Intracellular Phosphotyrosine Induction by Major Histocompatibility Complex II Requires Co-aggregation with Membrane Rafts

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Cross-linking MHC class II molecules human leukocyte antigen (HLA-DR) on the surface of THP-1 cells was found to induce their entry into the glycolipid-enriched membrane fraction of the plasma membrane. At the cellular level, this resulted in the synergistic co-aggregation of class II with cholera toxin, a marker of membrane rafts. The accompanying induction of intracellular protein tyrosine phosphorylation could be inhibited by treating cells with methyl-β-cyclodextrin, a drug that chelates membrane cholesterol and thereby disperses membrane rafts. Signaling could also be inhibited by treating cells with the Src-family kinase inhibitor PPI. Together, these results show that the induced association of class II molecules with membrane rafts can contribute to their aggregation on the cell surface and mediate an association with intracellular protein-tyrosine kinases.

The plasma membrane is known to contain distinct microdomains, which are enriched in glycosphingolipids, cholesterol, and specific membrane proteins (1, 2). Often referred to as membrane rafts, these domains can be distinguished from the rest of the plasma membrane by their relative insolubility in detergents and low buoyant density (3). They have been implicated in many cellular processes including membrane sorting in polarized cells (3–6), endocytosis (7, 8) and signal transduction from cell surface receptors (9–15).

Different membrane-associated proteins can partition into the raft fraction to varying degrees; for example, the FcR I is highly enriched within rafts (11, 16), whereas CD45 is excluded from them (12). This partitioning can profoundly affect protein function, as has been demonstrated using site-directed mutagenesis to relocate the raft-associated signaling proteins Lck (9) and LAT (14) within the fluid phase of the plasma membrane. Both of these molecules are targeted to membrane rafts by palmitoylation, a post-translational modification that is a good predictor of such targeting. To date, however, it has not proved possible to predict the partitioning characteristics of transmembrane proteins based on their primary sequence alone. It was therefore of interest to determine experimentally the degree to which major histocompatibility complex (MHC) class II molecules partition into membrane rafts and the influence such partitioning has on class II function.

MHC class II molecules are heterodimers composed of α and β polypeptides, both of which span the plasma membrane and have short cytoplasmic tails. High levels of class II are expressed on professional antigen-presenting cells, where they present processed exogenous antigen to CD4+ T cells. When recognition of the MHC-peptide complex by a specific T cell receptor (TCR) occurs, intracellular signals, including the induction of protein tyrosine phosphorylation, are transduced in the T cell through the TCR (17) and in the antigen-presenting cells through the MHC class II molecules (18–22). MHC signaling has pleiotropic effects on antigen-presenting cell function, affecting antigen presentation (23), adhesion (24–26), proliferation (19), apoptosis (27, 28), and cytokine release (29–32). The mechanism whereby class II initiates phosphotyrosine induction is currently unclear; surprisingly, the cytoplasmic tails of the class II polypeptides do not associate with any detectable tyrosine kinases (33), and the tails are in any case dispensable for the induction of tyrosine phosphorylation (22, 34, 35). Such signaling by class II is, however, critically dependent on the sequence of the highly conserved transmembrane regions of both the α and β polypeptides (33, 34). This suggests that interactions between class II molecules and components of the plasma membrane are likely to be important for signaling.

A precedent for intracellular signaling by proteins naturally lacking a cytoplasmic tail is afforded by glycosphosphatidylinositol-linked surface molecules. Signaling by these molecules has been found to be dependent upon the integrity of membrane rafts (13, 36–38), to which they are targeted by means of the glycosphosphatidylinositol tail (1). Signaling is thought to be dependent upon the aggregation of membrane rafts that accompany cross-linking of such raft-associated molecules. It is suggested that such aggregation facilitates the transactivation of raft-associated protein-tyrosine kinases and, hence, the initiation of intracellular signaling cascades (39). This led us to investigate whether membrane rafts play a role in the aggregation of MHC class II molecules and in facilitating an association protein tyrosine kinases.

