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Restricted Localization of the TNF Receptor CD120a to Lipid Rafts: A Novel Role for the Death Domain

Vincent Cottin,* Joyce E. S. Doan,* and David W. H. Riches2*†

The TNF-α receptor, CD120a, has recently been shown to be localized to both plasma membrane lipid rafts and to the trans Golgi complex. Through a combination of both confocal microscopy and sucrose density gradient ultracentrifugation, we show that amino acid sequences located within the death domain (DD) of CD120a are both necessary and sufficient to promote the appropriate localization of the receptor to lipid rafts. Deletion of the DD (CD120a.L321-425) prevented the receptor from being targeted to lipid rafts and resulted in a uniform plasma membrane localization. A similar loss of raft localization was also observed following pairwise deletion of the six α-helices that comprise the DD. In all situations, the loss of the ability of CD120a to become localized to lipid rafts following mutagenesis was paralleled by a failure of the receptor to initiate apoptosis. Furthermore, introduction of the lpr mutation into CD120a (CD120a.L351N) also resulted in both a loss in the ability of the receptor to signal apoptosis and to be appropriately localized to rafts. In contrast to CD120a, CD120b, which lacks a DD, is mainly expressed in the bulk plasma membrane and to a lesser extent in lipid rafts, but is absent from the Golgi complex. However, a chimeric receptor in which the DD of CD120a was fused to the cytoplasmic domain of CD120b was predominantly localized to lipid rafts. Collectively, these findings suggest that in addition to its role in CD120a signaling, an appropriately folded and functionally active DD is required for the localization of the receptor to lipid rafts. The Journal of Immunology, 2002, 168: 4095–4102.

The plasma membranes of most cell types contain cholesterol and glycosphingolipid-containing microdomains or “lipid rafts” that are enriched in a variety of receptors and signaling molecules (11, 12). They are characterized by their resistance to solubilization at low temperature in nonionic detergents and by a punctate or focal staining pattern as detected by confocal microscopy. Recent studies have shown that members of the TNFR superfamily, including CD40, the p75 neurotrophin receptor, and CD120a are also associated with lipid rafts (13–16), and that their localization to these structures is necessary for the initiation of signaling events. However, the mechanisms that mediate the localization of TNFR superfamily members to lipid rafts remain largely unknown.

In this study, we have investigated the mechanism of localization of CD120a to lipid rafts. In view of the importance of the DD in signaling programmed cell death, we hypothesized that this region of CD120a would also be important in localizing the receptor.
to rafts. Using a mutagenic approach combined with confocal microscopy and raft isolation strategies, we show that the DD of CD120a is necessary and sufficient for both the localization of the receptor to lipid rafts as well as for signaling apoptosis. In contrast, the DD was not found to be important in the localization of CD120a to the Golgi complex, implying that receptor localization to lipid rafts is an important event in the initiation of programmed cell death.

### Materials and Methods

#### Materials

The hamster monoclonal anti-CD120a (p55) antagonist Ab (no. 80-4005-01) was purchased from R&D Systems (Minneapolis, MN). The anti-γ-adaptin-1 mouse mAb was from Sigma-Aldrich (St. Louis, MO). The goat polyclonal anti-CD120a and anti-CD120b Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescent secondary Abs were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). FITC-conjugated wheat germ agglutinin was from Molecular Probes (Eugene, OR).

#### Expression vectors

The expression vector encoding CD120a has been described (14). All deletion and point mutants were constructed using overlapping PCR (17) and ligated into EcoRI/SalI-digested pFLAG-CMV-1. The CD120b-(1-462)/CD120a-(308-425) chimeric protein was created by fusing the full-length CD120b to the DD of mouse CD120a (aa 308–425). A 1762-bp BamHI/EcoRI cDNA fragment encoding the chimera was amplified by PCR, digested with BamHI and EcoRI, and ligated into BamHI/EcoRI-digested pcDNA3 (Invitrogen, Carlsbad, CA). The fidelity of all the constructs was verified by restriction enzyme analysis and nucleotide sequencing. The expression vector for CD120b was a generous gift from Dr. H.-B. Shu (National Jewish Medical and Research Center, Denver, CO).

