Lipid Raft-dependent and -independent Signaling through HLA-DR Molecules*

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Lipid rafts are plasma membrane microdomains that are highly enriched in signaling molecules and that act as signal transduction platforms for many immune receptors. The involvement of these microdomains in HLA-DR-induced signaling is less well defined. We examined the constitutive presence of HLA-DR molecules in lipid rafts, their possible recruitment into these microdomains, and the role of these microdomains in HLA-DR-induced responses. We detected significant amounts of HLA-DR molecules in the lipid rafts of EBV+ and EBV− B cell lines, monocytic cell lines, transfected HeLa cells, tonsillar B cells, and human monocytes. Localization of HLA-DR in these microdomains was unaffected by the deletion of the cytoplasmic domain of both the α and β chains. Ligation of HLA-DR with a bivalent, but not a monovalent, ligand resulted in rapid tyrosine phosphorylation of many substrates, especially Lyn, and activation of ERK1/2 MAP kinase. However, the treatment failed to induce further recruitment of HLA-DR molecules into lipid rafts. The HLA-DR-induced signaling events were accompanied by the induction of cell-cell adhesion that could be inhibited by PTK and Lyn but not ERK1/2 inhibitors. Disruption of lipid rafts by methyl-β-cyclodextrin (MβCD) resulted in the loss of membrane raft association with HLA-DR molecules, inhibition of HLA-DR-mediated protein tyrosine phosphorylation and cell-cell adhesion. MβCD did not affect the activation of ERK1/2, which was absent from lipid rafts. These results indicate that although all the HLA-DR-induced events studied are dependent on HLA-DR dimerization, some require the presence of HLA-DR molecules in lipid rafts, whereas others do not.

Although major histocompatibility complex (MHC) class II molecules do not possess any known signaling motifs in their cytoplasmic and transmembrane domains, they act as signal transducers in addition to playing a critical role in antigen presentation and autoimmune disease susceptibility and severity (1, 2). Corley et al. (3) reported that the recognition of MHC class II-peptide complexes by specific T cell receptors (TCR) leads to the activation of both T lymphocytes and antigen-presenting cells (APCs), suggesting that MHC class II molecules can act as signal transducers. To confirm this possibility, anti-MHC class II antibodies (Abs) and superantigens (SAgs), which act as natural MHC class II ligands, were used to mimic MHC class II-peptide complex recognition by specific TCRs. This ligation led to various cellular events such as homotypic and heterotypic cell-cell adhesion (4), B cell proliferation and differentiation (5), cytokine production, and expression of costimulatory molecules (6) and, under certain conditions, cell death (7). Like other ligand-receptor interactions, some of these events are dependent on MHC class II ligand dimerization (8, 9), generate cAMP and intracellular calcium flux (1), and are mediated by signaling pathways and secondary messengers including protein kinase C (PKC), protein tyrosine kinase (PTK), cyclooxygenase 2, cytosolic phospholipase A2, and phospholipase C.

A number of studies have addressed the structural basis by which MHC class II molecules carry out its multiple functions. It has been shown that a six amino acid region of the cytoplasmic domain of the β chain, in particular residues 225, 227, and 228, are required for antigen presentation, cAMP accumulation, PKCβ translocation, and the expression of CD80 molecules in murine B cells (10–14). Other MHC class II-induced biochemical events such as tyrosine phosphorylation, phosphoinositide hydrolysis, and calcium mobilization, are unaffected by the substitution or deletion of the cytoplasmic domains of the α and β chains (12, 13). It has been proposed that both transmembrane and cytoplasmic regions are involved in signaling via MHC class II molecules using different pathways. Nevertheless, the two pathways must be at least partially cooperative since disruption of either leads to a loss of MHC class II-induced B cell differentiation (13, 15).

The plasma membranes of eukaryotic cells contain microdomains enriched in cholesterol and sphingolipids (16–18), commonly called lipid rafts or glycolipid-enriched microdomain (GEMs). These microdomains are resistant to solubilization at low temperature by nonionic detergents and can be separated and isolated from the rest of the plasma membrane using sucrose density gradients (18, 19). They also harbor large quantities of proteins such as G proteins, kinases, and adaptor

ammonio]-1-propanesulfonic acid; HRP, horseradish peroxidase; SAgs, superantigens; TCR, T cell receptor; CIITA, class II transactivator; APC, antigen-presenting cells; BCR, B cell receptor; SEA, staphylococcal enterotoxin A; MAM, Mycoplasma arthritidis-derived mitogen.
molecules that act as intermediate transducers for many receptors, including TCR (20) and B cell receptor (BCR) (21). The relevance of lipid rafts with regard to MHC class II-induced signal transduction pathways and APC function is less clearly defined (22, 23). Anderson et al. (22) reported that large numbers (20–50%) of MHC class II molecules are located in the lipid rafts of murine and human B cell lines. Such localization seems to be critical for T cell activation when minimal numbers of relevant MHC class II-peptide complexes are available on APCs. However, Huby et al. (23) failed to detect MHC class II molecules in the lipid rafts of a human IFN-γ-treated THP-1 monocytic cell line unless the molecules were oligomerized by cross-linking with specific primary Abs followed by a secondary Ab. Indeed, the translocation of MHC class II molecules into lipid rafts seems to be a requirement for PTK activation.

