Membrane Interactions Influence the Peptide Binding Behavior of DR1

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Summary

We analyzed the binding of an influenza matrix protein-derived peptide, MAT(17-31), to cell surface and purified DR1. The pH dependence of peptide binding was dramatically influenced by the membrane environment. Cell surface binding was enhanced at low pH, with little or no binding detected at neutral pH and optimal binding at pH 4. By contrast, hydrogen ion concentration had minimal effect on peptide binding to purified DR1. Exposure to low pH in the absence of peptide did not affect the peptide binding capacity of cell-associated DR1. Purified DR1 was stable at low pH, excluding the possibility that enhanced binding was offset by a competing denaturation event at low pH. The striking effect of pH on peptide binding characteristic of cell surface DR1 was recovered after reconstitution of purified DR1 in B cell membranes by detergent dialysis. This behavior was partially recovered by reconstitution of full-length, but not truncated DR1 in vesicles containing purified lipid. Our results demonstrate that interactions involving membrane components influence the peptide-binding behavior of DR1.

CD4$^+$ T lymphocytes recognize antigen-derived peptides bound to class II MHC molecules on the surface of APC. Studies with purified MHC proteins have contributed greatly to our current understanding of the binding events leading to the formation of peptide–class II MHC complexes (1, 2). Binding is specific, although individual class II proteins are capable of binding peptides with many different sequences (3). The initial interaction of peptide with class II molecules to form unstable complexes may be rapid (4). However, the rate of formation of stable complexes is quite slow, consistent with a mechanism that may involve a change in class II conformation (2, 4). The rate of peptide dissociation from stable complexes is also slow, often less than the physiological turnover rate of class II proteins in viable APC (5). Studies with mouse class II proteins have indicated that peptide binding is generally enhanced at acidic pH values that approximate the hydrogen ion concentrations found in endosomes and lysosomes (6–10). Peptide binding is believed to occur in endosomal compartments in APC. Hydrogen ion concentrations in the range of pH 4–6 appear to enhance the association rate and maximal extent of binding without affecting the rate of dissociation of high affinity peptides (6–8, 10).

Studies to date suggest that the peptide-binding behavior of purified class II proteins in detergent micelles accurately reflects the behavior of molecules present in a normal membrane environment in APC. For example, the specificity of peptide binding observed with purified class II proteins corresponds to known specificities defined by T cells induced by in vivo immunization (11). The pH dependence of peptide binding observed with purified mouse class II proteins also corresponds to that observed in functional assays where T cells are used to measure peptide binding to cell surface class II (10, 12, 13). There is speculation, however, that cofactors present in APC may influence the rate or degree of peptide/class II binding (6, 14). It is possible that molecular chaperones or other components present in physiological compartments may participate in peptide loading.

Recent reports using several purified DR proteins raise the possibility that the peptide-binding activity of human class II molecules may be optimal at neutral pH (15–17). These results call into question the hypothesis that vesicular acidification is generally important in regulating peptide loading in human APC under physiological conditions (12).

In the present study, we analyzed the binding of the influenza matrix peptide, MAT(17-31), to purified DR1 and found that binding was only slightly enhanced at acidic pH. The use of a recently developed assay (18) allowed us to directly measure binding of MAT(17-31) to cell surface DR1 on LG2 B cells. Binding to cell surface DR1 was strikingly enhanced at low pH. Our results indicate that the peptide-binding behavior of DR1 can be influenced by interactions involving

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components of cellular membranes and raise the possibility that studies with soluble class II proteins may not always accurately represent their physiological behavior.

Materials and Methods

Purification and Reconstitution of DR1. DR1 was purified from the EBV-transformed homozygous LG-2 B cell line (DRB1*0101) using a monoclonal antibody LB3.1 (19) immunoaffinity column as previously described (6). Samples were stored in buffer containing 1% *N*-octylglucoside at 4°C and analyzed by SDS-PAGE and Coomassie blue staining. Protein was determined using the bicinchoninic acid (BCA) assay (Pierce Chemical Co., Rockford, IL). A soluble product of the purified DR1 membrane protein was generated by digestion with papain as described by Gorga et al. (20). Soluble recombinant DR1 was isolated from the conditioned culture media of Sf9 insect cells infected with baculoviruses carrying truncated α and β subunit genes as described (19).