#### Transfections and confocal immunofluorescence microscopy

HeLa cells were maintained in DMEM containing 10% (v/v) FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 2 mM glutamine. Approximately 6 × 10^5 cells/well were seeded in 12-well plates containing 18-mm glass coverslips and grown in 5% (v/v) CO_2. Cells were transfected with 250 ng of DNA the following day using the Lipofectamine reagent (Life Technologies, Rockville, MD). Fourteen hours after transfection, the cells were washed with PBS, fixed for 15 min at room temperature in a solution containing 3% (v/v) paraformaldehyde and 3% (w/v) sucrose in PBS (pH 7.5). Cells were incubated for 15 min with wheat germ agglutinin (1/2000) in PBS, washed again extensively, and permeabilized with 0.2% (v/v) Triton X-100 for 10 min. The cells were then washed, blocked for 30 min in HBSS (without Mg^2+ and Ca^2+), and phenol red, pH 7.2) containing 5% normal goat serum, and then incubated with the primary Ab (1/200) in blocking solution for 2 h. After washing with PBS, the cells were incubated for 1 h with Cy3- and/or fluorescein-conjugated F(ab')₂ goat secondary anti-hamster IgG (1/200). The coverslips were incubated overnight in PBS supplemented with 0.02% sodium azide, and mounted in a solution containing 90% glycerol, 10% Tris-HCl (0.1 M, pH 8.5), and 20 mg/ml α-phenylenediamine as an antifading agent. To visualize the nuclei, cells were incubated with 10 μg/ml Hoechst 33342 (Hoechst Sigma Chemical, St. Louis, MO) together with the secondary Abs. Cells were observed with a Leica DMRXA confocal immunofluorescence microscope (Leica Microsystems, Bannockburn, IL) using a ×100 Plan objective. Digital images were captured using a SensiCam camera, deconvolved using the software Slidebook 2.6 (Intelligent Imaging Innovations, Denver, CO) to remove out of focus fluorescence, and processed using Adobe Photoshop (Adobe Systems, Mountain View, CA).

For quantitative analysis, cells were transfected with the appropriate constructs, and stained in the absence of cell permeabilization as above. Analysis was performed using the software Slidebook 2.6. Briefly, stacks of 25 images were acquired in 0.4-μm steps throughout the cells. Stacks were deconvolved using the “nearest neighbors” algorithm, and the volume of the entire cell was defined manually. The amount of fluorescence was defined by the integrated intensity of fluorescence after correction for background fluorescence. Ten cells were analyzed per condition.

### Flow cytometry analysis

HeLa cells (10^6) were seeded in 100-mm dishes and transfected with 5 μg of DNA the following day using the Lipofectamine PLUS reagent as recommended by the manufacturer (Life Technologies). Eighteen hours after transfection, the cells were incubated for 15 min with 1 μg/ml of hamster monoclonal anti-CD120a Ab, or monoclonal hamster Ab anti-TCR H57 (generously provided by Dr. J. Freed, National Jewish Medical and Research Center) as a control, washed, and incubated for 15 min on ice in HBSS (without Mg^2+, Ca^2+, and phenol red, pH 7.2) containing 5 mM EGTA. Cells were lifted from the plates, washed, resuspended in 1 ml of cold PBS, and fixed for 15 min at room temperature by the addition of 1 ml of 2% fixative (6% w/v) parafomaldehyde and 6% (w/v) sucrose in PBS, pH 7.5). Cells were washed three times with PBS, incubated for 20 min at 4°C in 100 μl blocking buffer (HBSS containing 5% of normal goat serum), then incubated for 30 min with fluorescein-conjugated goat anti-hamster IgG (1/100 in blocking buffer). After washing with PBS, cells were resuspended in 100 μl of cold sample buffer (2% PBS in PBS containing 0.02% sodium azide). Cells (10^6) were analyzed on a FACSCaliber (BD Biosciences, Mountain View, CA) and data were processed by using the CellQuest software (BD Biosciences). Data are presented as a histogram of cell number vs. fluorescence intensity.

