CD8 Raft Localization Is Induced by Its Assembly into CD8αβ Heterodimers, Not CD8αα Homodimers*

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The coreceptor CD8 is expressed as a CD8αβ heterodimer on major histocompatibility complex class I-restricted TCRαβ T cells, and as a CD8αα homodimer on subsets of memory T cells, intraepithelial lymphocytes, natural killer cells, and dendritic cells. Although the role of CD8αα is not well understood, it is increasingly clear that this protein is not a functional homologue of CD8αβ. On major histocompatibility complex class I-restricted T cells, CD8αβ is a more efficient TCR coreceptor than CD8αα. This property has for the mouse protein been attributed to the recruitment of CD8αβ into lipid rafts, which is dependent on CD8β palmitoylation. Here, these divergent distributions of CD8αβ and CD8αα are demonstrated for the human CD8 proteins as well. However, although palmitoylation of both CD8α and CD8β chains was detected, this modification did not contribute to raft localization. In contrast, arginines in the cytoplasmic domain are crucial for raft localization of CD8ββ. Most strikingly, the assembly of a non-raft localized CD8β chain with a non-raft localized CD8α chain resulted in raft-localized CD8αβ heterodimers. Using chimeric CD8 proteins, this property of the heterodimer was found to be determined by the assembly of CD8α and CD8β extracellular regions. The presence of two CD8α extracellular regions, on the other hand, appears to preclude raft localization. Thus, heterodimer formation and raft association are intimately linked for CD8αβ. These results emphasize that lipid raft localization is a key feature of human CD8αβ that clearly distinguishes it from CD8αα.

The cell surface glycoprotein CD8 plays an important role in the development and function of MHC class I-restricted T cells. CD8 fulfills its function through interacting via its extracellular domain with MHC class I proteins and via its intracellular domain with the tyrosine kinase Lck. An interaction of both the T cell receptor (TCR) and CD8 with MHC proteins enhances the adhesion between the T cell and the antigen-presenting cell.

The concept of lipid rafts is based on the segregation of lipids into liquid-ordered and liquid-disordered phases. Raft formation at the plasma membrane is dependent on cholesterol, which tightly packs sphingolipids that contain saturated fatty acids, into a liquid-ordered domain. The phospholipids on the other hand, with at least one unsaturated fatty acid, are in the liquid-disordered phase. Detergent-insoluble membranes (DIMs), enriched in cholesterol and sphingolipids, can be isolated from mammalian cells and are believed to represent lipid rafts. These DIM fractions also contain glycosylphosphatidylinositol-anchored, acylated cytosolic and specific transmembrane proteins. Many different functions, ranging from signaling to protein transport to viral budding, have been ascribed to rafts. Although their existence has also remained an issue of debate because these domains cannot be visualized by normal microscopy, methods such as co-patching, fluorescence resonance energy transfer, and single particle tracking have all provided compelling evidence for the compartmentalization of the plasma membrane.

For T cells the segregation of proteins into lipid rafts provides an attractive model for the propagation of TCR signaling. According to this model, the sequestration of the TCR complex and signaling molecules into lipid rafts separates them from negative regulators such as phosphatases and thereby promotes efficient and prolonged signaling. Support for this model comes from the observations that proteins including the TCR, CD3γ chains, and crucial signaling molecules such as protein kinase Cθ and phospholipase Cγ relocate to detergent-insoluble fractions after ligand recognition. For other proteins such as linker for activation of T cells (LAT) and Lck, raft localization has been shown to be essential for T cell activation. Further support comes from the finding that T cell activation can be initiated by cross-linking of the lipid ganglioside 1.
lipid raft localization of CD4 and CD8 has been shown to enhance T cell activation-induced tyrosine phosphorylation through increasing coreceptor association with Lck and recruiting the TCR to rafts (8, 9, 31, 32). In the case of mouse CD8, targeting to lipid rafts has been demonstrated to depend on palmitoylation of the CD8β chain (8, 31). Disruption of this modification interfered with CD8 function. In contrast, palmitoylation and lipid raft localization were not observed for an alternative CD8 form, the CD8αβ homodimer. This is consistent with the view that CD8αβ is critically involved in T cell signaling and that CD8α is a functionally distinct protein (33).

CD8αα is transiently induced on some conventional MHC-restricted CD4+ and CD8+ T cells by antigen stimulation and constitutively expressed on a subset of thymocytes and unconventional oligoclonal TCRαβ and TCRγδ intraepithelial lymphocytes (33). Besides T cells, subpopulations of natural killer and dendritic cells express CD8αα. The function of the CD8αα homodimer is still poorly understood, but it is clear that CD8αα is a less effective TCR coreceptor than CD8αβ despite its capacity to interact with MHC class I proteins (8, 34–37). Indeed, CD8αα cannot support thymic development of MHC class I-restricted T cells (38, 39). In the mouse, CD8αα interacts with the non-classical MHC protein thymic leukemia antigen, suggesting that at least some of its functions may be TCR-independent (33, 40, 41).