In some experiments, DR1 (4.6 μM) was reconstituted into liposomes from solutions containing 0.5% *N*-octylglucoside and purified lipid [500 μM dipalmitoylphosphatidylcholine (DP-PC), 150 μM cholesterol (both from Sigma Chemical Co., St. Louis, MO)] by extensive dialysis against PBS. For other experiments, DR1 was reconstituted into mouse A20 B cell (21) membranes that were prepared after cell disruption by N_2 cavitation as previously described (22). The mouse class II proteins (I-A^d and I-E^d) that are expressed in A20 do not bind MAT(17-31) (data not shown). DR1 (4.6 μM) was mixed with 0.5% *N*-octylglucoside-solubilized membranes (10^8 cell equivalents/ml) followed by dialysis to form liposomes. Liposomes were used directly in binding assays in the presence of protease inhibitors. Each sample contained 2 pmol DR1, 2.5 nmol DP-PC, and 0.75 nmol cholesterol or 5 × 10^5 A20 membrane equivalents.

Peptide–Class II Binding Assay

Solutions. The solutions in this assay include the following: binding buffer = 100 mM citrate/phosphate, 1 mM *N*-ethylmaleimide, 5 mM EDTA, 1 mM iodoacetamide, 1 mM benzamidine, 1 mM PMSF, and 0.2% NP-40 for unreconstituted purified DR1 samples; TTBS = 500 mM Tris, pH 7.5, and 0.1% Tween-20; MTB = 5% skim milk, 1% BSA, 500 mM Tris pH 7.5 and 0.1% Tween-20; MTBN = MTB + 0.5% NP-40; neutralization buffer = 3.5% skim milk, 0.7% BSA, 335 mM Tris, pH 7.5, 0.07% sodium azide, 0.07% Tween-20, and 0.35% NP-40; lysis buffer = 0.5% NP-40, 0.15 M NaCl, 50 mM Tris, pH 8.0, and protease inhibitors; europium assay buffer = 100 mM Tris, 0.15 M NaCl, 1% sodium azide, 2 μM diethylenetriaminepentaacetic acid (Sigma Chemical Co.), 0.5% BSA, and 0.01% Tween-40; enhancement solution A = 1 M acetate, pH 3.1, 60 mM benzoyl trifluoroacetone, 850 μM Yttrium oxide (Y_2O_3; both from Sigma Chemical Co.), and 5% Triton X-100; and enhancement solution B = 2 M Tris, pH 7.0 and 200 mM 1,10-phenanthroline (Sigma Chemical Co.).

Peptide. MAT(17-31) (23) (sequence; SGPLKAEIAQRLEDV) was synthesized in the Emory University Microchemical Facility as previously described. The peptide was labeled with biotin by reaction with excess *N*-hydroxysuccinimide-biotin in dimethyl formamide followed by precipitation with acetone and HPLC purification (6).

Preparation of Assay Plates. Plates were coated with 100 μl of 20-μg/ml L243 mAb (DRα specific) in borate buffered saline, pH 8, overnight at 4°C. Plates were blocked with MTB for 30 min at 24°C, and washed with TTBS. 50 μl MTBN was added to the plates before sample addition.

Binding Reactions. The binding assays using purified DR1 or reconstituted DR1 were performed with 2 pmol DR1 and various concentrations of biotin-MAT(17-31) at 37°C in microfuge tubes in a 30-μl volume of binding buffer with (purified DR1) or without (reconstituted DR1) detergent. The binding reaction was allowed to proceed for at least 18 h, and the pH was then neutralized by the addition of an equal volume of neutralization buffer.