### TUNEL assay

HeLa cells grown on coverslips and transfected 18 h earlier with the appropriate expression vectors were washed with PBS, fixed and permeabilized as above, and incubated with terminal transferase reaction solution containing fluorescein-conjugated dUTP at 1 h at 37°C as recommended by the manufacturer (Roche Diagnostics, Indianapolis, IN). The cells were washed three times with 0.03 M sodium citrate (pH 7.4) containing 0.3 M sodium chloride to remove unbound nucleotides, then washed with PBS. Cells were then blocked and incubated with Abs as above. The percentage of TUNEL-positive cells among transfected cells was determined by counting at least 200 cells with a confocal microscope.

### Isolation of lipid rafts

Lipid rafts were isolated by sucrose density gradient centrifugation of Triton X-100 lysates using a modification of the method of Cheng et al. (18). Approximately 1.2 × 10^7 HeLa cells or ~8 × 10^7 COS-7 cells were transfected as described above. Twelve to 16 h posttransfection, the cells were washed once with either ice-cold PBS or MES-buffered saline (25 mM MES buffer, pH 6.5, containing 150 mM NaCl) and then harvested by scraping into PBS. The cells were collected by centrifugation and then lysed in 1 ml TMBS (25 mM MES (pH 6.5) containing 150 mM NaCl, 1% (v/v) Triton X-100, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM PMSF, 0.1 mM Na_2VO_3, and 1 mM NaF). The cell lysates were sonicated at full strength for 1 min at 4°C and the high-density insoluble debris was removed by centrifugation at 1,000 × g for 10 min at 4°C. Clarified lysates were combined with an equal volume of 80% (w/v) sucrose, transferred to 1.25 ml ultracentrifuge tubes, and overlaid with a discontinuous sucrose gradient comprised of 8 ml 35% sucrose, followed by 2 ml of 5% sucrose. Separation of the low-density lipid rafts was achieved by centrifugation at 37,000 rpm in an SW41 rotor for 18–22 h at 4°C. Following centrifugation, 1 ml fractions were harvested from the bottom of the gradients. Fractions were analyzed for the raft markers alkaline phosphatase activity and ganglioside GM₁, by catalytic assay using 5 mM p-nitrophenyl phosphate in 0.1 M 3-amino-2-methyl-1-propanol (pH 10.0) as buffer, and Western blotting with cholera toxin B-conjugated HRP, respectively (19, 20). Fractions were also blotted with anti-CD120a and anti-CD120b Abs, as indicated in Results.

Results shown are representative of at least three separate experiments unless indicated.

### Results

#### Flow cytometry analysis

The DD is required for the localized expression of CD120a to lipid rafts

In previously reported studies, we showed that CD120a is expressed at the cell surface at focal points, rather than being uniformly distributed in the plasma membrane (14). In addition, based on the insolubility of the receptor at low temperature in Triton X-100 and its low buoyant density in sucrose density gradients, Ko...
et al. (16) provided evidence to suggest that these focal sites represent lipid rafts. To investigate the structural requirements necessary for the localization of CD120a to rafts, we transfected wild-type CD120a into HeLa cells and analyzed the subcellular localization of the receptor by immunofluorescent staining with a hamster anti-mouse CD120a antagonistic mAb and confocal microscopy. As can be seen in Fig. 1, a and b, the wild-type receptor was consistently expressed at focal sites within the plasma membrane. The receptor was also detected in a juxtanuclear position that colocalized with the Golgi marker adaptin-1 (Fig. 1, i–n), as previously reported (8, 14, 21). A similar pattern was also observed when endogenous CD120a was stained in HeLa cells (14), ruling out the possibility of a transfection-associated artifact. We also conducted experiments in which CD120a-transfected HeLa cells were costained with anti-CD120a Ab and with fluorescein-conjugated wheat germ agglutinin in the absence of detergent permeabilization, the focal staining pattern of the receptor was found to be colocalized with areas of the plasma membrane that were stained by wheat germ agglutinin (Fig. 1, o–q). These data suggest that the focal pattern of expression of CD120a represents receptor localization to discrete areas of the plasma membrane, a finding consistent with its localization to lipid rafts (16).
To confirm this conclusion, we also isolated low density Triton X-100 insoluble lipid rafts by sucrose density gradient ultracentrifugation and investigated the distribution of CD120a and markers of lipid rafts (GM₁ and alkaline phosphatase) by Western blotting and catalytic assays. As can be seen in Fig. 1a, CD120a was detected in fractions containing low density lipid rafts as reflected by its colocalization with alkaline phosphatase and GM₁, as well as in fractions containing Triton X-100 soluble membrane material located at the bottom of the gradients. Thus, these findings support the conclusion that CD120a is present at the cell surface in structures consistent with the properties of lipid rafts.