In this study, we show that small but significant amounts of HLA-DR molecules were constitutively present in the lipid rafts of normal human B cells, human EBV+, and EBV− B cell lines, monocytoid HeLa cells, and monocytic THP-1 cells treated with IFN-γ or transfected with the MHC class II transactivator CIITA. The localization of HLA-DR in the lipid rafts was unaffected by the deletion of the cytoplasmic domains of the α and β chains. We further show that initiation of signal transduction such as activation of PTK, in particular Lyn, and homotypic cell-cell adhesion required the dimerization of MHC class II molecules in the lipid rafts. In contrast, activation of ERK1/2 was critically dependent on MHC class II dimerization, but did not require the presence of MHC class II molecules in the rafts.

MATERIALS AND METHODS

Reagents—The following antibodies were used: mAb L243 (mouse IgG2a, recognizes a conformational epitope of HLA-DR; ATCC, Manassas, VA), mAb DA6.147 (mouse IgG1, recognizes the C-terminal intracellular tail of the HLA-DR α chain; a generous gift from Dr. P. Cresswell, Yale University, New Haven, CT), mAb XD6 (mouse IgG1, recognizes the HLA-DR β chain), mAb W6/32 (mouse IgG2a, anti-MHC class I; ATCC, Manassas, VA), mAb 9.4 (mouse IgG2a, anti-CD45; ATCC, Manassas, VA), mAb 8C12 (mouse IgG2a, anti-CD8; produced in our laboratory), anti-phosphotyrosine mAb 4G10 (mouse; Upstate Biotechnology, Lake Placid, NY), rabbit anti-phospho-ERK 1/2 (New England Biolabs, Beverly, MA), rabbit anti-phospho-Lyn, goat anti-ERK1, and rabbit anti-Lyn (Santa Cruz Biotechnology, Santa Cruz, CA). The F(ab) fragment of L243 was prepared using a commercially available kit (Pierce). The secondary Abs included donkey anti-goat IgG coupled to HRP, goat anti-mouse IgG coupled to HRP, goat anti-rabbit IgG (Pierce). The secondary Abs included donkey anti-goat IgG coupled to HRP, goat anti-mouse IgG coupled to HRP, and goat anti-rabbit IgG coupled to HRP (Santa Cruz Biotechnology, Santa Cruz, CA). Reconstituted staphylococcal enterotoxin A (SEA) and Mycoplasma arthritidis derived monomeric protein (MAM; ATCC, Manassas, VA) were purchased from ATCC. THP-1 cells (5 × 10⁵ cells/ml) were incubated for 15 min at 37 °C in serum-free medium before stimulation. Stimulations were carried out as indicated in the figure legends and stopped by adding an equal volume of 2 × modified Laemmli sample buffer (29) preheated to 95 °C. Total cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% milk powder in TNE and incubated for 5 min at 37 °C with appropriate primary Ab and 0.15% Tween 20 in TBS, membranes were immunoblotted with anti- phospho-Lyn antibody mAb 4G10 and subjected to chemiluminescent detection with HRP-conjugated anti-mouse IgG Ab as described above. After stripping, the membranes were sequentially reprobed with anti- phospho-ERK 1/2, anti-ERK1, anti-phospho-Lyn, and anti-Lyn Abs. To detect the GM1 raft marker, 10-μl aliquots of each fraction were dotted on a polyvinylidene difluoride membrane, which was then incubated with CTB-HRP and developed with chemiluminescent reagents as described above.

Tyrosine Phosphorylation and Lyn and ERK1/2 Activation by Immunoblots—THP-1 cells (10⁵ cells/ml) were incubated for 15 min at 37 °C in serum-free medium before stimulation. Stimulations were carried out as indicated in the figure legends and stopped by adding an equal volume of 2 × modified Laemmli sample buffer (29) preheated to 95 °C. Total cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% milk powder in TNE and incubated for 5 min at 37 °C with appropriate primary Ab and 0.15% Tween 20 in TBS, membranes were immunoblotted with anti- phospho-Lyn antibody mAb 4G10 and subjected to chemiluminescent detection with HRP-conjugated anti-mouse IgG Ab as described above. After stripping, the membranes were sequentially reprobed with anti- phospho-ERK 1/2, anti-ERK1, anti-phospho-Lyn, and anti-Lyn Abs.