Here we have studied the human CD8 proteins, which are likewise found as either CD8αβ heterodimers or CD8αα homodimers. The data show that the raft targeting behavior of human CD8 proteins is similar to those of the mouse; CD8αβ is recruited to, and CD8αα is excluded from lipid rafts. Palmitoylation is regarded as a key factor to the raft localization of cytosolic proteins, but for transmembrane proteins the situation is far less clear. We, therefore, investigated the involvement of this modification and other determinants in raft localization of human CD8 proteins. We show that both human CD8α and CD8β are palmitoylated, but unlike for the mouse CD8 protein, this modification does not drive lipid raft localization. Rather, the localization to lipid rafts can be evoked by the pairing of CD8α and CD8β chains, even when targeting signals in the individual chains have been removed. This distribution requires the assembly of the CD8α and CD8β extracellular regions and is prevented by two CD8α extracellular regions. Thus, the CD8αβ heterodimer formation is intimately linked with recruitment into lipid rafts. CD8αα, on the other hand, resists raft targeting. These findings strengthen the premise that the distributions of CD8αβ and CD8αα at the plasma membrane are strictly controlled and important for the distinct functions of these proteins.

**EXPERIMENTAL PROCEDURES**

*Reagents—Chemicals were from Sigma-Aldrich unless otherwise indicated.*

**Lipid Raft Localization of Human CD8**

CD8αα and CD8β cDNAs—Human CD8α and CD8β cDNAs were amplified from human T cell lymphoblast cDNA using ThermalAce DNA Polymerase (Invitrogen) and cloned into the bicistronic vector pMX in front of an internal ribosomal entry site and green fluorescent protein (GFP) cDNA. Mutant CD8 cDNAs were generated by site-directed mutagenesis according to the Stratagene QuikChange II protocol. The chimeras CD8αbb, CD8bbbaa, and CD8babb were made by overlapping PCRs. CD8αabb contains amino acids (aa) 1–182 (counting from the first amino acid of the signal peptide) of CD8α, joined to aa 171–212 of CD8β. CD8bbbaa contains amino acids (aa) 1–170 of CD8β joined to aa 183–235 of CD8α. In CD8babb, amino acids (aa) 136–170 of CD8β are replaced by aa 134–182 of CD8α. The sequence of all constructs was verified by sequencing (Lark Technologies). For co-patching experiments with FITC-conjugated cholera toxin, CD8 α cDNAs were subcloned into pcDNA3.

**Cells and Transfections—Human embryonic kidney epithelial cells (HEK-293T) were maintained at 37 °C, 5% CO2 in Dulbecco’s modified Eagle’s medium (Cambrex) and SUP-T1 cells in RPMI 1640 (Cambrex). Media were supplemented with 2 mM L-glutamine (Invitrogen), 100 units/ml penicillin, 0.1 mg/ml streptomycin (Invitrogen), and 10% fetal calf serum (Helena BioSciences). HEK-293T cells were transfected using the calcium phosphate method with 30 μg of DNA/10-cm tissue culture dish.

**Antibodies and Horseradish Peroxidase (HRP)-coupled Reagents—** For fluorescence-activated cell sorting (FACS) and immunofluorescence microscopy, phycoerythrin (PE)-conjugated mouse mAb 2ST8.5H7 (Immunotech) was used to detect CD8αβ. PE-Cy5-conjugated mAb DK25 (Dako Cytomation) and PE-conjugated RPA-T8 (eBioscience) were used for CD8α in FACS and immunofluorescence, respectively. CD8β was detected with rabbit antibody H-149 (Santa Cruz) followed by rhodamine-conjugated goat anti-rabbit antibodies (Pierce). In immunoprecipitations, 2ST8.5H7 was used for CD8αβ, H-149 for CD8β, and mAb UCHT4 for CD8α (Ansell). For immunoblotting, mAb 5F2 (Serotec) was used for CD8β, mAb H68.4 (Zymed Laboratories Inc.) was used for transferrin receptor, mouse mAb 3A5 (Santa Cruz) was used against Lck, and a mixture of mouse mAbs 7.1 and 13.1 (Roche) was used against GFP. HRP-conjugated goat anti-rabbit and rabbit anti-mouse antibodies, peroxidase-conjugated cholera toxin B subunit (CTX-HRP), and streptavidin-HRP were from Pierce. Rabbit antimouse IgG and rabbit polyclonal antibody against cholera toxin were from Sigma.

**FACS Analysis—** 1 x 10⁵ SUP-T1 cells were stained with antibodies diluted in ice-cold FACS buffer (2.5% fetal calf serum in PBS) for 30–45 min. After washes in PBS, cells were resuspended in 200 μl of FACS buffer and fixed by the addition of 100 μl of 3% (w/v) paraformaldehyde for 30 min on ice. Acquisition was performed on a BD Biosciences FASCalibur, using CellQuest software (BD Biosciences) and analysis on WinMDI (Scripps Institute). Viable lymphocytes are shown after gating based on forward and side scatter properties.