To begin to dissect the structural requirements that target CD120a to lipid rafts, we constructed a C terminus deletion mutant of CD120a (aa 321–425) in which the entire DD was removed. When compared with the wild-type receptor, removal of the DD (CD120a.Δ321-425) resulted in a complete loss of the focal staining pattern (Fig. 1, c and d). Instead, the receptor was consistently detected as a diffuse continuous band that circumnavigated the cell periphery both in the presence and absence of detergent permeabilization (Fig. 1, g and h). The continuous staining pattern of the truncated receptor was also observed to be colocalized with FITC-labeled wheat germ agglutinin (Fig. 1, r–t) confirming its localization to the peripheral plasma membrane. In addition, juxtanuclear staining of the receptor corresponding to the presence of the mutant CD120a in the Golgi complex was also observed (Fig. 1, c, d, and l–n), suggesting that the DD is not required for the localization of CD120a to this organelle. We also subjected CD120a.Δ321-425-transfected HeLa cells to an analysis of the partitioning of the mutant receptor to lipid rafts by sucrose density gradient ultracentrifugation. As can be seen in Fig. 2c; the CD120a.Δ321-425 mutant receptor was present in the Triton X-100 soluble membrane fractions at the bottom of the sucrose density gradients, but was absent from fractions containing the lipid rafts. In contrast, the mutant CD120a.Δ208-308, in which the membrane proximal region was deleted but the DD was preserved, was localized to lipid rafts in a fashion similar to the wild-type receptor (Fig. 2, a and b). Therefore, these results indicate that the DD is required for the appropriate expression of CD120a in lipid rafts.

Next, we questioned whether the localization of CD120a to rafts was associated with a quantitative change in the level of expression of the receptor on the membrane. Wild-type CD120a and the deletion mutant CD120a.Δ321-425 were transfected into HeLa cells, stained for CD120a in the absence of detergent permeabilization, and analyzed by flow cytometry. Membrane expression of the endogenous receptor was not detectable under the conditions used. As expected, transfection of wild-type CD120a was associated with an increase in membrane expression of the receptor (Fig. 1v). As compared with wild-type CD120a, membrane expression of CD120a.Δ321-425 was markedly increased indicating that removal of the DD may lead to increased receptor expression at the cell surface. To confirm these data, we performed a quantitative analysis of the membrane expression of CD120a using immunofluorescence confocal microscopy. Cells were transfected with wild-type CD120a or the deletion mutant CD120a.Δ321-425 and stained for CD120a in the absence of detergent permeabilization. Stacks of 25 images were acquired, deconvolved, and the integrated intensity of fluorescence was determined. The intensity of fluorescence was significantly higher (~17-fold) in cells transfected with CD120a.Δ321-425 than in cells transfected with wild-type CD120a (Mann-Whitney U test = 2, p < 0.0001), consistent with the flow cytometry results. Thus, the DD is involved in the localization of CD120a to membrane rafts, and its deletion is associated with a striking up-regulation of receptor expression at the cell surface.