Measurement of Fluorescence—Cells were stimulated with FITC-labeled L243 mAb or FITC-labeled isotype control mAb (8C12) for 5 min. Washed cells were lysed and fractionated on a sucrose gradient as described above. Fractions 3, 4, and 5 (raft) and fractions 9, 10, and 11 (soluble) were pooled. FITC-labeled L243 and FITC-labeled 8C12 were quantified in the two pools using a microplate fluorescence reader (BIO-TEX, FL600). The results were normalized with respect to the isotype control and expressed as percentages.

Disruption of Lipid Rafts—To alter the cholesterol content of the plasma membrane, cells were washed three times in serum-free RPMI 1640 and resuspended at a concentration of 10⁶ cells/ml in serum-free RPMI 1640 containing 10 mM MβCD for 30 min at 37 °C. After extensive washing at room temperature, the cells were stimulated with either isotype-matched control mAb 8C12 or mAb L243 at a concentration of 0.5 μg/10⁶ cells for 5 min at 37 °C. To reverse the effect of cholesterol depletion, MβCD-treated cells were incubated at 37 °C for 2 h in RPMI 1640 containing 50 μg/ml of cholesterol water-soluble.

Cell-Cell Adhesion Assays—FITC-transfected THP-1 cells were re-suspended in RPMI 1640 containing 5% fetal bovine serum and seeded in 96-well microtiter plates at 1 × 10⁵ cells/well. Cells were stimulated at 37 °C for 1 h with mAb W6/32, mAb L243, or the F(ab) fragment of L243 at 1 μg/ml. The cells were pretreated with pertussin (5 μg/ml) or FP2 (10 μl) at 1 h at 37 °C with or without MβCD for 30 min at 37 °C in serum-free RPMI 1640 when indicated. Cell-cell adhesion was detected using a light microscope, and photographed.

RESULTS

HLA-DR Molecules Are Constitutively Present in Lipid Rafts of Human B Cells and Monocytic Cells—To determine whether HLA-DR molecules were present in lipid rafts in APCs and to study the role of their presence in HLA-DR-induced responses, we first examined the baseline distribution of these molecules in normal human B cells using sucrose gradient fractions derived from 1% Triton X-100 lysates. HLA-DR mole-
The presence of EBV proteins in human B cell lines does not interfere with constitutive residency. A, human tonsillar B cells were lysed in 1% Triton X-100 for 30 min on ice and then subjected to sucrose density gradient ultracentrifugation. Fractions were collected beginning from the top of the centrifuge tube. Samples from $8 \times 10^6$ (fractions 1–7) and $2 \times 10^6$ (fractions 8–11) cell equivalents were resolved by SDS-PAGE and analyzed by Western blot using anti-HLA-DR α chain mAb DA6.147. The membrane was then stripped and reprobed with a primary antibody to reveal CD45. Aliquots (10 μl) of each fraction were dot-spotted, and the lipid raft marker GM1 ganglioside was detected using HRP-conjugated CTB. The relative positions of the raft and soluble fractions are indicated based on the distribution of GM1 ganglioside and CD45. B, human EBV-negative and -positive BJAB cells, and C, human EBV-negative and -positive BL28 cells were fractionated as described above. Raft (fractions 2–4) and soluble protein (fractions 9–11) samples from $2 \times 10^6$ and $5 \times 10^5$ cell equivalents, respectively, were resolved by SDS-PAGE and analyzed by immunoblot using anti-HLA-DR α chain mAb DA6.147.

Molecules were mainly detected in soluble fractions (Fig. 1A), ~2% were found in the lipid rafts of resting human tonsillar B cells as determined by quantitative densitometry. GM1 ganglioside, a well-known constituent of lipid rafts, was recovered in fractions 2–4, whereas CD45 phosphatase was found exclusively in fractions 9–11, indicating that our conditions were appropriate for separating raft and non-raft membrane fractions. When Triton X-100 was titrated or other detergents were used such as CHAPS, Brij 58, and Nonidet P-40, we found that 1% Triton X-100 is the only condition that allow us to isolate GM1 without any detectable levels of CD45 molecules (data not shown). Based on these results, 1% Triton X-100 cell lysates were used in all the following experiments.

Because it is well established that MHC class II molecules and BCR signaling share numerous similarities (30), and the presence of LMP2A in the lipid rafts of EBV-immortalized human B cell lines has recently been shown to affect the distribution of BCR (31), we examined the effect of the EBV transformation on the distribution of HLA-DR molecules. To this end, we used two B cell lines (BL28 and BJAB) and their EBV-transformed counterparts. Fig. 1B and C shows that 4% (BJAB) to 5% (BL28) of HLA-DR molecules were found in the fractions corresponding to lipid rafts, and the presence of EBV gene products did not affect the partitioning of HLA-DR molecules.