**Cell Surface Biotinylation—** Cells were biotinylated using the membrane-impermeable EZ-Link Sulfo-NHS-LC-biotin (Pierce) for 30 min on ice at 1 mg/ml in PBS (5 mg/10-cm dish). After incubation, the reaction was stopped by the addition of glycine.
primary antibodies, secondary HRP-conjugated antibodies, CTX-HRP, and streptavidin-HRP were for 1 h at room temperature.

Immunofluorescence and Co-patchning—HEK-293T cells were seeded on 13-mm diameter glass coverslips coated with poly-L-lysine (1 mg/ml in PBS) in 24-well plates. After 16 h of culture, cells were transfected, cultured overnight, and fixed with 3% paraformaldehyde. Incubations with primary and rhodamine-conjugated secondary antibodies were in 2.5% fetal calf serum in PBS for 1 h at room temperature. Coverslips were mounted in Mowiol (Calbiochem-Novabiochem) and observed with a Leica TCP SP2 AOBS™ confocal microscope. For co-patching experiments, HEK-293T cells were stained in suspension on ice with either PE-conjugated 2ST8.5H7 for CD8αβ or PE-conjugated RPA-T8 for CD8α together with FITC-conjugated cholera toxin B (CTX-FITC) for 20 min. Next, cells were incubated with rabbit anti-mouse IgG for 10 min at 37 °C. Cells were then fixed and seeded on poly-L-lysine-coated coverslips that were subsequently mounted in Mowiol. Alternatively, cells were stained in suspension with Alexa Fluor 647-conjugated cholera toxin (CTX-A647) together with either PE-2ST8.5H7 against CD8αβ or PE- RPA-T8 against CD8α for 20 min on ice. Subsequently, patching was induced with anti-cholera toxin antibodies for 10 min at 37 °C followed by fixing, seeding, and mounting of the cells.

\[^{3}H\]Palmitate Labeling—HEK-293T cells were labeled 20 h after transfection on 6-cm dishes. Cells were preincubated in labeling medium (Dulbecco’s modified Eagle’s medium, 2% fetal calf serum, 5 mM sodium pyruvate (Invitrogen Ltd.) and 4× nonessential amino acids (Invitrogen)) for 60 min. n-[9,10-\[^{3}H\]]Palmitic acid in ethanol (Amersham Biosciences) was dried under nitrogen and added to the cells at 1 mCi/ml for 6 h. After labeling cells were washed once with PBS and processed for immunoprecipitation and SDS-PAGE. Samples were transferred to nitrocellulose membranes and visualized by autoradiography using Kodak Biomax MS film and a Biomax Transcreen-LE (Kodak) intensifying screen or by Western blotting.

RESULTS

CD8αβ, but Not CD8αα, Localizes to Lipid Rafts in T Cells—The raft localization of human CD8 was analyzed in SUP-T1 cells, a T cell leukemia cell line that expresses multiple T cell lineage markers, including CD4 and CD8. First, the subunit composition of CD8 on these cells was determined by FACS analysis. Staining with anti-CD8α, which detects both CD8αα and CD8αβ, showed that most (91%) SUP-T1 cells were CD8-positive (Fig. 1A). However, staining with an antibody that exclusively detects the CD8αβ heterodimer by recognizing an

(50 mM in PBS) for 5 min, and the cells were washed 3 times in PBS. SUP-T1 cells were biotinylated in suspension with 1 mg of sulfo-NHS-LC-biotin per 25 × 10⁶ cells in 1 ml. For sucrose gradient fractionation, transfected HEK-293T cells (on 10-cm dishes) were scraped off the plate into 1 ml of 1% Triton X-100 (Pierce) MNE buffer (25 mM MES, pH 6.5, 150 mM NaCl, 5 mM EDTA) supplemented with the protease inhibitors phenylmethylsulfonfonyl fluoride (1 mM), chymostatin, pepstatin A, antipain hydrochloride (each at 50 μg/ml), and leupeptin hemisulfate (10 μg/ml) and lysed for 30 min on ice. SUP-T1 cells were lysed in the same buffer at 10⁶ cells/ml. Lysates were homogenized with 10 strokes in a Dounce homogenizer (Wheaton Scientific) and mixed with an equal volume of 80% (w/v) sucrose in MNE buffer. The resulting 2 ml of lysate in 40% sucrose was overlaid in a centrifuge tube with 6 ml of 30% sucrose and 40 ml of 5% sucrose in MNE and centrifuged at 220,000 × g for 20 h at 4 °C using a Sorvall TH-641 swing-out rotor and a Sorvall Discovery™ 100 ultracentrifuge. After centrifugation, 12 1-ml fractions were collected from the top of the gradient.