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** The DD of CD120a is required for raft localization. HeLa cells were transfected with the either a DD deletion mutant (CD120a.Δ321-425), a membrane proximal region deletion mutant (CD120a.Δ208-308) or with wild-type CD120a as a control. The cells were then lysed in 1% Triton X-100 and subjected to raft isolation by sucrose density gradient ultracentrifugation. Fractions were analyzed for CD120a and GM₁ by Western blotting. a, Wild-type CD120a; b, CD120a.Δ208-308; c, CD120a.Δ321-425; d, empty vector used as a transfection control.

An intact CD120a DD is required for localization to rafts and for signaling death

Previous studies on the structural requirements for CD120a signaling have emphasized the importance of specific residues and sequences within the DD. Therefore, we investigated whether the requirement of the DD for CD120a trafficking and raft localization was attributable to a particular region within the DD. The nuclear magnetic resonance structure of the DDs of FADD, Fas, the p75 neurotrophin receptor, and CD120a have shown a conserved three-dimensional structure of the DD comprising six well-conserved α-helices (22, 26). Based on these findings and as shown in Fig. 3a, we designed and created a series of deletion mutants based on homologies between the published amino acid sequences of the various DDs (22, 27). The mutants were then transfected into HeLa cells and their localization was determined by immunofluorescence confocal microscopy in nonpermeabilized cells. As expected, deletion of the entire cytoplasmic domain (CD120a.Δ211-425) prevented the receptor from being focally expressed but promoted its diffuse expression at the plasma membrane (Fig. 3c). Removal of two (CD120a.Δ386-425) or four (CD120a.Δ353-425) of the predicted α-helices within the DD of CD120a also resulted in a complete loss of the focal staining pattern and in the redistribution of the receptor to the bulk plasma membrane (Fig. 3c). Similarly, partial deletion of the two C-terminal DD α-helices (constructs CD120a.Δ391-425, CD120a.Δ399-425, and CD120a.Δ405-425) resulted in the diffuse membrane expression of CD120a and...
a complete loss of the focal expression pattern. In contrast, a short deletion of the C terminus of the receptor (CD120aΔ413-425) in which the DD was preserved did not alter the expression of the receptor as compared with the wild-type CD120a (Fig. 3c).

To test whether deletions within the intracellular domain of CD120a were associated with a decreased ability of the receptor to induce cell death, HeLa cells were transfected with expression vectors encoding wild-type CD120a, or the CD120a deletion mutants. Fourteen hours later, cells were stimulated with TNF-α and cycloheximide (50 ng/ml and 10 μg/ml, respectively) for 4 h, fixed, permeabilized, and the percentage of apoptotic cells among CD120a-transfected cells was quantified by confocal fluorescence microscopy. As expected, transfection of wild-type CD120a induced a robust increase in the proportion of apoptotic cells, both in the presence and in the absence of TNF-α and cycloheximide (Fig. 3b). In contrast, transfection of any of the DD deletion mutants completely abolished apoptosis as compared with that induced by the transfected wild-type receptor, consistent with previous reports (27). Induction of apoptosis was not affected in the short C-terminal deletion mutant in which the DD was still present (CD120aΔ413-425; Fig. 3b). Collectively, these results indicate that the appropriate expression of CD120a in lipid rafts requires the presence of the entire DD, and that this property of the DD parallels its ability to signal apoptosis.

The selective localization of CD120a to lipid rafts is contingent on a functional DD