We next analyzed the localization of HLA-DR molecules in normal human monocytes and in a human THP-1 monocytic cell line. Monocytes were purified from peripheral blood mononuclear cell by adherence to plastic Petri dishes precoated with heat-inactivated autologous plasma. The THP-1 cells, which are known to express very low levels of HLA-DR molecules, were treated with IFN-γ for 48 h to induce high levels of HLA-DR expression and to allow them to respond to HLA-DR ligation in a manner similar to that observed in human monocytes. Western blot analyses (Fig. 2, A and B) revealed the presence of HLA-DR molecules in the lipid raft fractions of human monocytes (3%) and IFN-γ-treated THP-1 cells (3.5%). Although the purity of the monocytes used exceeded 90% (OKM1-positive) and no CD20-positive cells were observed in the preparation (data not shown), a possible contribution to the response by contaminating B cells could not be ruled out. Indeed, it could be argued that the presence of HLA-DR molecules in lipid rafts might result from the priming effect of IFN-γ and/or cell activation that occurred during purification process. To overcome this problem, we generated stable THP-1 cells transfected with the human MHC class II transactivator gene CIITA and tested the distribution of HLA-DR molecules. Quantitative densitometry of the immunoblots (Fig. 2C) revealed that significant amounts of HLA-DR molecules were constitutively associated with the lipid rafts of these CIITA-transfected cells (7.5%).

Our results indicate that all the cell types tested constitutively express significant amounts of HLA-DR molecules in the lipid rafts, that the proportion of these molecules does not show gross variations among the cell types, and that the expression of EBV proteins in B cell lines failed to affect the constitutive localization of the HLA-DR molecules.

**Ligation of HLA-DR Molecules with a Bivalent Ligand Failed to Induce Recruitment of HLA-DR Molecules into Lipid Rafts**—We next looked at whether the distribution of HLA-DR molecules would change following ligation with specific Abs, with SEA, or with MAM. SEA and MAM are members of the SAg family that are known to bear two MHC class II binding sites, the first of which interacts with the α chain and the second, with the β chain of another MHC class II molecule (32, 33). CIITA-transfected cells were stimulated for 5 min at 37 °C with 8C12 isotype-matched control mAb, anti-HLA-DR mAb L243, SEA, or MAM. Cell lysates were prepared and fractionated as described above for the analysis of HLA-DR partitioning.

**Fig. 1.** HLA-DR molecules are constitutively present in lipid rafts of human tonsillar B cells. A, normal human monocytes; B, IFN-γ-treated THP-1 cells or C, CIITA-transfected THP-1 cells were fractionated as described in the legend to Fig. 1. Samples from $2 \times 10^6$ (fractions 1–7) and $5 \times 10^5$ (fractions 8–11) cell equivalents for THP-1 cells and $8 \times 10^5$ and $2 \times 10^6$ cell equivalents for monocytes were resolved by SDS-PAGE and analyzed by immunoblot using anti-HLA-DR α chain mAb DA6.147.

**Fig. 2.** HLA-DR molecules are constitutively present in lipid rafts in purified human monocytes and human monocytic cell lines. A, normal human monocytes; B, IFN-γ-treated THP-1 cells or C, CIITA-transfected THP-1 cells were fractionated as described in the legend to Fig. 1. Samples from $2 \times 10^6$ (fractions 1–7) and $5 \times 10^5$ (fractions 8–11) cell equivalents for THP-1 cells and $8 \times 10^5$ and $2 \times 10^6$ cell equivalents for monocytes were resolved by SDS-PAGE and analyzed by immunoblot using anti-HLA-DR α chain mAb DA6.147.
Fig. 3. Ligation of HLA-DR molecules with a bivalent mAb L243 failed to induce additional recruitment of HLA-DR molecules into rafts. A, CIITA-transfected THP-1 cells were stimulated for 5 min at 37°C with: (i) 8C12 isotype-control mAb or anti-HLA-DR mAb L243 (0.5 μg/10⁶ cells), or (ii) SEA or MAM at a concentration of 1 μg/10⁶ cells. Cells were then lysed in 1% Triton X-100 and fractionated as described in the legend to Fig. 1. Raft (fractions 2–4) and soluble protein (fractions 9–11) samples from 2 × 10⁶ and 5 × 10⁵ cell equivalents, respectively, were resolved by SDS-PAGE, and analyzed by immunoblot using anti-HLA-DR mAb L243. C, disruption of lipid rafts by MβCD treatment abolished the association of HLA-DR molecules with membrane rafts. Cells pretreated or not with 10 mM MβCD for 30 min at 37°C were stimulated as above. After lysis in 1% Triton X-100 and fractionation, samples of pooled raft and pooled soluble fractions from 2 × 10⁶ and 5 × 10⁵ cell equivalents, respectively, were subjected to SDS-PAGE and immunoblot analysis.