Immunoprecipitation, SDS-PAGE, and Western Blotting—For immunoprecipitation samples were precleared with 40 μl of a 50% protein A-Sepharose slurry (Amersham Biosciences) for 30 min. Subsequent antibody incubations were for 3 h on ice followed by a 2-h incubation with protein A-Sepharose. Protein A-Sepharose beads were washed 4 times with 1% Triton MNE buffer before resuspension in reducing 5% (v/v) β-mercaptoethanol) SDS-PAGE sample buffer. Samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). To detect the lipid GM1, 0.5 μl of sucrose gradient fractions was directly pipetted on nitrocellulose membranes and left to dry. Nitrocellulose membranes were blocked in 10% (w/v) skimmed milk or in 10% (w/v) bovine serum albumin when streptavidin-HRP was used for development. Incubations with
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epitope on CD8β that is dependent on association with CD8α (42) demonstrated that only 43% of the cells expressed the CD8αβ heterodimer. Thus, these SUP-T1 cells are heterogeneous for CD8, with approximately half of the CD8+ cells expressing CD8αα only, and the other half expressing CD8αβ. Whether the latter population expresses CD8αα as well cannot be distinguished by this analysis. Importantly, these cells allow the comparison of lipid raft localization between CD8αα and CD8αβ proteins.

To determine the lipid raft distribution of CD8, the presence of this protein in detergent-insoluble membranes was investigated. SUP-T1 cells were lysed in ice-cold 1% Triton-X100 and separated into detergent-soluble and -insoluble fractions on discontinuous sucrose gradients. The cells were surface-biotinylated before solubilization to selectively analyze the fraction of CD8 at the plasma membrane. Western blotting for Lck and transferrin receptor (TfR), a raft and non-raft marker, respectively, identified the detergent-insoluble and soluble fractions (Fig. 1B). To distinguish between CD8αβ and CD8αα, parallel immunoprecipitations with anti-CD8αα, anti-CD8β, and anti-CD8α antibodies were performed. Anti-CD8αα immunoprecipitation revealed that the CD8αβ heterodimer was completely detergent-insoluble, a result that was confirmed with anti-CD8β immunoprecipitations (Fig. 1B). In contrast, the anti-CD8α antibody recovered CD8 proteins from both the detergent-soluble and -insoluble fractions. Given that CD8β chains were exclusively detected in the detergent-insoluble fractions, it can be concluded that the CD8α chains in the soluble fractions 10 to 12 are not associated with CD8β and, thus, represent CD8αα homodimers. Thus, the CD8αβ heterodimer, but not the CD8αα homodimer, localizes almost exclusively to lipid rafts in the T cell line SUP-T1.

Lipid Raft Localization of CD8 Proteins in HEK-293T Cells—To investigate this difference between CD8αβ and CD8αα in lipid raft localization, transient transfection experiments in HEK-293T cells were employed. The use of these cells allows the efficient expression of CD8 constructs and has the added advantage of the absence of Lck, which has been shown to recruit CD4 into lipid rafts (9), and may, therefore, do the same to CD8.

Transfection of CD8α on its own into HEK-293T cells resulted in cell surface expression of this protein, detected by immunofluorescence microscopy (Fig. 2A). Similarly, CD8β transfected on its own was detected at the cell surface, in agreement with a previous report (43) but in contrast to the murine CD8β chain, which requires CD8α for transport to the plasma membrane (44). Non-reducing SDS-PAGE showed that CD8α and CD8β expressed on their own are present as disulfide-linked homodimers (Fig. 2B). CD8α and CD8β transfected together led to the formation of CD8αβ heterodimers at the cell surface that could be detected with the anti-CD8β heterodimer antibody 2ST8.5H7 (Fig. 2A). This antibody recognizes an epitope on CD8β that is only formed after assembly with CD8α and, in agreement with this, showed no reactivity...
with CD8α or CD8β chains expressed separately (data not shown). Hence, using HEK-293T cell transfections, the lipid raft distribution of CD8αβ, CD8αα, and CD8ββ at the plasma membrane could be analyzed. Similar to the SUP-T1 cell experiments, the HEK-293T cells were surface-biotinylated before fractionation on sucrose gradients. In the absence of Lck, the lipid GM1, detected with HRP-conjugated cholera toxin subunit B, was used as a marker for the detergent-insoluble fractions (Fig. 2C). Analysis of CD8 proteins revealed that, like in SUP-T1 T cells, CD8αβ heterodimers were exclusively detergent-insoluble. In contrast, CD8αα was completely soluble in 1% Triton-X100 (Fig. 2C). Moreover, these differences in distributions were also observed with immunofluorescence co-patching experiments. Antibody-induced patching of CD8αβ, but not that of CD8αα, resulted in co-patching of the raft lipid GM1, detected by cholera toxin staining (Fig. 3A). Similarly, patching of GM1 with cholera toxin and anti-cholera toxin antibodies led to co-patching of CD8αβ but not that of CD8αα (Fig. 3B). Thus, the lipid raft distributions of CD8αβ and CD8αα in HEK-293T cells mimic those in SUP-T1 cells. These experiments also show that the raft localization of CD8αβ is independent of Lck.