Several point mutations in the DD of Fas or CD120a have been shown to inactivate the ability of these death receptors to initiate apoptosis. The lymphoproliferation (lpr) mutation of mouse Fas (V238N) (28) has been shown to cause a structural alteration of the molecule, locally unfolding the protein in the region corresponding to the α3 helix of the wild-type protein (29), a region that has been shown to be important for the self association of Fas and for binding to FADD. The lpr mutant is more soluble than wild-type Fas (29), but its phenotype of membrane expression has not been studied. L351 in murine CD120a corresponds to the lpr mutation in Fas, and point mutagenesis of L351 to either Asn or Ala inhibits the cytotoxic signal of CD120a (27). To test the hypothesis that the localization of CD120a to rafts was altered by mutations that inactivate signaling of the DD of CD120a, HeLa cells were transfected with the CD120aL351N point mutant, stained for CD120a, and analyzed by confocal microscopy. As expected, the L351N mutation abolished the apoptosis signal induced by CD120a (Fig. 3b). As compared with wild-type CD120a, CD120aL351N was more diffusely expressed on the cell membrane and the focal pattern was lost in >50% of transfected cells (Fig. 4, a–d). The continuous staining pattern of the receptor was colocalized with the staining of the plasma membrane with wheat germ agglutinin (Fig. 4, h–j). However, the CD120aL351N mutant was still present in the Golgi apparatus, as shown by the colocalization with adaptin-1 (Fig. 4, e–g). These data thus show that the L351N point mutation is sufficient to abolish death signaling and to alter the pattern of CD120a membrane expression, redistributing the receptor from rafts to a more diffuse membranous localization. This suggests that proper folding of the DD is required for the adequate targeting of CD120a to rafts as well as for the initiation of CD120a-mediated apoptosis.

FIGURE 3. Requirement of the DD of CD120a for raft localization and apoptosis. a, C-terminal truncations of CD120a. TM, transmembrane domain; L, Leucine 351. b, Lack of apoptosis induction by DD truncations of CD120a. HeLa cells were transfected with C-terminal truncations of CD120a. Fourteen hours after transfection, cells were stimulated for 4 h with TNF-α (50 ng/ml) and cycloheximide (10 μg/ml), fixed, permeabilized, and stained using anti-CD120a Abs and TUNEL as described in Materials and Methods. The percentage of apoptotic cells was quantified by confocal fluorescence microscopy. Results are mean ± SD. c, Confocal fluorescence microscopy showing punctate or continuous plasma membrane staining of C terminus truncations of CD120a.
The DD is sufficient for the targeting of CD120a to lipid rafts

We next questioned if the DD was sufficient for the targeting of CD120a to lipid rafts. Reported studies have shown that CD120a is expressed throughout the plasma membrane (21). To further investigate the role of the DD in targeting receptors to rafts, we created a chimeric protein, CD120b-(1-462)-CD120a-(308-425), or "CD120b-DD" in which the DD of mouse CD120a (aa 308–425) was fused to the C terminus of CD120b. Transfection of CD120b-DD into COS-7 cells resulted in the expression of a protein of the expected size as detected by Western blotting of whole cell lysates (data not shown) confirming that the chimeric receptor was appropriately expressed. HeLa cells were then transfected with vectors encoding wild-type CD120b or the CD120b-DD chimera, stained using Abs specific for the extracellular domain of CD120b or the C terminus of CD120a, and examined by confocal microscopy. As shown in Fig. 5a, wild-type CD120b was expressed at the cell surface in a predominately peripheral but somewhat punctate fashion, consistent with previous reports (8, 21). In contrast, the CD120b-DD chimeric protein was not uniformly expressed in the plasma membrane and the staining pattern was replaced by a more focal membranous staining pattern similar to that seen with wild-type CD120a (Fig. 5b). In addition, CD120b-DD formed intracytoplasmic tubular structures, similar to those seen following phosphorylation of CD120a by p42mapk/erk2 (14). We also examined the localization of wild-type CD120b and the CD120b-DD chimera to lipid rafts by sucrose density gradient ultracentrifugation. As shown in Fig. 5c, the majority of CD120b was present in the Triton X-100 soluble membrane fractions at the bottom of the gradient with a smaller proportion present in the
lipid rafts. In contrast, the majority of the CD120b-DD chimera was localized to fractions containing the lipid rafts with markedly reduced amounts being found in the Triton X-100 soluble fractions at the bottom of the gradients (Fig. 5c). Thus, the localized expression of CD120b to lipid rafts was greatly enhanced by the presence of the CD120a DD.