Having determined that HLA-DR was constitutively present in the lipid rafts of CIITA-transfected THP-1 cells and that anti-HLA-DR mAb failed to induce further HLA-DR recruitment, we extended our analysis to confirm the specificity of this interaction. Under these conditions, there was no additional recruitment of HLA-DR molecules into the raft compartment when CIITA-transfected THP-1 cells were stimulated with anti-HLA-DR (Fig. 3A) (7.5% for isotype control versus 8% for L243). In contrast, stimulation with SEA or MAM (Fig. 3B) led to a slight but reproducible increase in HLA-DR in the lipid rafts (10% for SEA and 11% for MAM). Similar results were obtained with IFN-γ-treated THP-1 and B cell lines (data not shown).

Having determined that HLA-DR was constitutively present in the lipid rafts of CIITA-transfected THP-1 cells and that anti-HLA-DR mAb failed to induce further HLA-DR recruitment, we extended our analysis to confirm the specificity of this localization. Cells were left untreated or were treated with MβCD, a drug that disrupts rafts by extracting cholesterol (34). Washed cells were then stimulated for 5 min at 37°C with isotype-matched control mAb 8C12 or anti-HLA-DR mAb L243. Cell lysates were fractionated, pooled as indicated in the figure legend, and analyzed for HLA-DR partitioning by immunoblotting. Treatment with MβCD did not affect the interaction of HLA-DR with the specific Abs as determined by fluorescence-activated cell sorting (FACS) analysis, and cell viability was not compromised as measured by trypan-blue exclusion (data not shown). Fig. 3C shows that MβCD treatment led to the complete disappearance of HLA-DR molecules from these microdomains of both stimulated and unstimulated cells.

MβCD Inhibits HLA-DR Dimerization-mediated Proximal Tyrosine Phosphorylation Events but Not ERK Activation—As mentioned above, Huby et al. (23) reported that oligomerization of HLA-DR molecules by cross-linking with primary and secondary Abs leads to HLA-DR recruitment into the lipid rafts of human IFN-γ-treated THP-1 monocytic cells, an event that seems to be required for PTK activation. On the other hand, Mehindate et al. (8, 9, 35) demonstrated that dimerization of HLA-DR molecules by SAgs is sufficient for various HLA-DR-induced cellular events to occur, particularly PTK-dependent cytokine expression. We therefore hypothesized that while most HLA-DR-induced events require HLA-DR dimerization, some are raft-dependent while others are raft-independent, and none require an additional recruitment of HLA-DR molecules into lipid rafts.

To investigate this possibility, we stimulated CIITA-transfected THP-1 cells with isotype-matched control mAb, mAb L243, and the F(ab) fragment of L243. Total cell lysates were resolved by SDS-PAGE and analyzed by immunoblot using anti-phosphotyrosine mAb 4G10. We detected tyrosine phosphorylation of several proteins ranging in molecular mass from 50 to 150 kDa within 1 min in L243-stimulated samples (data not shown). Maximum phosphorylation occurred at 5 min and persisted at least for 15 min (Fig. 4A). In contrast, stimulation with the F(ab) fragment of L243, the isotype-matched control mAb, or the PMA induced no detectable tyrosine phosphoryla-
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The detection of heavily phosphorylated proteins (~53–55 kDa) migrating in samples stimulated with the anti-HLA-DR mAb suggested that they might be members of the Src family of kinases, in particular Lyn. To test this possibility, antibodies directed against the phosphorylated and total forms of Lyn were used. Fig. 4B shows that only the bivalent anti-HLA-DR mAb triggered the phosphorylation of Lyn, a response that was detected after 1 min (data not shown) and persisted for at least 15 min.

Our findings suggest that tyrosine phosphorylation is probably the first step in HLA-DR-induced signaling cascades and that the proximal pTyr events associated with ligation of HLA-DR molecules are critically dependent on their dimerization.

We conducted additional experiments to identify other signaling pathways induced by the ligation of HLA-DR molecules. To that end, we analyzed the phosphorylation status of two members of the MAPK family (ERK1/2 and p38), each requiring phosphorylation at tyrosine and threonine residues for activation (36). CIITA-transfected THP-1 cells were stimulated as above, and cell lysates were sequentially immunoblotted with the phosphorylated ERK1/2 and ERK1 Abs. ERK1/2 activation was observed after 5 min of stimulation with mAb L243 and persisted up to 15 min (Fig. 4C). Once again, dimerization of HLA-DR molecules was required to trigger the phosphorylation of ERK1/2. We detected no activation of the p38 MAPK under our conditions (data not shown). Stimulation with PMA-induced ERK1/2 activation (Fig. 4C), but had no effect on p38 (data not shown).