It was further observed by transfecting CD8β in the absence of CD8α that CD8ββ homodimers predominantly localized to the detergent-insoluble fractions (Fig. 2C). Hence, it can be concluded that the CD8β chain contains raft-localizing determinants.

**CD8 Is Targeted to Lipid Rafts in the Absence of Palmitoylation**—The lipid raft targeting of several transmembrane proteins has been correlated to their modification by palmitoylation (27, 45). Palmitoylation involves the post-translational esterification of palmitic acid to a free thiol group of cysteine residues (46). For murine CD8, the CD8αβ chain, but not the CD8αα chain, was shown to be palmitoylated on a single cysteine located at the boundary of the transmembrane and cytoplasmic domain (8, 31). The human CD8αβ chain contains two cysteines in the corresponding region (Fig. 4A) that were experimentally substituted by alanines. The usage of these cysteines as palmitoylation sites was investigated in [3H]palmitate cell-labeling experiments. Indeed, labeled bands were detected in cells transfected with wild type CD8αβ and CD8αα in HEK-293T cells. These experiments also show that the raft localization of CD8αβ is independent of Lck.
FIGURE 4. CD8α and CD8β are palmitoylated. A, amino acid sequences for the complete cytoplasmic domains of mouse CD8β (mCD8β), human CD8β (CD8β), and part of the cytoplasmic domain of human CD8α (CD8α). In mCD8β, palmitoylation occurs on the juxtamembrane cysteine. The potential palmitoylation sites in CD8β and CD8α are boxed and were mutated as shown. TM, transmembrane domain. B, HEK-293T cells transfected with wild type (wt), or mutant CD8α and CD8β chains, as indicated, were labeled with [3H]palmitic acid for 6 h. Anti-CD8αβ immunoprecipitates (IP) were analyzed by SDS-PAGE and blot transfer followed by either autoradiography (top) or immunoblotting with anti-CD8αβ (bottom). C, HEK-293T cells transfected with wild type or mutant CD8α were labeled with [3H]palmitic acid for 6 h. Anti-CD8α immunoprecipitates were analyzed by autoradiography (top). In the absence of antibodies that detect CD8α by Western blotting, transfection efficiencies were verified by blotting lysates for GFP, which is expressed from a bicistronic vector together with CD8αβ constructs as indicated. Cells were biotinylated and fractionated on sucrose gradients, and CD8 molecules were immunoprecipitated from the DIM and soluble fractions with anti-CD8αβ (B and C) or anti-CD8β (D). CD8 was detected with streptavidin-HRP.

FIGURE 5. CD8 palmitoylation is not required for detergent insolubility. A, palmitoylation-deficient CD8α (C1A) and CD8β (C1/2A) chains were transfected separately or together into HEK-293T cells. Cell surface expression of these proteins was determined by staining with antibodies against CD8α (top), CD8β (middle), or CD8αβ (bottom) without permeabilization. GFP is expressed from a bicistronic vector together with CD8 chains. Cells were observed by confocal microscopy. Scale bar, 16 μm. B–D, HEK-293T cells transfected with wild type (wt) or mutant CD8α and CD8β constructs as indicated. Cells were biotinylated and fractionated on sucrose gradients, and CD8 molecules were immunoprecipitated from the DIM and soluble fractions with anti-CD8αβ (B and C) or anti-CD8β (D). CD8 was detected with streptavidin-HRP.

The intermediate band may contain alternatively processed carbohydrate chains compared with the highest molecular weight CD8β band. When either the mutant CD8βC1A or CD8βC2A (Fig. 4A), which each lack one of the potential palmitoylation sites, were expressed together with CD8α, labeled CD8β chains were still detectable (Fig. 4B, lanes 2 and 3). However, the absence of both cysteines in mutant CD8βC1/2A resulted in the disappearance of CD8β labeling (Fig. 4B, lane 4), demonstrating that CD8β is palmitoylated. The persistence of [3H]palmitate incorporation in CD8αβ heterodimers composed with mutant CD8βC1/2 suggested that CD8α is palmitoylated as well. Indeed, palmitoylated CD8α chains could be detected when this protein was transfected on its own (Fig. 4C). The CD8α cytoplasmic domain contains one juxtamembrane cysteine in addition to the two cysteines that are involved in binding to Lck (Fig. 4A) (47, 48). The substitution of this cysteine in the mutant CD8αC1A completely abolished palmitoylation (Fig. 4C). In the absence of antibodies that detect CD8α by immunoblotting, the transfection efficiency in this experiment was monitored by the expression of GFP (Fig. 4C), which is expressed with CD8α from a bicistronic vector. The co-expression of CD8αC1A with the palmitoylation-deficient CD8β chain CD8βC1/2A resulted in a complete lack of CD8αβ palmitoylation (Fig. 4D, lane 4).