Discussion

Although much has been learned about the mechanism of signaling following ligand and Ab-induced cross-linking of CD120a, the findings of the present study suggest that the spatial distribution of this receptor represents a critical component of signaling that was heretofore not fully appreciated. Studies in endothelial cells by Bradley et al. (8) and by Jones et al. (21) have shown that CD120a is predominantly expressed in the trans Golgi network, with only low levels being detected at the plasma membrane. Using differences in staining patterns in nonpermeabilized and detergent-permeabilized HeLa cells, we have shown that CD120a is present both at the cell surface and has confirmed its localization to the Golgi complex (14). Cell surface CD120a was not uniformly expressed in the membrane, but rather was detected at focal sites as reflected by a punctate staining pattern reflective of the staining pattern of proteins that are localized to lipid rafts, an interpretation confirmed by sucrose density gradient separations of Triton X-100 cell lysates. The major finding of the present study is that the appropriate localization of CD120a to lipid rafts is solely attributable to the presence of an intact DD and that the residues within the DD that are necessary for its ability to signal cell death were inseparable from those required for the localization of the receptor to lipid rafts.

The ability of some members of the TNFR superfamily, including CD120a, CD40, and the p75 neurotrophin receptor, to be localized to rafts has only recently been recognized (13–15). Studies by Ko et al. (16) were the first to suggest that TNF-α-induced apoptosis was reduced in cells grown under lipoprotein-deficient conditions, indicating that lipid rafts may be necessary for CD120a-induced apoptosis. It has been suggested that once clustered, receptors may associate more effectively with rafts, thereby enhancing their interaction with raft-associated signaling molecules (12, 30). Thus, the recruitment and/or localization of CD120a to rafts may facilitate its interaction with adaptor molecules and the recruitment of other signaling molecules. Indeed, several components of the CD120a-stimulated signaling pathways have been shown to be activated in lipid rafts, including Ras, c-Raf-1, and p42\textsuperscript{mapk}/erk2 (31). Elegant studies conducted by Liu and Anderson (32) have also revealed that the production of ceramide in response to IL-1β occurs in lipid rafts. In addition, the low-affinity p75 neurotrophin receptor, which also contains a DD, was recently shown to be enriched in caveolin-containing rafts and was coimmunoprecipitated with caveolin (32). Furthermore, neurotrophin-induced hydrolysis of sphingomyelin has been shown to be localized to caveolin-enriched rafts, suggesting that the ligand-induced production of ceramide might also occur in caveolae-like rafts (13). Taken together, these observations suggest that the localization of CD120a to lipid rafts may be important in receptor signaling.

The region of the DD that was necessary for the targeting of the receptor to rafts could not be distinguished from the sequences and individual amino acid residues that account for the induction of cell death. The results of the original studies by Tartaglia et al. (27) suggested that continuous amino acid sequences were not involved in the initiation of signaling by the DD of CD120a, but that individual amino acids became spatially clustered in the three-dimensional structure to provide a “patch” capable of mediating signaling. Recent nuclear magnetic resonance studies have revealed the importance of the electrostatic charge conferred by solvent-exposed basic residues in mediating the interactions between the DDs of CD120a and TRADD (26). Thus, it is tempting to speculate that the intact three-dimensional structure of the DD is also necessary to enable CD120a to be appropriately localized to lipid rafts. This interpretation is consistent with the data reported herein showing that deletions within the six α-helices of the DD of CD120a inhibited the receptor both from associating with rafts and from inducing apoptosis. Similarly, Hsu et al. (4) found that a C-terminal deletion that removed part of the α6 helix, or an internal deletion that removed the α1 helix and part of the loop between the α1 and α2 helices at the N-terminal end of the DD likewise inhibited TRADD binding, implying that small deletions in the DD can have a major effect on signaling. In addition, we have shown that the introduction of the lpr mutation into the DD of CD120a similarly blocked both the induction of apoptosis and localization of the receptor to rafts. Nuclear magnetic resonance studies of the V238N (lpr) mutation of Fas have shown that the α3 helix of the DD becomes unfolded, thereby abolishing the interaction of Fas with FADD (23). Other studies have shown the lpr mutation of Fas also renders the receptor susceptible to solubilization in nonionic detergents in contrast to the relative insolubility of the wild-type Fas protein (29). Interestingly, and unlike the situation in HeLa cells, CD120a is principally localized to the trans Golgi network and is only poorly expressed at the plasma membrane in vascular endothelial cells (8). Recent studies by Gaeta et al. (33) have also shown that the DD is necessary but not sufficient to localize CD120a to the Golgi complex. Our results differ from those of Gaeta et al. (33) in that we found that deletions of or within the DD did not affect the accumulation of CD120a in the Golgi complex. Although seemingly at odds, these divergent findings may suggest that sequences necessary for the spatial localization of CD120a to different cell compartments may differ in divergent cell types, and therefore, may further contribute to the observed heterogeneity of TNF signaling in different cell populations.