Having confirmed the requirement of HLA-DR dimerization for the signaling events described above, we then investigated the role of raft integrity in these responses. We stimulated MβCD-treated and untreated CIITA-transfected THP-1 cells for 5 min at 37 °C with 8C12 control mAb and mAb L243. Fig. 5A shows that treatment with MβCD led to a significant inhibition of HLA-DR-induced tyrosine phosphorylation in total cell lysates. Because Lyn is enriched in lipid rafts (16, 19) and Lyn phosphorylation is triggered by the anti-HLA-DR mAb, we then examined the phosphorylation status of Lyn. The increased phosphorylation of Lyn resulting from the stimulation by the anti-HLA-DR mAb was also inhibited following pretreatment of cells with MβCD (Fig. 5B). In contrast, HLA-DR-mediated activation of pERK1/2 was unaffected by MβCD (Fig. 5C).

To further confirm that the presence of HLA-DR molecules in lipid rafts was crucial for their capacity to initiate tyrosine phosphorylation, especially of Lyn, we pretreated CIITA-transfected THP-1 cells with MβCD before stimulation and fractionation on a sucrose gradient. Although most tyrosine-phosphorylated proteins following L243 mAb stimulation were recovered in the soluble fractions, significant tyrosine phosphorylation was also observed in the lipid raft fractions (Fig. 6A). Tyrosine phosphorylation events in rafts were undetectable after the MβCD treatment, whereas phosphorylation in soluble fractions was markedly inhibited. As expected, the phosphorylation of Lyn in rafts was abolished following raft disruption (Fig. 6B). However, ERK1/2 were not present in the lipid rafts and their activation by mAb L243, as measured by immunoblotting of their phosphorylated forms, was not affected by the MβCD treatment (Fig. 6C).

Taken together the above results indicate that although lipid rafts act as a platform for HLA-DR-induced PTK and Lyn activation, they are not involved in the activation of ERK pathway, which is a strong support for the involvement of two separate HLA-DR signaling pathways in APCs. To further confirm this hypothesis, and to demonstrate that ERK activation is independent of Lyn function, the effects of PP2 and PP1 on HLA-DR-induced ERK activation was analyzed. Cells were pretreated with PP2 or PP1 for 30 min prior to HLA-DR stimulation. Cells were then lysated and analyzed by Western blot for PTK and ERK activation. Fig. 7 shows that although tyrosine phosphorylation is completely blocked by treatment with both inhibitors, activation of ERK is not affected by this treatment.

**The Cytosplasmic Domain of Both α and β Chains Is Not Required for HLA-DR Association with Lipid Rafts**—The results presented above indicate that HLA-DR-mediated tyrosine phosphorylation requires raft integrity while ERK1/2 activation does not. Based on these observations and studies showing that the deletion of the cytoplasmic domains of both the α and β chains of HLA-DR does not affect HLA-DR-induced tyrosine phosphorylation (37), we hypothesized that the cytoplasmic domains are not involved in the localization of HLA-DR molecules in the lipid rafts. To verify this hypothesis, HeLa cells transfected with wild-type HLA-DR or truncated HLA-DR (cytoplasmic domains of both the α and β chains deleted) and that expressed comparable levels of these molecules at the cell surface were used (Fig. 8A). Fractions of Triton X-100 cell lysates were isolated by sucrose gradient and analyzed by Western blot...
Fig. 6. MβCD abolishes the association of HLA-DR molecules with membrane rafts, which results in the inhibition of their ability to induce protein tyrosine phosphorylation and Lyn phosphorylation in a CIITA-transfected THP-1 monocytic cell line. Cells were pretreated or not with 10 mM MβCD for 30 min at 37 °C, then stimulated with the isotype-control mAb or the anti-DR mAb for 5 min at 37 °C. After lysis in 1% Triton X-100 and fractionation, samples of pooled raft and pooled soluble fractions from 5 × 10⁶ and 1 × 10⁵ cell equivalents (A and B) or 2 × 10⁶ and 5 × 10⁵ cell equivalents (C), respectively, were subjected to sequential Western blot analysis as described in the legend to Fig. 5.

Fig. 7. The Src family kinase inhibitors PP1 and PP2 inhibit HLA-DR-induced tyrosine phosphorylation but fail to affect activation of ERK1/2 in CIITA-transfected THP-1 monocytic cell line. Cells were either untreated or pretreated with 10 μM PP1 or PP2 for 30 min at 37 °C and then stimulated for 15 min at 37 °C with 8C12 isotype control mAb or anti-DR mAb L243. Total cell lysates from 5 × 10⁶ cell equivalents were subjected to Western blot analysis with Abs specific for either: A, pTyr; B, p-ERK1/2 and ERK1.