Next, the contribution of CD8 palmitoylation to detergent insolubility was tested. Immunofluorescence showed that palmitoylation-deficient CD8αα, CD8ββ, and CD8αβ were normally expressed at the cell surface (Fig. 5A). Moreover, the detergent insolubility of CD8αβ was not significantly affected by the absence of palmitoylation on either CD8βC1/2A (Fig. 5B), CD8αC1A, or CD8αC1A and CD8βC1/2A together (Fig. 5C). Similarly, the CD8βC1/2A chain expressed on its own was mostly detergent-insoluble (Fig. 5D). Thus, non-palmit-
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Subsequent mutagenesis of the four arginines individually showed reduced detergent insolubility compared with the wild type CD8ββ for all four mutants (Fig. 6B). These results suggested that the detergent insolubility of CD8ββ depends on the presence of specific arginine residues rather than on a consecutive stretch of arginines. The biggest impact was observed for the substitution of Arg-180 and Arg-182 in CD8βR1A and CD8ββR3A, respectively, implying a dominant role for these residues. To determine whether the contribution of these arginines is charge-dependent, Arg-182 was replaced with the positively charged lysine. Comparable with the alanine mutant, the CD8ββR3K mutant was completely soluble in Triton-X100 (Fig. 6B), indicating that an arginine, rather than a positive charge at position 182, is essential for the detergent insolubility of CD8ββ.

Although the determinants for palmitoylation are not yet understood, it has been suggested for several proteins that positively charged amino acids promote palmitoylation at neighboring cysteines (46, 49). We, therefore, investigated whether the arginine mutations interfered with palmitoylation. Palmitate incorporation was determined for CD8ββR1A/R3A co-expressed with a palmitoylation-deficient CD8α chain. Immunoprecipitation detected labeled CD8αβ and anti-GFP immunoprecipitations (IP) were analyzed by autoradiography after SDS-PAGE and transfer to nitrocellulose membranes.

Oxylated CD8αβ and CD8ββ localized to lipid rafts, indicating that palmitoylation is not absolutely required for this distribution of CD8 proteins.

An Arginine Motif Targets CD8ββ into Lipid Rafts—The cytoplasmic domain of CD4 contains a stretch of five positively charged residues (RHRRR) (Fig. 6A), the mutation of which was shown to abolish lipid raft localization despite palmitoylation and Lck binding being intact (11). A similar region of positively charged residues (RRRR) can be found close to the transmembrane domain in the cytoplasmic domain of CD8β (Fig. 6A). To investigate the contribution of this region to detergent insolubility, arginine to alanine mutants were generated. First, the detergent insolubility of these CD8β chains was investigated in the absence of CD8α. The mutation of three consecutive arginines in CD8βR2A/R3A/R4A, largely eliminated detergent insolubility (Fig. 6B). Similarly, the mutant CD8ββR1A/R3A with two arginine substitutions was predominantly soluble in Triton-X100. Thus, arginines in the cytoplasmic domain play an important role in the detergent insolubility of CD8ββ. The arginine mutations did not appear to affect intracellular transport given that the mutants could be efficiently detected at the cell surface by biotinylation (Fig. 6B) and immunofluorescence (not shown). Furthermore, the ability to form homodimers was not affected and was as efficient for CD8ββR1A/R3A as for wild type CD8β (Fig. 2B, lanes 4 and 5 and lanes 10 and 11).

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shown). Thus, it is most likely that determinants outside the cytoplasmic domain of CD8α and CD8β are involved in the detergent insolubility of CD8αβ heterodimers.

To determine the involvement of CD8 domains in this, chimeric CD8 proteins were generated. In CD8bbaa, the transmembrane and cytoplasmic domains of CD8β are replaced by those of CD8α. This resulted in reduced detergent insolubility of this protein compared with CD8ββ, consistent with the loss of raft-targeting signals in the CD8β cytoplasmic domain (Fig. 8A, top panel). In Fig. 2B it can be seen that the CD8bbbaa chimera is able to form homodimers to the same extent as CD8ββ. Cotransfection of CD8bbaa with CD8α led to the formation of heterodimers that were largely detergent-insoluble (Fig. 8A, middle panel). Thus, again the pairing of two detergent soluble chains resulted in the formation of detergent-insoluble heterodimers, and it appears that this solely requires the presence of CD8αα and CD8ββ extracellular regions. To test this further, a chimera composed of the CD8αα extracellular region and the CD8β transmembrane and cytoplasmic domains, CD8aabb, was generated. Expressed on its own, CD8aabb protein was predominantly found in detergent-soluble fractions despite the presence of raft localizing signals in the cytoplasmic domain of CD8ββ (Fig. 8B, top panel). Analysis under non-reducing conditions showed the efficient homodimerization of CD8aabb (Fig. 2B). The detergent insolubility of CD8aabb compared with CD8ββ suggests that the pairing of two CD8αα extracellular regions actively prevents lipid raft localization.

