopentin (TP5) represents the residues 32−36 of thymopoietin, and it was well documented as an immunomodulator for the treatment of primary immunodeficiencies, such as AIDS,[14] rheumatoid arthritis,[15] autoimmune diseases,[16] etc. Recently, we have established experimental and computational methods to verify the capability of TP5 to associate with human leucocyte antigen DR (HLA-DR).[17] In our previous works, we provided a better understanding to the interaction pattern between TP5 and DR proteins.

Since short peptides with structural flexibility do not require internalization for presentation, such peptides bind almost exclusively to MHC II proteins on cell surface rather than in intracellular of APC.[18,19] To explore the possibility that TP5 loading on HLA-DR occurs by surface binding, we examined the occurrence and nature of surface complexes of TP5 bound to DR proteins in living APC.

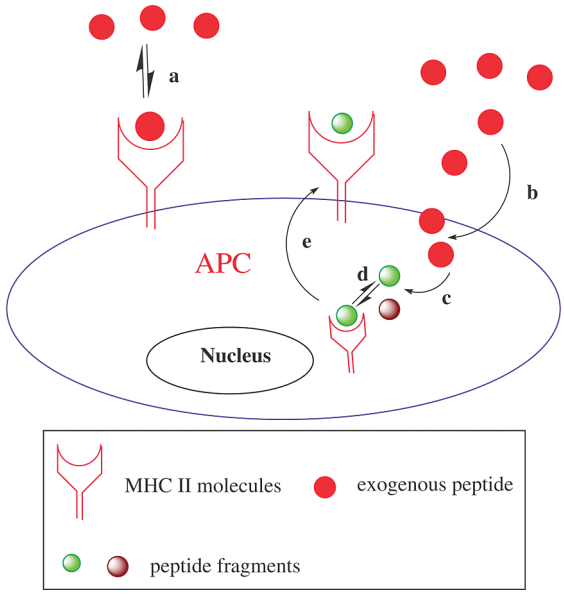

Figure 1. Pathways of exogenous peptide loading of MHC II molecules in living cells. Exogenous peptide loads onto MHC II molecules either by directly extracellular pathway on the living cells surface (a) or by basic intracellular pathways. In intracellular pathways, exogenous peptide is endocytosed by APC (b) and cleaved into peptide fragments by proteases (c), then loaded onto MHC II molecules in a process involving the peptide exchange (d). Once loaded, the MHC−peptide complex is transported to the cell surface (e).

2. Experimental Methods

2.1. Cell Culture. Epstein−Barr virus (EBV)-transformed B cells were maintained in RPMI 1640 containing 2 mM L-glutamine (HyClone Laboratories, UT) supplemented with 10% standard FBS (HyClone Laboratories, UT) at 37 °C and

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in 5% CO_2. Hela cells were maintained in DMEM supplemented with 10% standard FBS (HyClone Laboratories, UT) at 37 °C and in 5% CO_2. The surface DR expression was detected using anti-DR mAb L243 labeled with FITC (Pharmingen, San Diego, CA), by incubation for 45 min on ice followed by FCM.

2.2. Binding Specificity of FITC-TP5 to APC. EBV-transformed B cells and Hela cells (10^6 cells per sample) were washed twice with phosphate-buffered saline (PBS) buffer (pH 7.4) supplemented with 0.1% albumin bovine (BSA) and 0.05% NaN_3, respectively. Then 100 μM FITC-TP5 prepared as described previously[17] was added to the treated cells in PBS (pH 7.4) containing 1% BSA (binding buffer), and further incubated for 2 h at 37 °C. The fluorescent signal in living cells could be monitored qualitatively;[20,21] however, FCM may be applied to detect quantitatively the binding affinity. Thereafter, both the cells were subjected to flow cytometric analysis using a FACSAria analyzer (Becton Dickinson Immunochemical Systems, Mountain View, CA), respectively. A total of 10 000 events were acquired for each sample. Background binding in the presence of alone PBS was 0.9% for EBV-transformed B cells or 1.0% for Hela cells.

In addition, EBV-transformed B cells (10^6 cells per sample) were preincubated with either a 30-fold excess of TP5 and a 25-fold excess of HA peptide for 2 h at 37 °C or mAb DR (20 μL) and mAb DQ (20 μL) for 45 min on ice. The cells were washed twice with PBS (pH 7.4) and coincubated in the presence of FITC-TP5 (50 μM) for 2 h at 37 °C. Thereafter, the washed cells were subjected to flow cytometric analysis. A total of 10 000 events were acquired for each sample. Background binding in the absence of FITC-TP5 was 1.1%. Percent inhibition was calculated as [(1 − binding percent in the presence of inhibitors/binding percent in the absence of inhibitors) × 100%].