Peptide binding to cell surface DR1 was measured using 1 × 10^6 LG2 cells/sample. The cells were washed 2× in HBSS and fixed with 0.5% paraformaldehyde for 10 min at 24°C. After extensive washing in RPMI 1640 with 10% serum followed by PBS, the cells were incubated with biotin-MAT(17-31) in binding buffer lacking detergent. After an 18-h incubation at 37°C, cells were washed twice with HBSS and lysed for 40 min on ice in 60 μl lysis buffer. The lysates were then cleared by centrifugation for 10 min at 20,000 *g*.

Samples (purified, reconstituted, or cell lysates) were applied to prepared microtiter plates for DR capture and quantitation of bound biotin-peptide. Samples were allowed to bind to the antibody-coated plates for 2 h at 4°C.

Detection of Peptide–Class II Complexes. Bound biotinylated peptide was detected by washing the plate with TTBS and incubating with 100 ng/ml europium-labeled streptavidin (Wallac Oy, Turku, Finland) in assay buffer for 1 h at 4°C. After washing, 100 μl of enhancement solution A was added per well and the plates were shaken gently for 3 min at 24°C (24). 10 μl of enhancement solution B was added per well, and the plate was shaken again for 1 min at 24°C. Fluorescence of each sample well was measured at 615 nm using a 1230 ARCUS time-resolved fluorometer (LKB Wallac, Turku, Finland). The data points represent the mean fluorescent counts per second/1,000 (cps × 10^{-3}) of duplicate samples. The procedures used allow comparison of relative fluorescence within individual experiments but not between experiments. The assays involving cells especially showed day to day variation in signal magnitude, possibly due to fluctuations in DR1 surface expression or variation in reagents. The results shown in this communication are representative of at least three separate experiments.

Western Blot Assay. DR1 (0.5 μg/lane) was incubated in a 10 μl volume with 0.2% NP-40, 22 mM citrate/phosphate buffer at the indicated pH overnight at 37°C. Samples were neutralized by the addition of 3 μl 1 M Tris, pH 7.0 before the addition of 4 μl nonreducing 4% SDS sample buffer and incubated for 20 min without heating (Fig. 3, lane *8*, sample was boiled). The samples were separated on linear 12% SDS polyacrylamide gels by electrophoresis. Gels were run at 200 V for 50 min, and protein was transferred to nitrocellulose membranes for 18 min at 12 V in 25 mM Tris, 192-mM glycine, pH 8.3, and 20% methanol. DR1 was detected with anti-DR rabbit serum, donkey anti–rabbit horseradish peroxidase and chemiluminescent substrate (ECL kit; both from Amersham International, Amersham, UK). The antisera used is fully cross-reactive with DR dimers and the dissociated monomers. The monomeric α and β subunits co-migrate on the mini-gels used in these experiments.

Results

A Difference in the Peptide-binding Behavior of Cell Surface and Purified, Detergent-solubilized DR1. The results of experiments measuring the binding of biotin-labeled MAT(17-31) to purified DR1 indicate that binding is relatively insensitive to hydrogen ion concentrations in the range of pH 7–4 (Fig.

1 *a*), confirming results with a similar peptide previously reported by Mouritsen et al. (16). The data are representative of more than 15 experiments and a similar pH dependence of binding was observed over a range of peptide concentrations from 40 nM to 10 μM.

The fluorescence assay used to measure the binding of biotin-labeled peptide to purified DR1 (Fig. 1 *a*) allowed us to also measure binding of peptide to cell-associated DR1. We have previously demonstrated that this assay is specific, highly sensitive, and linear over a large concentration range of peptide–class II complexes (18). Fixed LG2 B cells (homozygous for DR1) were incubated with biotin-MAT(17-31) in 0.15 M citrate/phosphate buffer containing protease inhibitors. After washing, detergent lysates were prepared and transferred to microtiter plates coated with the mAb, L243, to selectively capture DR–peptide complexes. Bound biotin-peptide was detected with streptavidin-europium followed by measurement of time-resolved fluorescence. Binding was inhibited in the presence of excess unlabeled peptide and only small background signals were observed with samples lacking biotin-peptide or captured with inappropriate mAb (Fig. 1 *b*). Significant signals were obtained with biotin-peptide concentrations <1 μM after incubation for 18 h at 37°C. We conclude that the fluorescence immunoassay specifically measures the binding of peptide to DR1.