EXPERIMENTAL PROCEDURES

Cell Lines—The human myelomonocytic cell line THP-1 was cultured in RPMI 1640 containing 5% fetal calf serum and antibiotics. To increase surface HLA-DR expression as required, cells at 106 ml−1 were cultured with human interferon (IFN-γ (Genzyme, Cambridge, MA). Antibodies and Chemicals—Antibodies L243, CR3/43, and T436, which recognize HLA-DR, were prepared as hybridoma supernatants. CR3/43 was affinity-purified using protein A-Sepharose from Amersham Pharmacia Biotec. 4G10, which recognizes phosphotyrosyl residues within peptides, was purchased from Upstate Biochemical Inc, Lake Placid, NY. HI30, which recognizes CD45, was from Pharmingen, San Diego, CA. Anti-caveolin was from Transduction Laboratories, Lexington, KY. Anti-Lyn rabbit antiserum was a gift of V. Tybulewicz (National Institute for Medical Research, UK). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies for Western blotting were from Pierce. Texas Red conjugated goat anti-mouse and goat
anti-rabbit antibodies for immunofluorescence staining were from Jackson Laboratories, Maine, PA. R-Phycocerythrin-conjugated rabbit anti-mouse antiserum, fluorescein isothiocyanate (FITC)-conjugated cholera toxin B subunit, methyl-β-cyclodextrin (MβCD), and cholesterol-loaded methyl-β-cyclodextrin were from Sigma Aldrich. 4-Amino-5-(chloro-phenyl)-7-(4-hexyl)-pyrazolo[3,4-d]pyrimidine (PP1) was a gift from Y. Xu and R. Munshauer (BASF, Bioresearch Corp., Worcester, MA).

Cell Treatment and Stimulation—To alter the cholesterol content of the plasma membrane, cells were washed three times in RPMI and resuspended at 10^5 cells ml^-1 in the appropriate mix of 10 mM MβCD/cholesterol-loaded methyl-β-cyclodextrin for 30 min at 37 °C. The "percent cholesterol" refers to the percent cholesterol-loaded methyl-β-cyclodextrin in the mix. Cell were then washed into ice-cold RPMI. To stimulate cells, they were resuspended in L243 or CR3/43 supernatant or HI30 at 2 μg ml^-1 in RPMI containing 5% fetal calf serum for 15 min on ice. Controls were resuspended in RPMI containing 5% fetal calf serum or 2 μg ml^-1 isotype-matched control antibody. Cells were then washed in cold RPMI, resuspended in rabbit anti-mouse antiserum for 5 min at 37 °C, and centrifuged at 15,000 × g for 30 s before lysis. 

Stimulation by pervanadate was carried out for 5 min at 37 °C by adding 100 μM stock solution created 15 min beforehand by mixing 50 mM Na3VO4 and 100 mM H2O2 in water. P1P stock at 10 μM in Me2SO was used at a final concentration of 10 μM for 30 min at 37 °C to inhibit Src-family kinases.

Sucrose Gradients and Western Blotting—For analysis of whole cell protein, cells were lysed on ice for 15 min in a standard lysis buffer (25 mM Tris-HCl, pH 6.5, 150 mM NaCl, 100 mM Na3VO4 and 1 μg ml^-1 each of chymostatin, leupeptin, and pepstatin) containing 1% Nonidet P-40 and cleared of insoluble matter by centrifugation at 20,000 × g for 30 min. For the enrichment of glycolipid-enriched membrane fractions (GEMs), cells were lysed in MNE buffer (25 mM MES, pH 6.5, 150 mM NaCl, 100 mM Na3VO4, and 1 μg ml^-1 each of chymostatin, leupeptin, and pepstatin) containing 1% Triton X-100 for 30 min on ice. Lysates were mixed with an equal volume of 80% sucrose in MNE, placed at the bottom of a 40%, 30%, 5% discontinuous sucrose gradient, and centrifuged at 100,000 × g for 1 h. The insoluble GEM fraction was recovered from the 30%, 5% interface, mixed with an equal volume of MNE buffer, and pelleted at 20,000 × g for 30 min. GEM pellets were resuspended in Laemmli buffer including 5% β-mercaptoethanol, and soluble proteins in the 40% sucrose layer were mixed with an equal volume of 2× Laemmli buffer. For detection of CD45, no β-mercaptoethanol was included. After heating to 95 °C for 5 min, samples were resolved by SDS-PAGE, and analyzed by standard Western blotting. Primary antibodies were used at 1 μg ml^-1 or 1/1,000 for antisera, and secondary antibodies were used at 1/25,000. Staining was developed using SuperSignal substrate from Pierce. For densitometric analysis, autoradios were scanned using a Sharp 330 flatbed scanner, and results were analyzed using SharpTwin 1-D analysis software.