The extracellular regions of CD8αα and CD8ββ each consist of an Ig-like domain attached to a heavily glycosylated proline-rich domain, the stalk. A third chimera was generated, CD8bbαβ, in which the stalk domain of CD8ββ was replaced by that of CD8αα. In contrast to CD8aabb, CD8bbαβ did localize to detergent-insoluble fractions (Fig. 8C). Thus, the CD8αα stalk region by itself is not sufficient to prevent detergent insolubility. Rather, the CD8αα Ig-like domain appears to play an important role in this.

In agreement with the incompatibility of two CD8αα extracellular regions with raft localization, the cotransfection of CD8aabb with CD8αα did not increase lipid raft localization (Fig. 8B, second panel). However, cotransfection of CD8aabb with CD8ββ resulted in the formation of anti-CD8αββ recognizable dimers that were almost exclusively detected in detergent-insoluble fractions (Fig. 8B, third panel). Moreover, cotransfection of CD8aabb with the equally detergent-soluble CD8bbbaa also led to the recruitment of anti-CD8αββ-detectable proteins to the detergent-insoluble membranes (Fig. 8B, fourth panel). Thus, it can be concluded that the assembly of extra- cellular CD8αα and CD8ββ regions is sufficient and critically important for lipid raft localization. Conversely, the presence of two CD8αα extracellular regions is incompatible with this localization.

**DISCUSSION**

The TCR coreceptor CD8αβ shares the ability to localize to lipid rafts with its functional homologue CD4 but not with the structurally similar CD8αα. To understand the basis for these divergent localizations, we analyzed here the raft tar-
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targeting properties of human CD8 proteins. Our data demonstrate a powerful capacity of CD8αβ to target to lipid rafts, driven to a large extent by the assembly of CD8α and CD8β extracellular regions. On the other hand, CD8α alone appears to have an extreme resistance for this localization.

Various studies have demonstrated an involvement of lipid raft localization in the amplification of TCR signaling by CD8 and CD4 (8, 9, 31, 32). Our findings are supportive of an important role for this localization in several ways. First, the raft partitioning, previously demonstrated for mouse CD8αβ, was found to be conserved for the human protein. Moreover, raft localization of human CD8αβ was shown to be controlled by multiple factors, an arginine motif in the cytoplasmic domain of CD8β and the pairing of CD8α and CD8β extracellular regions. A similar multifactorial situation exists for the lipid raft distribution of CD4, which is determined by palmitoylation, an intracellular arginine motif, and by binding to Lck. Most striking for CD8 is the tight connection between lipid raft localization and the formation of CD8αβ heterodimers, whereas palmitoylation per se is not important. Indeed, the pairing of CD8α and CD8β extracellular domains can confer lipid raft association even when CD8β raft targeting signals are not present. In accordance, we have been unable to generate non-raft associated CD8αβ despite extensive mutagenesis.

In contrast, a complete lack of lipid raft association was observed for the CD8αα homodimer, both in transfected HEK-293T cells and in the T cell line SUP-T1. This distribution is remarkable given that CD8α contains several potential raft-targeting determinants. We demonstrate that CD8α is palmitoylated and able to confer raft localization on a mutant non-raft-associated CD8β chain. Additionally, an arginine motif is present in the cytoplasmic domain similar to the one in CD8β, and CD8α can associate with Lck. Yet, even the combined occurrence of these factors does not recruit CD8α into lipid rafts. To the contrary, it was observed that the presence of a CD8α extracellular region prevents the raft localization of a chimera that contains CD8β raft targeting signals.

In transfection experiments the exclusion of CD8αα from lipid rafts results in less efficient TCR signaling (8, 31). Whether this also plays a role in cells where CD8αα is normally expressed remains to be shown. The role of CD8αα could be completely TCR-independent, as suggested by the TCR-independent recognition of the non-classical MHC protein thymic leukemia antigen by mouse CD8αα (33, 40). This interaction may correlate with the reduced proliferation of activated unconventional intraepithelial lymphocytes, but the signaling pathways involved are not yet known. It will be interesting to investigate the association of CD8αα with other non-raft-associated proteins and, in particular, the effects of forced CD8αα raft localization in these cells if that can be achieved. The apparent reluctance of CD8αα to enter lipid rafts provokes the hypothesis that such a localization may compromise CD8αα function. On the other hand, the apparent pressure on the CD8αβ heterodimer to enter rafts suggests that for this protein such a localization is essential for function.