Binding of biotin-MAT(17-31) to cell surface DR1 was markedly enhanced at low pH (Fig. 1 *c*). Binding was essentially undetectable at neutral pH and increased as the hydrogen ion concentration was increased to reach a maximum at pH 4 or pH 4.5. Similar results were obtained in nine experiments and with biotin-peptide concentrations ranging from 0.1 to 10 μM.

Peptide Binding Is Unaffected by Pretreatment of DR1 at Low pH. Pretreatment of fixed LG2 cells in buffer at pH 5 for up to 3 d had no appreciable effect on the degree of peptide binding measured at pH 7 (Fig. 2). These results rule out the possibility that enhanced binding at low pH on cells is due to an increase in binding sizes resulting from the acid-induced dissociation of loosely bound peptides.

We have previously proposed that murine class II molecules undergo a conformational change at acidic pH, resulting in a flexible molecule that is better able to bind peptide (6, 7). It is possible that the flexible form of purified DR1 at

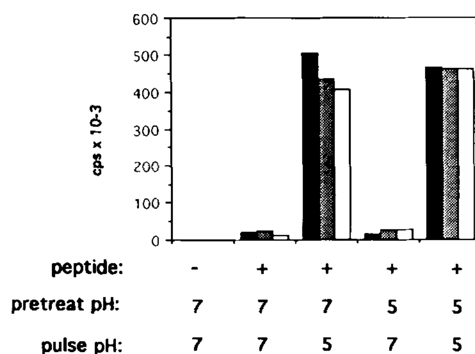

Figure 2. Pretreatment of fixed cells at acidic pH does not enhance peptide binding at neutral pH. Fixed LG2 cells were incubated at 37°C for 1 (*open bars*), 2 (*gray bars*), or 3 d (*solid bars*) in 150 mM citrate/phosphate at pH 5 or 7. After washing, the cells were incubated with 1 μM biotin-MAT(17-31) in 150 mM citrate/phosphate at pH 5 or 7 at 37°C for 18 h. Binding of peptide to DR1 was determined as described in Materials and Methods.

low pH is unstable and rapidly denatures. The dissociation of the class II heterodimer at low pH may counteract enhanced peptide binding, resulting in the relatively flat pH curve observed with purified DR1.

To address this possibility, the stability of purified DR1 was measured by incubation at various pH for 18 h at 37°C (Fig. 3 *a*). Unboiled samples were resolved on SDS-PAGE to determine the extent of subunit dissociation to form monomers. The fraction of monomers was only marginally increased with incubation under acidic conditions as low as pH 4. Thus, no gross destabilization of purified DR1 is observed at low pH.

It is likely that only "empty" DR1 molecules are available for peptide binding, and this subpopulation is believed to be unstable in SDS (19, 25, 26). The small fraction of empty molecules in the total pool of purified DR1 may not be detected by SDS-PAGE analysis. The potential loss of functional DR1 molecules was therefore analyzed by a 48-h treatment of purified DR1 at low pH in the absence of peptide followed by measurement of residual binding activity in an 18-h assay (Fig. 3 *b*). Binding activity was completely retained after incubation in acidic buffer as low as pH 4. All activity was