The mechanism through which the DD of CD120a promotes raft localization remains to be determined. Studies in B cells have begun to suggest that the recruitment of B cell receptors (BCR) to rafts is initiated by BCR aggregation but occurs in a fashion that is independent of the cytoskeleton (34), possibly occurring as a result of the increased affinity of the aggregated hydrophobic BCR transmembrane regions for the cholesterol/sphingolipid-rich rafts (35). In contrast, the subsequent aggregation of small receptor-containing rafts into larger aggregates has been proposed to require an intact actin-based cytoskeleton (34). A similar view has also been proposed to explain the initial recruitment of the TCR complex into rafts and the subsequent formation of the so-called “immunological synapse” (36, 37). Recent studies with CD120b have suggested that caveolin forms a complex with TNFR-associated factor 2 which in turn interacts with CD120b (38). However, it remains to be determined how the DD of CD120a promotes raft localization of CD120a and what role, if any, is played by the cytoskeleton.

Recent reports have suggested that the signaling of apoptosis by death receptors may be regulated in part by the subcellular localization of these receptors. Studies by Bennett et al. (9) have suggested that Fas traffics between the Golgi complex and the plasma membrane in a p53-dependent fashion, but that signaling competence is only initiated through cell surface Fas. The TNF-related apoptosis-inducing ligand receptors DR4 and DR5 have also been found on the cell surface and in the trans Golgi network, and following exposure to ligand, the receptors traffic to endosomes (10). In addition, TRADD has been shown to rapidly translocate from the Golgi complex to the plasma membrane in response to stimulation with TNF-α (21). In the case of CD120a, we have
demonstrated that phosphorylation of the intracellular domain of CD120a by p42/p44MAPK induces a redistribution of the receptor from the plasma membrane and the Golgi complex to tubular structures located within the endoplasmic reticulum (14). In addition, the recruitment of phosphorylated CD120a to the tubular structures results in the corecruitment of Bcl-2 (39). Phosphorylation of CD120a by p42/p44MAPK occurs within the membrane proximal region (40), indicating that different regions of the receptor affect its intracellular trafficking and localization. Of note, caveolin has been shown to directly traffic from the plasma membrane to the endoplasmic reticulum in response to cholesterol oxidation in a microtubule-dependent manner (41). Conceivably, the presence of CD120a within lipid rafts might account for the yet unexplained retrograde trafficking of phosphorylated CD120a from the plasma membrane and Golgi complex to the endoplasmic reticulum.

In conclusion, the results of the present study indicate that CD120a is expressed in lipid rafts as well as in the Golgi complex and we have shown that an intact DD is both necessary and sufficient for the restricted expression of CD120a within rafts. Therefore, these findings suggest that: 1) an appropriately folded DD is necessary for raft localization and signaling, and 2) that the previously observed residues defined as being necessary for apoptosis also participate in the localization of the receptor to lipid rafts. Recent studies have also shown that the sequences necessary for the initiation of programmed cell death are indistinguishable from those required for DD self association and TRADD binding (26). Thus, it is conceivable that these signaling components are assembled in lipid rafts following ligand binding (4, 42, 43), and that these structures represent the site of signaling at least for the induction of apoptosis.

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