2.3. Mutual Inhibition of Binding to APC of TP5. EBV-transformed B cells (10^6 cells per sample) were coincubated in the presence of varying concentrations of TP5 and FITC-TP5 (100 μM) in binding buffer for 2 h at 37 °C. Then, cells were washed twice and analyzed by FCM. In each analysis, 10 000 cells were examined. Background binding in the absence of FITC-TP5 was 1.0%. Mean fluorescence intensity (MFI) was used in the assay to evaluate the binding affinity, and percent inhibition was calculated as [(1 − MFI in the presence of TP5/MFI in the absence of TP5) × 100%].

2.4. Cellular Peptide Loading Assays. EBV-transformed B cells (10^6 cells per sample) were incubated with FITC-TP5 (100 μM) in binding buffer for various incubation periods at 0 and 37 °C, respectively. In addition, cells were preincubated either in 0.1 M chloroquine (Sigma, St. Louis, MO) at the final concentration of 170 μg/mL or in 10 mM ammonia chloride (Sigma, St. Louis, MO) for 30 min at 37 °C before the addition of FITC-TP5, and the same concentration of regents was existed throughout the subsequent procedures. In the experiments involving fixation, the cells (10^6 cells per sample) were washed twice in PBS and fixed with 1% paraformaldehyde (Sigma, St. Louis, MO) for 10 min at room temperature. After extensive washing in PBS, they were incubated with 100 μM FITC-TP5 at 37 °C for 2 h. For the experiments involving pH regulation, cells (10^6 cells per sample) were incubated in 0.15 M citrate/phosphate buffer at various pH in the presence of 100 μM FITC-TP5 at 37 °C for 2 h and washed by buffer with respective pH. Thereafter, the cells mentioned above were washed twice and followed by flow cytometric analysis. A total of 10 000 events were acquired for each sample. Background binding in the absence of FITC-TP5 was 1.0%. The binding percent was used to evaluate the binding affinity.

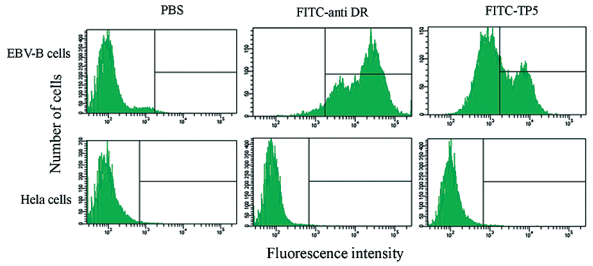

Figure 2. Binding of FITC-TP5 to EBV-transformed B cells and Hela cells. Both the cells were incubated with FITC-TP5 (100 μM) for 2 h at 37 °C and then analyzed by FCM. One representative experiment of the three performed is shown.

TABLE 1: Binding of FITC-TP5 in the Presence of Various Inhibitors[a]

inhibitor	% binding above background	% inhibition
none	24.4	
anti-DR (10 μg)	18.0	28
anti-DQ (10 μg)	23.5	3
TP5 (150 μg)	16.6	33
HA (125 μg)	15.0	40

[a] The cells were incubated with FITC-TP5 (5 μg) as described in Experimental Methods and subjected to flow cytometric analysis. Background binding in the absence of FITC-TP5 was 1%. Results are expressed as mean values of percent of binding from three experiments. Typical SD values were <10%.

3. Results

3.1. Binding Specificity of TP5. In our previous work, we have provided a better insight into the interaction between TP5 and HLA-DR.[17] To further assess the binding specificity, we here measured the binding of FITC-TP5 to EBV-transformed B cells and Hela cell lines, respectively. It could be seen clearly in Figure 2 that there appeared to be a positive fluorescence shift in the presence of EBV-transformed B cells expressing HLA-DR, suggesting FITC-TP5/DR complexes formation. However, no fluorescent signal was observed on Hela cell line with deficient DR molecules. Comparing with Hela cells, 43% of EBV-transformed B cells were stained above background, with a 2.8-fold increase in the MFI. This observation indicates that FITC-TP5 is genuinely bound to HLA-DR expressed in living EBV-transformed B cells.