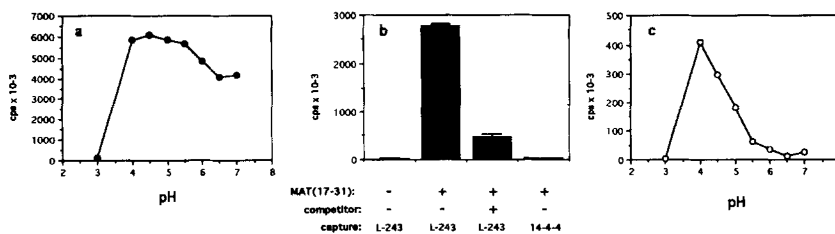

Figure 1. Effect of pH on binding of MAT(17-31) to purified, detergent solubilized DR1 and cell surface DR1. (*a*) Purified DR1 was incubated with 1 μM biotin-MAT(17-31) in 0.2% NP-40 and 100 mM citrate/phosphate at the indicated pH for 18 h at 37°C. (*b*) Fixed LG2 cells were incubated with or without 1 μM biotin-MAT(17-31) in 150 mM citrate/phosphate, pH 5 for 18 h at 37°C. In one sample, 100 μM unlabeled MAT(17-31) was added to show specificity of binding. After washing, the cells were lysed and added to microtiter assay plates coated with L-243 or 14-4-4 (control mAb). (*c*) Fixed LG2 cells were incubated with 1 μM biotin-MAT(17-31) in 150 mM citrate/phosphate at the indicated pH for 18 h at 37°C. Biotinylated peptide binding to DR1 was measured with europium-streptavidin fluorescence as described in Materials and Methods.

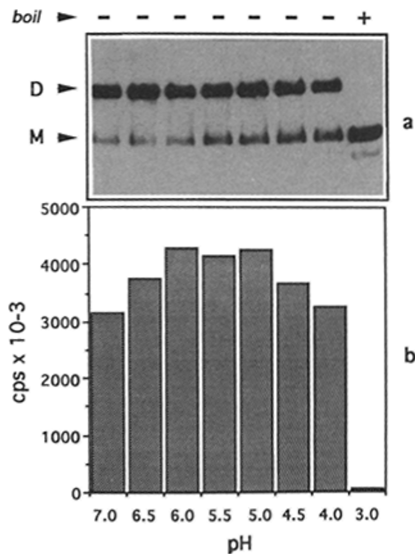

Figure 3. Pretreatment at low pH does not affect the stability or peptide binding activity of purified DR1. (*a*) Purified DR1 was incubated for 18 h in a 10-μl volume of 20 mM citrate/phosphate at the indicated pH. Samples were neutralized with 3 μl, 1 M Tris, pH 7.0 for 10 min before the addition of 4% SDS sample buffer. One sample, pretreated at pH 7.0, was boiled for 5 min before electrophoresis on a 12% polyacrylamide gel. A Western blot was used to measure the relative proportion of class II dimers (*D*) and dissociated monomers (*M*) as described in Materials and Methods. (*b*) Purified DR1 was incubated in 10 mM citrate/phosphate at the indicated pH for 2 d at 37°C. The pH was brought to 5.0 by adding 100 mM (final) citrate/phosphate pH 5.0, and 1 μM biotin-MAT(17-31) was added. After 18 h at 37°C, peptide binding was measured as described in Materials and Methods.

lost after incubation at pH 3, consistent with irreversible denaturation of functional molecules. We conclude that the relatively small effect of low pH on peptide binding by purified DR1 is not accounted for by a competing reaction where functional molecules are irreversibly denatured.

Reconstitution of pH-dependent Peptide Binding. The observed difference in the peptide-binding behavior of purified DR1 as compared to cell surface DR1 could reflect the selection of a subpopulation of molecules during the purification procedure, which involves exposure to various detergents, high concentrations of salt, and high pH. Alternatively, the protein may undergo irreversible structural alteration during isolation resulting in altered behavior.

These latter possibilities were excluded by the observation that the pH-dependence of peptide binding that is characteristic of cell surface DR1 was recovered after reconstitution of purified DR1 in liposomes containing B cell membrane components (Fig. 4 *a*). Purified DR1 was added to detergent-solubilized membranes that were isolated from murine A20 B cells and liposomes were formed by detergent dialysis. Similar results were obtained in five experiments. Maximal binding after reconstitution ranged from ~45 to 140% as compared with control DR1 preparations in 0.2% NP-40 detergent. Inaccessibility of a fraction of the reconstituted DR1 resulting from orientation in the membrane bilayer of the liposomes may partially account for this variation. The observed recovery of pH-dependent binding activity in these experiments also excluded the formal possibility that the fixation procedure used in experiments with intact LG2 B cells artifactually altered peptide binding behavior.