The observation that palmitoylation does not contribute to detergent insolubility of human CD8 was unexpected given that this modification is involved in raft localization of mouse CD8αβ (8, 31). For the latter protein, disruption of the CD8β palmitoylation site impairs raft localization, and the CD8α chain is not palmitoylated. For human CD8, we demonstrate that non-palmitoylated mutant CD8ββ and CD8αβ forms can still localize to rafts and that human CD8αα is palmitoylated but does not target to rafts. Thus, palmitoylation is neither absolutely required nor sufficient for the lipid raft distribution of human CD8 chains. In light of this it is noteworthy that CD8β palmitoylation sites are not conserved and, for example, do not occur in cat and chicken orthologues. Indeed, a role for palmitoylation of human CD8 has not been detected; in addition to lipid raft targeting, the subunit assembly and transport to the plasma membrane appeared normal for palmitoylation-deficient forms, although a more detailed analysis may reveal subtle changes.

Palmitoylation is generally considered a raft-targeting signal, and such a role has been demonstrated clearly with acylated cytosolic proteins (45, 46, 50). For these, palmitoylation provides the only membrane anchor, and lipid raft localization is most likely achieved by the preferential integration of the saturated palmitic acid into the liquid-ordered domains. For transmembrane proteins, the situation is obviously more complex since a transmembrane region needs to be accommodated into rafts as well. Palmitoylation has been shown to occur for several raft-associated transmembrane proteins and has been predicted for many others based on the presence of cysteines in the cytoplasmic domain. For only a few of these proteins has its role been investigated directly and found to be essential for raft targeting. The key T cell adaptor LAT and the influenza virus protein hemagglutinin, for instance, do not target to rafts in the absence of palmitoylation (27, 45). Yet even for these proteins other factors such as interactions via the cytoplasmic and transmembrane domains contribute to raft localization as well (51–53). For CD4, palmitoylation plays a role, but is not sufficient for raft targeting (11). Other transmembrane proteins, including the transferrin receptor and vesicular stomatitis virus G protein, are palmitoylated but not raft-associated (45, 54, 55). Thus, it is clear that a simple equation of “palmitoylation equals raft-localization” does not exist for transmembrane proteins but, rather, that palmitoylation influences raft-localization to different extents dependent on other protein characteristics.

The involvement of an arginine-containing motif in lipid raft localization was detected for CD8ββ in transfected HEK-293T cells. This motif is absolutely required for lipid raft localization of CD8β expressed on its own but redundant for the CD8αβ heterodimer. Thus far, CD8ββ homodimers have not been observed on human T cells, and in the mouse the CD8β chain is retained intracellularly in the absence of CD8αα (44). Nevertheless, the identification of this motif, similar to the one found in CD4, is intriguing and suggests that it may be more generally present on raft-localized transmembrane proteins. For CD4, the substitution of three consecutive arginines in HRNRR abolishes lipid raft localization, but the contribution of individual residues was not addressed (11). For CD8ββ, the substitution of each arginine in RRRR individually reduced raft distribution even when an arginine triplet was still intact. The most drastic effect was seen with mutations of either the first or third arginine of this motif, which virtually abolished raft targeting.
The observation that a positively charged lysine at the third position could not replace an arginine indicates that the function of this motif is not merely charge-dependent. More likely, the conformation of this area is important for protein-protein or protein-lipid interactions.

The use of non-raft-associated CD8β arginine mutants allowed the detection of the most potent signal for raft targeting, heterodimerization. Because raft-associated dimers could be formed from non-raft CD8α and CD8β chains, a property of the combined chains has to be involved. With chimeric CD8 proteins, detergent-insoluble heterodimers could also be formed from non-raft CD8α and CD8β extracellular domains but does not require the pairing of CD8α and CD8β transmembrane and cytoplasmic regions. It is clear that the folding of the CD8αβ extracellular domains differs from that of the individual chains given the selective presence of the 2ST8.5H7 CD8β epitope on heterodimers. Whether the heterodimeric extracellular regions influence lipid raft localization through interactions with other proteins or with lipids is not yet clear.

Striking was also the observation that CD8α extracellular domains were able to prevent lipid raft localization of a chimera that contained CD8β raft targeting signals. This was found to require the presence of the CD8α Ig-like domain since replacing only the CD8β stalk with that of CD8α did not impede raft targeting. We tried to address this further by exchanging only the Ig-like domain of CD8β with that of CD8α, but the resulting chimera was not detected by anti-CD8α or CD8β antibodies.

In summary, this study on human CD8 shows that raft localization of transmembrane proteins is a multifaceted process and that a role for palmitoylation should not be assumed until experimentally proven. Indeed, palmitoylation cannot be considered a conserved determinant of raft localization despite it being a conserved feature of CD8β in human and mouse. Importantly, the data identify a novel determinant, the assembly of a protein from its subunits, as a raft-targeting signal. The involvement of heterodimerization is a powerful way to ensure the raft localization of CD8αβ. Equally striking is the strict exclusion of CD8αβ from these domains; this should be considered when reviewing the function of this protein.

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