More compelling evidence for the binding specificity is given by the data in Table 1. These data show that the binding of FITC-TP5 to DR molecules is inhibited by an anti-DR mAb. By contrast, an anti-DQ mAb irrelevant to DR fails to affect the binding of FITC-TP5 to EBV-transformed B cells. Comparing both the results, we conclude that the binding is specific, since it is inhibited by a relevant anti-DR mAb but not by a nonrelevant anti-DQ mAb. To ascertain the binding site for TP5, we further examined the blocking effect of DR molecules to APC surface in the inhibition assay of TP5 binding. As shown in Table 1, preincubation of APC with a 30-fold excess of unlabeled TP5 inhibited 33% of FITC-TP5 binding. In addition, HA peptide, a competitor for DR molecules,[22] inhibited the binding of FITC-TP5 by 40% at a 25-fold excess. These data are compatible with the fact that the binding is specific; i.e., both the peptides bind onto APC at the same site, HLA-DR proteins.

3.2. Mutual Inhibition of FITC-TP5 Binding. To ascertain that unlabeled TP5 and FITC-TP5 bind to the same site, cells

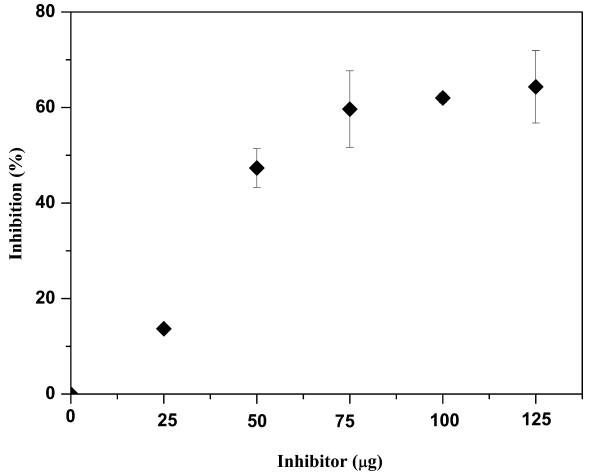

Figure 3. Inhibition of the binding of FITC-TP5 by unlabeled TP5. EBV-transformed B cells were coincubated with FITC-TP5 (100 μM) for 2 h at 37 °C at the indicated concentration, then analyzed by FCM. Results are expressed as mean inhibition of triplicates ±SD. Typical SD values were <10%.

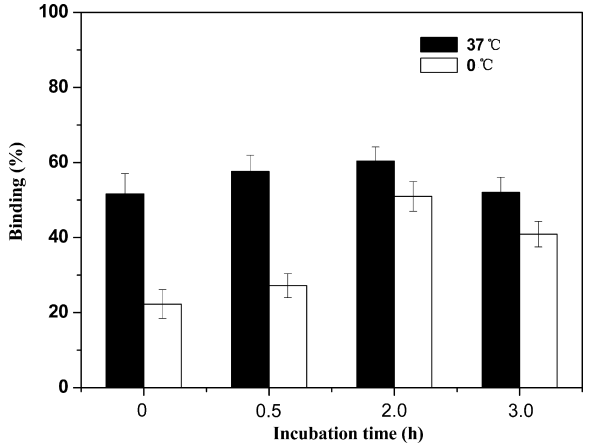

Figure 4. Kinetics of FITC-TP5 binding EBV-transformed B cells at 0 and 37 °C. The cells were incubated for the indicated periods in the presence of FITC-TP5 (100 μM) at 0 and 37 °C, respectively, then subjected to flow cytometric analysis. Results are the mean percentage binding ±SD of three experiments. The SD values were <10%.

were coincubated with FITC-TP5 in the presence of an excess of unlabeled TP5. As shown in Figure 3, the binding level decreased with increasing the concentration of the competitor and reached 59.7% inhibition at an excess of 20-fold by unlabeled TP5. The unlabeled TP5 considerably inhibits the binding of FITC-TP5 in a dose-dependent manner. As a result, the mutual inhibition indicates that they bind to the same binding site.