Cell Staining and Confocal Microscopy—THP-1 cells were pretreated with 10 μM MβCD for 30 min at 37 °C as appropriate, washed, then stained with primary antibody (neat L243 supernatant, purified HI30 at 2 μg ml^-1 or isotype-matched antibody control) on ice for 15 min, washed once, stained with Texas Red conjugated goat anti-mouse antibody at 20 μg ml^-1 on ice for 5 min, then warmed to 37 °C for a further 5 min. Cells were then washed in cold RPMI and fixed for at least 4 h in 3.7% paraformaldehyde. Fixed cells adhered to 3-amino-propyltriethoxysilane (TESPA) (Sigma)-coated coverslips were stained as appropriate with cholera toxin B subunit conjugated to fluorescein isothiocyanate (CT-FITC) at 1 μg ml^-1 for 10 min before washing and mounting and viewed with an MRC 600 confocal microscope. For dual color analysis, cells were excited at 488 and 568 nm, with FITC (green) and Texas Red (red) fluorescence detected simultaneously. All images represent Z-series piles of 15–18 transverse sections acquired at 0.5-μm intervals, from the top of a cell to its center.

RESULTS

Cross-linking HLA-DR Induces Its entry into the Glycolipid-enriched Membrane Fraction—To test whether HLA-DR (MHC class II) is associated with the GEM fraction of the plasma membrane, THP-1 cells were treated with cross-linking antibodies as appropriate, lysed in MNE buffer containing 1% Triton X-100, and fractionated by discontinuous sucrose gradient centrifugation. Three discrete subcellular fractions were isolated: the GEMs from the 5%, 30% sucrose interface, soluble proteins from the 40% sucrose layer, and a pellet from the bottom of the tube. These fractions were analyzed by SDS-PAGE and Western blotting. HLA-DR molecules belonging to both the immature intracellular pool and the mature surface-bound pool were identified in the soluble fraction, the latter distinguished by their greater apparent molecular weight. No HLA-DR could be detected in the GEM fraction. However, when cells were treated with cross-linking anti-HLA-DR antibodies (either L243 or CR3/43 followed by a goat anti-mouse secondary antibody) before lysis, a significant fraction of the mature HLA-DR molecules were detected within the GEM fraction. (Fig. 1A). The immature, intracellular HLA-DR, being inaccessible to antibodies, showed no change in its distribution, although in some experiments a small fraction was constitutively associated with the GEM fraction, presumably reflecting an association with intracellular raft structures (3). HLA-DR could not be detected within the nuclear pellet under any circumstances (data not shown).

As a control, we determined the fate of the transmembrane molecule CD45 following its cross-linking on the surface of THP-1 cells. In contrast to HLA-DR, CD45 could not be detected in the GEM fraction either before or after cross-linking, even when gels were heavily overloaded (Fig. 1B). This demonstrates that cross-linking with antibody per se does not cause the indiscriminate entry of transmembrane proteins into the GEM fraction. Thus, cross-linking specifically enhances the partitioning of HLA-DR into GEMs. It is notable that, like other leukocytes (40), THP-1 cells expressed no detectable CD45 (data not shown). Thus caveolae, which can form when caveolin associates with membrane rafts (41), do not contribute to the GEMs isolated here (42).

Cross-linked HLA-DR Co-aggregates with Membrane Rafts—To test whether the association of HLA-DR with membrane rafts affects its distribution on the surface of intact cells, THP-1 cells were used that had been induced to express high levels of HLA-DR by exposure to IFN-γ for 48 h. First, membrane rafts were revealed by incubating paraformaldehyde-fixed cells with the CT-FITC. This binds to GM1, a glycolipid concentrated within membrane rafts (43). Because CT-FITC has pentamerenic valency for GM1 (44), it induced some aggregation of membrane rafts, giving a punctate staining pattern (Fig. 2a) similar to that reported elsewhere (39). Next, HLA-DR was cross-linked on cells before fixation using the primary antibody L243 and a Texas Red labeled goat anti-mouse sec-
ondary antibody. Large patches of HLA-DR formed within 5 min of exposure to the secondary antibody at 37 °C, which co-localized with CT-FITC (Fig. 2b). This indicated that most cross-linked HLA-DR was associated with membrane raft aggregates. These aggregates were larger than those seen on the surface of control cells, suggesting that association with cross-linked HLA-DR contributed to membrane raft aggregation.