Interactions between DR1 and specific cofactors in B cell membranes are not required for partial reconstitution of pH-dependent binding behavior. The relative pH dependence of binding was partially, although not completely, recovered after reconstitution into lipid vesicles containing only phosphatidyl choline and cholesterol (Fig. 4 *b*). Direct addition of lipid to detergent-containing solutions of DR1 did not significantly influence peptide-binding behavior (Fig. 4 *c*), indicating that restoration of the pH dependence requires the presence of a lipid bilayer. These results suggested that the insertion of DR1 into a lipid bilayer is sufficient to confer enhanced binding activity at acidic pH. This conclusion was further substantiated in control experiments using truncated forms of DR1. Recovery of pH-dependent peptide binding was not observed after mock-reconstitution of recombinant (19) or papain-digested (20) DR1, lacking the transmembrane and cytosolic domains (data not shown).

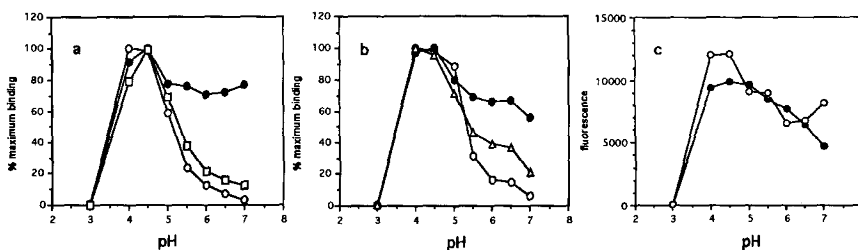

Figure 4. The pH dependence of MAT(17-31) binding can be recovered by reconstitution of purified DR1 into membrane vesicles. (*a*) 1.5 × 10^6 fixed LG2 (*open circles*), 2 pmol purified DR1 in NP-40 micelles (*closed circles*), or 2 pmol purified DR1 reconstituted into mouse B cell (A20) membranes (*open squares*) were incubated with 1 μM biotin-MAT(17-31) in 100 mM citrate/phosphate at the indicated pH for 18 h at 37°C. Peptide binding was measured as described in Materials and Methods and expressed as percent of maximum binding for each preparation. Maximum binding for fixed cells, 328; detergent micelles, 920; and reconstituted DR1, 418 cps × 10^{-3}. (*b*) Binding of biotin-MAT(17-31) to DR1 in fixed LG2 (*open circles*), detergent micelles (*closed circles*), and vesicles containing DP-PC and cholesterol (*open triangles*) was measured at various pH and expressed as described above. Maximum binding for fixed cells, 282; detergent micelles, 277; and reconstituted DR1, 552 cps × 10^{-3}. (*c*) Purified DR1 was incubated with (*open circles*) and without (*closed circles*) 2.5 nmol DP-PC and 0.75 nmol cholesterol in 100 mM citrate/phosphate, 0.2% NP-40 and 1 μM biotin-MAT(17-31) at the indicated pH. After 18 h at 37°C, bound peptide was measured as described in Materials and Methods.

Discussion

In this report we have shown that the pH dependence of binding of MAT(17-31) to DR1 is drastically different depending on the presence of a lipid bilayer. The peptide binding activity of purified DR1 in detergent micelles is relatively independent of pH in the physiological range. By contrast, cell-associated DR1 or purified DR1 that has been reconstituted into B cell membranes shows a striking pH dependence with optimal binding at pH 4–4.5.

The increase in peptide binding observed at low pH with cell-associated DR1 could not be attributed to an increase in available binding sites after acid stripping of loosely associated peptides. No major enhancement or decrease in binding activity was observed after extensive pretreatment of fixed B cells with low pH. This was further confirmed by the observation that pH-dependent binding could be recovered after reconstitution of purified DR1 into lipid membranes. The latter result also excluded potential effects of fixation on cell surface DR1 binding behavior and the possibility that pH-dependent peptidase activity associated with the cell membrane was responsible for decreased peptide–DR1 complex formation at neutral pH.