3.3. Kinetics of Binding to APC. To examine the temperature kinetic requirement for DR molecules binding of TP5, EBV-transformed B cells were incubated with FITC-TP5 at 37 and 0 °C, respectively. Although both the binding of FITC-TP5 were clearly detectable after 5 min incubation, a decrease of the binding degree was seen as the incubation exposure to 0 °C as compared with 37 °C (Figure 4). The kinetic data suggest an effect of temperature on the association kinetics of TP5 with surface DR molecules. According to the transition state theory ($K = K_0 \exp[-\Delta F/K_B T]$),[23] the rate of binding increases with the increasing of temperature. In this assay, the rate of binding, K, is in direct proportion to MFI. Our results are consistent with the qualitative predication of transition state theory in the sense that the rate is higher when the temperature

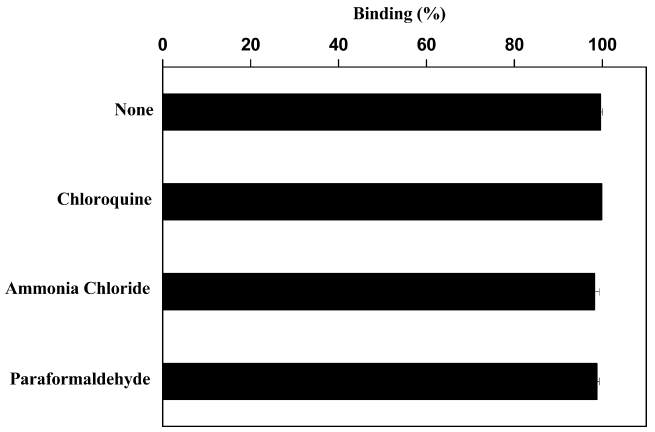

Figure 5. FITC-TP5 binds to cell-surface MHC II molecules in the presence of various metabolic inhibitors. The cells were treated as described in Experimental Methods, and analyzed by FCM. Results are the mean percentage binding ±SD of three experiments. The SD values were <10%.

is higher. These results have already shown that the qualitative behavior changing with temperature is the same as with transition state theory.[24−26]

As mentioned above, this peptide binding is specific, which can be therefore applied for analyzing the time course of the binding. Of particular importance is the finding that peptide loading on the cell surface was halved at 0 °C relative to at 37 °C during the incubation time period from 0 to 0.5 h. With increasing the incubation time period the binding degree increased gradually, which reflects the kinetics of association of this peptide with DR molecules.[27] Of interest is the observation that both the incubations at 37 and 0 °C for 2 h achieved their maximal percent binding (60.4 ± 3.74% and 51.0 ± 3.95%, respectively), which is much shorter than the time needed for maximum binding of peptides to purified class II MHC molecules.[28−30] However, the binding ratio was reduced when the incubation time lasted out from 2 to 3 h. This phenomenon is in agreement with the previous report of influenza matrix peptide 17−29 binding to living cells.[22] In that case, the authors suggested that the MHC loading time must be shorter than the intracellular transit time of either the newly synthesized or the recycling MHC II molecules. Simultaneously, the slight reduction in cell surface binding suggests that the binding failed to approach a true equilibrium or steady state over the time of the assay and that little dissociation exists in the time scale of binding, washing, and detecting procedures.[27] Additional support for the reduction of binding ratio came from the fact that peptide/MHC complexes in living cells were less stable.[1] Collectively, the immediate binding and the time courses of FITC-TP5 binding strikingly suggest that TP5 does not undergo processing before binding to MHC II molecules and exclusively loads on DR at the cell surface.[31]

3.4. Nonrequirement for Processing of TP5 Binding with HLA-DR. To determine whether the formation of TP5-DR complexes was relative to intracellular metabolic processes, a variety of metabolic inhibitors were employed in this assay. Fixation procedure was widely used in the assessment of processing requirements due to inhibiting the activity of a cell-surface cofactor that facilitates peptide binding to MHC II molecules.[32] To address this possibility, fixed and unfixed APC were used to compare the degree of TP5 binding to MHC II molecules. The fixation failed to inhibit TP5 binding (Figure 5), which confirms the nonrequirement for a processing step in the presentation of TP5 by APC. We further studied the effect

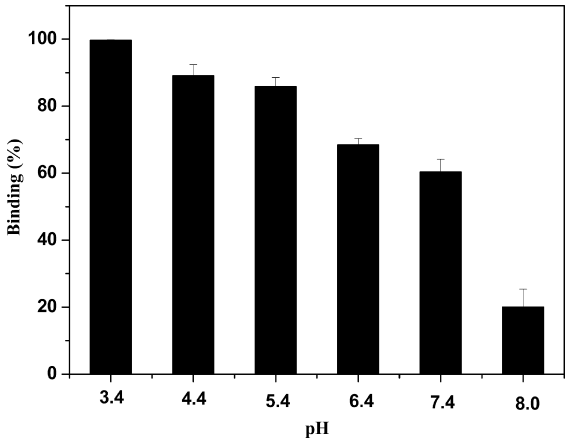

Figure 6. pH-dependent loading between FITC-TP5 and EBV-transformed B cells. The cells were incubated in 0.15 M citrate/phosphate buffer at the indicated pH in the presence of FITC-TP5 (100 μM) for 2 h at 37 °C, then washed by buffer with respective pH and followed by flow cytometric analysis. Results are the mean percentage binding ±SD of three experiments. The SD values were <10%.