As a control, the patching of CD45, shown above to be excluded from GEMs, was investigated. Typically of a surface-bound protein, CD45 also patched following cross-linking with antibodies. However, CD45 patches did not co-localize with CT-FITC, and their presence had no effect on the aggregation of membrane rafts (Fig. 2c).

To determine whether membrane rafts conversely contributed to the patching of HLA-DR, cells were pre-treated with MβCD, a drug that chelates cholesterol and thereby disrupts membrane rafts (1). This had little effect on the distribution of CT-FITC on control cells (Fig. 2d), suggesting that GM-1 can be cross-linked by CT-FITC irrespective of membrane rafts. Treatment with MβCD did, however, reduce the size of HLA-DR patches as well as the degree of co-patching with CT-FITC (Fig. 2e). This suggests that HLA-DR patches are qualitatively affected by their association with membrane raft aggregates. Consistently, the patching of CD45 was unaffected by MβCD (Fig. 2f).

**Intact Membrane Rafts Are Required for Protein Phosphotyrosine Induction by Cross-linked HLA-DR**—When IFN-γ-treated THP-1 cells were exposed to cross-linking anti-HLA-DR antibodies, tyrosine phosphorylation of multiple proteins was induced, which was maximal within 5–10 min. Similar results were seen using three different antibodies, L243, CR3/43, and TU36; isotype-matched control antibodies induced no detectable tyrosine phosphorylation (data not shown). Qualitatively similar, but stronger, signaling was seen when the primary antibodies were hyper-cross-linked with a goat-anti-mouse secondary antibody. As a standard, cells were therefore stimulated by exposure to L243 on ice for 15 min, washed once in RPMI, then exposed to goat anti-mouse antibody for 5 min at 37 °C (Fig. 3A).

To test whether phosphotyrosine induction by HLA-DR was dependent upon intact membrane rafts, cells were pretreated with MβCD to disperse the rafts. This inhibited the inducible tyrosine phosphorylation of all protein substrates detectable in total cell lysates by an average of 90% (Fig. 3, B and C). MβCD was not in itself toxic, since if it was partially loaded with cholesterol, its ability to inhibit phosphotyrosine induction was completely reversed (Fig. 3, B and C). Interestingly, excessive loading with cholesterol, which increased the size and protein content of the GEM fraction approximately 2-fold (data not shown), also inhibited the induction of tyrosine phosphorylation. This suggests that phosphotyrosine induction by HLA-DR is critically dependent upon the composition of the plasma membrane and that the cholesterol content of the plasma membrane of THP-1 cells is normally optimal for signaling.

To test whether changes to the cholesterol content of cells compromised the integrity of phosphotyrosine signaling pathways, cells were stimulated with the phosphatase inhibitor pervanadate, which can activate intracellular tyrosine phosphorylation with no requirement for membrane-associated receptors. Pretreatment of cells with MβCD had no effect on their ability to respond to pervanadate, demonstrating that cholesterol modulation did not affect cell viability with respect to the ability to signal via phosphotyrosine induction (Fig. 4).

**Membrane Rafts Are Required for Very Proximal Events in HLA-DR Signaling**—The above established that intact membrane rafts are required for the induction of all tyrosine phosphorylation events detectable in total cell lysates. Fractionation of HLA-DR-stimulated cells revealed that most inducible tyrosine phosphorylation occurred within the soluble fraction, although some occurred within the GEM fraction (Fig. 5A).

These events, undetectable in total cell lysates, could also be inhibited by MβCD (Fig. 5A). There are therefore no detectable
tyrosine phosphorylation events that are not inhibited by the disruption of membrane rafts, implying their involvement in the very earliest stages of HLA-DR induced phosphoryrosine induction. Following MβCD treatment, HLA-DR was undetectable in the residual GEM fraction (Fig. 5B), despite about 20% of its total protein remaining, as judged on gold-stained blots. This is consistent with the possibility that MβCD ablates phosphotyrosine induction by preventing the physical association of HLA-DR with residual membrane raft structures.