We also considered the possibility that purified DR1 may be relatively unstable at low pH with enhanced α/β subunit dissociation and irreversible denaturation. Dornmair et al. (27) have provided evidence that IE^k may be more sensitive to acid-induced denaturation in the absence of a lipid bilayer. Subunit dissociation could offset any advantage in peptide binding provided by a low pH environment. However, the major fraction of purified DR1 dimers was found to be relatively stable during incubation in acidic buffer as low as pH 4 (Fig. 3). The capacity of DR1 to bind peptide, reflecting the functionally relevant subset of molecules, was unaffected by exposure to low pH.

The pH dependence of peptide binding observed with cells but not with detergent-solubilized protein can be substantially recovered by reconstitution of purified DR1 into lipid vesicles. It is possible that full recovery of the peptide binding behavior characteristic of cell surface DR1 may be achieved by altering the constituent lipids used in detergent dialysis. Thus, a mechanism involving pH-dependent interaction between DR1 or peptide and a specific cofactor(s) present in B cell membranes is not required to account for differences in peptide binding between cell surface and purified DR1. However, the potential contribution of a specific cofactor cannot be excluded and we are addressing this possibility with ongoing experiments. Roof et al. (28) reported that a variety of lipids, including PC, enhance the binding of peptides to purified class II glycoproteins in detergent. This effect does not appear to account for the enhanced binding observed at low pH after reconstitution of DR1 in lipid vesicles. Direct addition of equivalent amounts of lipid to DR1 in detergent had no effect on peptide binding. Furthermore, the peptide-binding activity of reconstituted DR1 was not enhanced at neutral pH in contrast to the results of Roof et al. (28). However, mouse class II proteins were used in their study and it is possible that altering the lipid of composition or increasing lipid concentration may result in formation of specific class II/lipid interactions that mimic conditions found in cellular membranes.

Our data support the conclusion that interactions between the transmembrane domain of DR1 and the lipid bilayer influence the overall structure of the protein with an effect on the conformation or flexibility of the spatially distant $\alpha 1$ and $\beta 1$ domains that directly interact with peptide. pH-dependent peptide binding was not recovered after mock-reconstitution with soluble forms of DR1 lacking the transmembrane and cytosolic domains. This suggests that the presence of detergent, per se, does not account for the altered peptide binding behavior of purified full-length DR1. Our data are consistent with a model in which the lipid bilayer and the transmembrane domain of DR1 interact to stabilize a rigid conformation that does not favor peptide binding. Low pH may be required to induce a more flexible conformation in membrane-bound, but not soluble, DR1 molecules.

Interactions involving the transmembrane domains do not appear to affect the peptide-binding behavior of several mouse class II proteins that have been studied. The pH dependence of peptide binding observed in experiments with purified IA^d, IA^k, IE^d, and IE^k in detergent (6, 7) is very similar to that inferred from experiments in which binding to cell surface class II was indirectly measured using functional assays (12). The characteristic pH dependence of binding is also conserved in studies with a recombinant phosphatidylinositol-anchored form of IE^k (8, 13). We speculate that interactions involving the transmembrane domains may have greater or lesser influence on peptide binding activity depending on stability of interactions between the extracellular domains. Alternatively, the interactions involving the transmembrane domains of DR1 may be more sensitive to changes in hydrogen ion concentration. This could be tested by analysis of chimeric class II proteins after exchange of transmembrane domains.

Sette et al. (15) reported that the binding of several different peptides to purified DR1, DR5, and DR7 was relatively unaffected by hydrogen ion concentration in the range of pH 4–7. This may be a property of many DR proteins in detergent. However, a marked enhancement of peptide binding at low pH was observed with DR3 in detergent (29). It is possible that peptide binding is generally optimal at low pH for DR proteins expressed in their normal membrane environment in APC. This is consistent with the hypothesis that peptide loading occurs in acidic endosomal compartments under physiological conditions. We have preliminary evidence that cell-associated DR1 preferentially binds peptides other than MAT(17-31) at low pH. Further studies will be required to test the generality of this observation. However, the data presented in the present report clearly demonstrate that interactions between DR1 and membrane components can influence peptide-binding behavior.

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