Fig. 8. The cytoplasmic domains of the α and β chains of HLA-DR are not required for HLA-DR association with lipid rafts. A, analysis of the expression of HLA-DR in transfected HeLa cells by flow cytometry. B, transfected-HeLa cells were lysed in 1% Triton X-100 for 30 min on ice, subjected to sucrose density gradient ultracentrifugation and fractionated as above. Samples from 7 × 10⁶ (fractions 2–4) and 2 × 10⁶ (fractions 9–11) cell equivalents were resolved by SDS-PAGE and analyzed by Western blot using anti-HLA-DR α chain mAb DA6.147 or XD5 for the HLA-DR β chain.

for 1 h led to cell-cell adhesion (Fig. 9). However, the F(ab) fragment of L243 and anti-MHC class I mAb failed to induce any detectable response. Pretreatment of CIITA-transfected THP-1 cells with MβCD abolished HLA-DR-induced cell-cell adhesion, indicating that lipid raft integrity and HLA-DR dimerization is essential for the HLA-DR-induced response. We then attempted to reverse the inhibitory effect by adding cholesterol. MβCD-treated cells were incubated in RPMI 1640 containing 50 μg/ml of cholesterol for 2 h and then stimulated with L243 or W6/32. Our results clearly demonstrate that such a treatment reverses the inhibitory effect induced by MβCD treatment.

To further support our conclusion, we treated cells with the
After extensive washing, the cells were resuspended in RPMI 1640 containing 5% fetal bovine serum and seeded in 96-well microtiter plates at 1 × 10⁴ cells/well. The cells were then stimulated for 37 °C with mAb W6/32, mAb L243, or the F(ab) fragment of L243 at 1 μg/ml. To restore the integrity of rafts, MβCD-treated cells were resuspended in RPMI 1640 containing 50 μg/ml of cholesterol water-soluble (cholesterol) and incubated for 2 h at 37 °C. Cells were then stimulated at 37 °C for 1 h with mAb W6/32, mAb L243, or the F(ab) fragment of L243 at 1 μg/ml as described. Cell aggregation was monitored using a light microscope, and photographs were taken using a camera coupled to the microscope.

**DISCUSSION**

Cholesterol/sphingolipid-rich plasma membrane microdomains, commonly called lipid rafts, are known to play a pivotal regulatory role in various cellular processes, including membrane trafficking and signal transduction initiation pathways (17). Although their role in BCR and TCR signaling is well established, investigation of their possible involvement in MHC class II-induced signal transduction pathways is just beginning. Anderson et al. (22) reported that significant amounts of MHC class II molecules (20−50%) are constitutively present in the lipid rafts of murine and human B cell lines. In contrast, Huby et al. (23) failed to detect any MHC class II molecules in the lipid rafts of a human IFN-γ-treated THP-1 monocytic cell line. Data obtained in the course of the present investigation showed that small but significant amounts (3−8%) of HLA-DR molecules were constitutively present in the lipid rafts of various cell types. This is consistent with the results reported by Kropshofer et al. (42), who showed that ∼3% of HLA-DR molecules are constitutively located in the lipid rafts of human B cell lines and dendritic cells. The variability in the amount of HLA-DR molecules in the lipid rafts reported in these various studies is likely to be due to the cholesterol and/or sphingolipid content of the cell culture medium as reported in another cell system (43), and to different detergents and/or concentration of the used detergents.

The constitutive localization of HLA-DR in the lipid rafts is not limited to B and monocytic cell lines. These molecules have also been detected in the lipid rafts of both normal human tonsillar B cells and monocytes. Although MHC class II molecules and BCR share numerous similarities (30), and the presence of EBV viral proteins is reported to block the translocation of BCR into rafts, the localization of HLA-DR molecules in these domains is unaffected by the EBV transformation (31). However, like BCR (21), HLA-DR localization in rafts was unaffected by the deletion of the cytoplasmic tails of the α and β chains.

All the HLA-DR-induced events studied required HLA-DR dimerization on the cell surface. None was dependent on the recruitment of additional HLA-DR molecules, indicating that the small amount of HLA-DR constitutively present in the lipid rafts of CIITA-transfected THP-1 cells was sufficient for various cellular events. This was strongly supported by our results showing that ligation of HLA-DR with a bivalent ligand failed to induce additional recruitment of HLA-DR molecules into raft microdomains but induced rapid protein tyrosine phosphorylation of many substrates, including Lyn, a member of the Src family tyrosine kinase (16). However, we cannot rule out that additional recruitment of HLA-DR molecules into lipid rafts can increase the HLA-DR-induced response, and such recruitment can be required if the studied cells express low levels of HLA-DR on their surfaces.