**Src-family Kinases Are Proximally Involved in HLA-DR Signaling**—The above suggested that tyrosine kinases physically associated with the inner leaflet of membrane rafts are likely to be involved in the initiation of HLA-DR-induced phosphoryrosine cascades. The prominent phosphorylated doublet enriched in the GEM fraction (Fig. 5A) was found by sequential Western blotting to have identical mobility to the Src-family tyrosine kinase Lyn (Fig. 5C). To test whether Src-family kinases (which are targeted to membrane rafts by N-terminal palmitoylation (45)) play a role in HLA-DR signaling, cells were treated with the Src-family specific kinase inhibitor PP1 (46) prior to cross-linking HLA-DR with antibodies. This inhibited all inducible tyrosine phosphorylation events, including those within the GEM fraction, to below detectable levels (Fig. 6). Thus, Src-family kinase(s), most probably Lyn in THP-1 cells, are required for the induction of all detectable phosphoryrosine events induced by cross-linking HLA-DR.

**DISCUSSION**

We have shown biochemically that cross-linking of HLA-DR induces its entry into GEMs, which are thought to be derived from membrane rafts. At the cellular level, this is accompanied by the co-aggregation of HLA-DR with membrane rafts. This contrasts with events following the cross-linking of CD45, which shows neither entry into GEMs nor co-aggregation with membrane rafts. It has been proposed elsewhere that raft aggregation could provide a mechanism to initiate signaling cascades through the clustering and transactivation of associated signaling molecules (39). Consistently, we found that the induction of intracellular tyrosine phosphorylation following HLA-DR cross-linking is entirely dependent upon the presence of intact membrane rafts, this being inhibited by MβCD. Using
possibility is that the transmembrane domains of the HLA-DR polypeptides are re-oriented within the plasma membrane when class II molecules are dimerized or undergo higher multimerization. Multimerization is suggested to occur in vivo when MHC/Ag+ and TCR interact at sufficiently high concentrations, as has been demonstrated in vitro (52). Such reorientation could lead to the partitioning of (multimerized) class II into the raft phase of the plasma membrane. Partitioning could also result from conformational changes induced in individual HLA-DR heterodimers following engagement of the TCR.

Extrapolating our findings to the likely course of events in vivo, we suggest that HLA-DR normally occupies the fluid phase of the plasma membrane, where it has a high degree of lateral mobility and has no association with intracellular tyrosine kinases. When engaged by the T cell receptor, HLA-DR bearing specific antigen may be induced to associate with membrane rafts, linking it to raft-associated tyrosine kinases including members of the Src family. Engaged T cell receptor is known to be actively aggregated at the site of cell-cell contact, driven by an actin-dependent mechanism (53, 54). This may lead to the passive aggregation of bound class II bearing specific antigen on the surface of the antigen-presenting cells. Significantly, we found that treating THP-1 cells with cytochalasin D to disrupt the actin cytoskeleton had no effect on the induction of tyrosine phosphorylation by HLA-DR. Passive aggregation of HLA-DR may ensure that this process is dependent upon the specificity of the T cell receptor expressed by the interrogating T cell. Engaged, aggregated class II may then be laterally stabilized as the associated membrane rafts fuse together. Thus, only class II bearing cognate antigen for the specific TCR would be aggregated into a stable antigen presenting array. Raft aggregation may also facilitate the activation of raft-associated tyrosine kinases, as has been suggested elsewhere (39).

We therefore suggest that membrane rafts may contribute to the function of MHC class II in two discrete ways: first, stabilizing localized aggregates, and second, mediating an association with intracellular tyrosine kinases. Future work will determine whether raft aggregation plays a critical role in antigen presentation by HLA-DR.

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The Src-family kinase inhibitor PP1 inhibits the induction of all detectable tyrosine phosphorylation events following the cross-linking of HLA-DR. IFN-γ-treated THP-1 cells were either left untreated or were pretreated with 10 μM PP1 for 30 min at 37 °C, then either left unstimulated or stimulated (Stim.) with cross-linking anti-HLA-DR antibodies for 5 min. Cells were lysed in Triton x-100 buffer, and subcellular fractions were resolved by discontinuous sucrose gradients. Samples were analyzed by SDS-PAGE and immunoblotted with primary anti-phosphotyrosine antibody 4G10.

| MW (kDa) | Cont | 10 μM PP1 |
|----------|------|-----------|
| 175      | +    | +         |
| 83       | -    | +         |
| 62       | +    | -         |
| 47.5     | -    | -         |
| 37.5     | +    | -         |
| 25.5     | -    | +         |
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