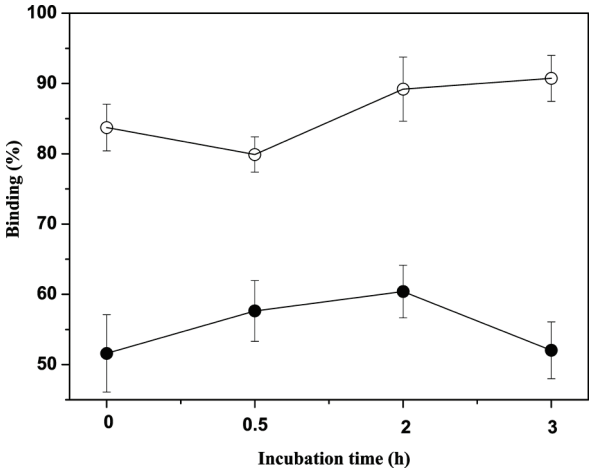

Figure 7. Effect of pH on the kinetics of TP5/MHC II complexes formation. EBV-transformed B cells were incubated with 0.15 M citrate/phosphate buffer containing FITC-TP5 (100 μM) for the indicated time periods at pH 5.4 (open symbols) or pH 7.4 (closed symbols). Then the cells were measured by FCM. Results are the mean percentage binding ±SD of three experiments. The SD values were <10%.

of metabolic inhibitors, ammonium chloride and chloroquine, on the capacity of APC to present TP5. Generally, acidic compartments are thought to correlate to antigen processing because these agents may raise the endosomal pH and further inhibit antigen processing.[33] Although each concentration of these inhibitors was shown to adequately disrupt surface binding, there was no decrease with any inhibitor in the amount of TP5-DR complex formed on the cell surface. The lack of effect on TP5 uptake with the metabolic inhibitors or the cell fixation confirms that TP5 loading onto surface DR is occurring exclusively at the cell surface.

3.5. Effect of pH on the Loading of TP5 on APC. The binding of TP5 to live APC is clearly pH dependent as measured by flow cytometry (FCM) in Figure 6. A stepwise increase of surface binding of FITC-TP5 is seen as the pH of this peptide exposure is lowered. Thus, it is clear that TP5/MHC II complex formation is favored in acidic environment. The result of pH-dependent binding suggests that pH directly regulates the binding of TP5 to DR molecules.[34,35]

The characteristics of TP5 binding HLA-DR at acidic pH were further evaluated. Binding of TP5 was already detected after 5 min of incubation at pH 5.4 and 7.4, with almost maximal binding at 2 h. As shown in Figure 7, a marked increase in the rate and extent of complex formation was observed after incubation of APC with TP5 at pH 5.4 as compared with pH 7.4 for all time points. Although the rate of TP5/MHC II complex formation appears to be faster at pH 5.4 than at pH 7.4 during the binding, the relative kinetics of peptide binding is strikingly similar. Collectively, these findings strongly suggest that pH may be generally important in regulating the formation of TP5/ MHC II complex.

4. Discussion

From this study, it can be clearly seen that pH has a marked effect on TP5 binding by DR molecules. As shown in the Results, the rate and extent of functional TP5 binding were markedly increased at pH 5.4 as compared with pH 7.4. The pH dependence of complex formation may be attributed to an increase in available peptide-binding sites, since the dissociation of endogenous peptide bound to MHC II molecules can be induced by exposure to lower pH (Figure 8). Although interpretation of the effect of pH on available binding sites is complicated,[36] the possibility could not be excluded.