The slight but reproducible increase of HLA-DR recruitment
observed with SEA and MAM can be explained by the ability of these two SAgs to oligomerize HLA-DR molecules on cell surface (33, 44). Upon binding to HLA-DR molecules, these two SAgs are capable of inducing additional recruitment of HLA-DR, which strongly supports the observation by Huby et al. (23) of HLA-DR translocation using a combination of primary and secondary antibodies.

The induction of protein tyrosine phosphorylation following HLA-DR dimerization was found to be critically dependent on raft integrity since disruption by MβCD resulted in the loss of HLA-DR molecules from the raft compartment and the complete abrogation of phosphorylation of pTyr and Lyn. One of the consequences of pTyr and Lyn activation was the induction of cell-cell adhesion, an event that was critically dependent on the dimerization of HLA-DR molecules and their presence in lipid rafts. These results indicate that membrane rafts provide a microenvironment crucial for proximal HLA-DR-mediated pTyr events. In fact, these events, particularly Lyn activation, must occur in membrane rafts to allow the proper association of HLA-DR molecules with components that are essential for transducing signals initiated by HLA-DR given that these molecules are devoid of classic signaling motifs (15). What then is the mechanism involved in the induction of PTK and Lyn activation? It is possible that the dimerization of HLA-DR molecules promotes the formation of an immune signaling complex involving HLA-DR, co-receptors, adaptor molecules, and Src family tyrosine kinases. This complex would then be able to transactivate raft-associated tyrosine kinases, thereby initiating signal transduction cascades. The high degree of transspecies conservation of the transmembrane sequences of the α/β chains of MHC class II molecules and the dependence on intact transmembrane domains for proper MHC class II-mediated tyrosine phosphorylation (12, 13, 15, 37) point to interactions between HLA-DR and partner molecules. This is strongly supported by studies demonstrating, at least in B cells, the existence of a physical association between MHC class II molecules and cell surface receptors, including CD20, CD40, CD19, CD79a/CD79b, and several members of the tetraspan family such as CD81 and 82. The role of the CD79a/CD79b association in MHC class II-induced signaling has been elegantly demonstrated in murine B cells (30), and the involvement of CD19 and CD20 has been demonstrated in HLA-DR-induced PTK activation in human B cells (45, 46). Since none of these molecules is expressed in monocytes and monocytic cell lines, studies are currently underway to determine whether HLA-DR molecules in human monocytes exist in association with other cell surface molecules and whether HLA-DR molecules are directly or indirectly associated with signaling partners.

In addition to the activation of PTK signaling pathways, the ligation of HLA-DR molecules with bivalent mAb L243 resulted in the activation of ERK 1/2, but not p38. This pathway was delayed compared with the PTK pathway (maximum at 15 min versus 5 min). The HLA-DR-mediated activation of ERK1/2 and p38 has been reported in human monocytes (47) using solid-phase mAb L243, which is equivalent to a high order of cross-linking. The ERK1/2 pathway was probably a parallel signal transduction pathway in our model since raft disruption had no impact on its activation. The dimerization of HLA-DR molecules by a bivalent ligand was necessary and sufficient to initiate ERK1/2 activation despite the disruption of raft integrity, indicating that the dimerization of non-raft HLA-DR molecules might be sufficient to trigger certain cellular events. These results point to a separate HLA-DR signaling pathways in antigen presenting cells, as previously proposed (12, 13). This possibility is strongly supported by the results showing that the cytoplasmic domains of the α and β chains are not required for HLA-DR localization in lipid rafts.

Anderson et al. (22) reported that efficient antigen presentation at low ligand densities is another important function of the enrichment of MHC class II molecules in the lipid rafts of murine and human B cell lines. Watts (48) used MHC class II molecules immobilized on planar membranes to demonstrate that individual MHC class II-peptide complexes must be less than 20-nm apart for proper T cell activation. Both studies highlight the importance of the localization and concentration of MHC class II molecules for antigen presentation. Given the relatively small number of MHC-peptide complexes required to activate T cells (49), our results with monocytes suggest that it is not merely the number of MHC class II molecules in individual rafts but rather their spatial organization that determines T cell activation potential. Dimerization and/or oligomerization MHC class II molecules at the cell surface might allow cross-activation, thereby circumventing the need for large numbers of MHC molecules at the site of the immunological synapse.

Since bivalent but not monovalent SAgs are able to induce cytokine gene expression (8, 9), it will be interesting to determine the roles played by lipid rafts in this event. If lipid rafts are indeed involved, it will reinforce the notion that they are of immunological relevance for APCs. Studies are currently underway to address this question and to determine the regions as well as the HLA-DR residues involved in the constitutive and/or additional recruitment of HLA-DR into these microdomains.

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