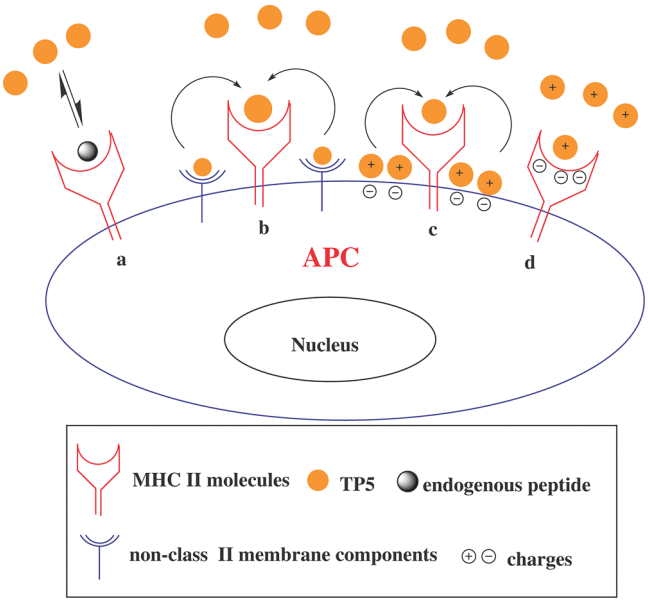

Figure 8. Model for the regulation of TP5/DR complex formation by acidic pH. (a) An increase in available peptide-binding sites is achieved, since the dissociation of endogenous peptide bound to MHC II molecules can be induced by exposure to low pH. (b) A potential pH-dependent interaction of TP5 with nonclass II membrane components increases the effectively local concentration of TP5. (c) The nonspecific electrostatic interactions between TP5 and the negatively charged plasma membrane accelerate the specific binding of TP5 to DR molecules due to increasing the effective local concentration of TP5 in the plasma membrane environment. (d) The effect of pH on the interaction of TP5 with DR molecules is of major importance through affecting the electrostatic interactions between ligand and receptor.

In addition, a potential pH-dependent interaction of peptide with nonclass II membrane components, such as the HSP-70-like peptide receptor and PBP 72/74 protein,[37] may facilitate the specific binding of TP5 to DR molecules by increasing the effective local concentration of the peptide.[36,38] It is therefore possible that pH-dependent interactions involving nonclass II membrane proteins may account for the observed phenomenon (Figure 8).

In the light of this study pointing to the marked pH dependence of complex formation, we are particularly concerned

about the possibility that pH directly affects the nonspecific electrostatic interactions between TP5 and the negatively charged plasma membrane in living APC. From insight into the molecular features, TP5 contains two basic amino acids, Arg and Lys. It is therefore possible that there appears to be nonspecific electrostatic interactions between TP5 and the negatively charged APC membrane (Figure 8). As proposed, binding interactions could be altered through altering the net charge of critical groups in peptide as a function of hydrogen ion concentration.[34] Especially, TP5 does contain an aspartic acid residue ($pK_R = 3.86$), which is likely to contribute toward a change in net charge in the relevant pH range. Since the alteration is of importance in the pH dependence of MHC II molecules combinations, it seems reasonable that nonspecific electrostatic interactions are responsible for the observed pH-dependent binding. As a result of nonspecific electrostatic interactions, the specific binding of TP5 to DR molecules is accelerated through increasing the effective local concentration of TP5 in the plasma membrane environment.

According to our previous modeling, the surrounding microenvironment of TP5 in the binding cleft was shown to be rich in polar amino acid residues. It therefore could be deduced that the electrostatic interactions possibly play a central role in the binding cleft.[39−43] Moreover, the fast kinetics shown in Figure 4 further confirms that electrostatic interactions are essential for the peptide binding.[34] Thus, we further deduce that pH mostly affects the electrostatic interactions between TP5 and DR molecules due to altering their net charges (Figure 8).

Especially, we must consider the possibility that a lone effect fails to fully account for TP5 binding observed in the present study in living APC. The potential requirement for pH-dependent interactions using purified MHC II molecules is obligatory for further defining this regulation of TP5 presentation by acidic pH. Whatever the mechanism, the knowledge generated from the proposed study will help design TP5 analogues, which may stably associate with MHC II molecules and further leads to T-cell stimulation in vivo.

5. Conclusions

The main findings of the present study are that the loading of TP5 in living APC occurs through specific interaction of this peptide with cell surface DR molecules; and the process is independent of cell metabolism or intracellular protein trafficking. In addition, temperature-specific inhibition is observed for living APC, suggesting that temperature has a direct effect on the binding of TP5 to DR molecules. The kinetic rate decreases as temperature decreases, which is consistent with what transition state theory predicts. Thus, we conclude that surface binding of TP5 by DR molecules on APC is the principal pathway of TP5/DR complex formation.

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