ABSTRACT

The lateral border recycling compartment (LBRC) is a reticulum of perijunctional tubulovesicular membrane that is continuous with the plasmalemma of endothelial cells and is essential for efficient transendothelial migration (TEM) of leukocytes. The LBRC contains molecules involved in TEM, such as PECAM, PVR and CD99, but not VE-cadherin. Despite its importance, how membrane proteins are included in or excluded from the LBRC is not known. Immunoelectron microscopy and biochemical approaches demonstrate that inclusion into the LBRC is the default pathway for transmembrane molecules present at endothelial cell borders. A chimeric molecule composed of the extracellular domain of VE-cadherin and cytoplasmic tail of PECAM (VE-CAD/PECAM) did not enter the LBRC, suggesting that VE-cadherin was excluded by a mechanism involving its extracellular domain. Deletion of the homophilic interaction domain EC1 or the homophilic interaction motif RVDAE allowed VE-CAD/PECAM and even native VE-cadherin to enter the LBRC. Similarly, treatment with RVDAE peptide to block homophilic VE-cadherin interactions allowed endogenous VE-cadherin to enter the LBRC. This suggests that homophilic interactions of VE-cadherin stabilize it at cell borders and prevent entry into the LBRC.

KEY WORDS: VE-cadherin, Lateral border recycling compartment, Membrane compartmentalization, Sorting signals, Endothelial cells

INTRODUCTION

Efficient cell function depends upon segregation and compartmentalization of membrane domains (Furuse et al., 2002; Shin et al., 2006). The specialized membrane composition of the apical and basolateral surfaces of polarized epithelial cells is maintained by selective membrane delivery and retention at these surfaces. Even within a membrane, microdomains such as lipid rafts and caveolae efficiently compartmentalize specific functions locally (Mukherjee and Maxfield, 2004; Ushio-Fukai, 2009). The lateral borders of endothelial cells are specialized regions containing adhesion and signaling molecules related to maintaining barrier function (Dejana and Vestweber, 2013; Mehta and Malik, 2006), sensing shear (Tarbell, 2010; Tzima et al., 2005) and allowing immune surveillance (Ley et al., 2007; Muller, 2011). There is a subcompartment of junctional membrane, the lateral border recycling compartment (LBRC), which is crucial for promoting transendothelial migration (TEM) of leukocytes.

Previous studies have demonstrated that the LBRC plays a crucial role in TEM under all inflammatory conditions studied (Mamdouh et al., 2003; Mamdouh et al., 2008; Mamdouh et al., 2009). The LBRC is a reticulum of interconnected membrane below the cell borders. It is defined by its function, as there are no identified unique markers (Sullivan et al., 2014). Membrane constitutively cycles between the border and this compartment evenly along the junctions between endothelial cells. However, when leukocytes transmigrate, LBRC membrane is actively trafficked along microtubules to surround the leukocyte at the site of TEM (Mamdouh et al., 2003; Mamdouh et al., 2008; Mamdouh et al., 2009; Muller, 2011). Another unique feature of the LBRC is that major molecules regulating TEM, including platelet/endothelial cell adhesion molecule (PECAM, CD31), CD99, poliovirus receptor (PVR; CD155) and junctional adhesion molecule A (JAM-A), all reside in the LBRC and display the same targeted recycling pattern during leukocyte TEM. Blockade of PECAM, PVR or CD99, or inhibition of kinesin molecular motors, disrupts the targeted recycling of the LBRC and thus blocks TEM (Dasgupta and Muller, 2008; Mamdouh et al., 2003; Mamdouh et al., 2008; Mamdouh et al., 2009; Muller, 2011; Sullivan et al., 2013).

Vascular endothelial cell specific cadherin (VE-cadherin), a major component of the endothelial cell border, is not present in the LBRC (Mamdouh et al., 2009; Sullivan et al., 2014). VE-cadherin plays a central role in the formation and maintenance of adherens junctions and barrier function in endothelium (Dejana and Giampietro, 2012; Dejana et al., 2008; Vestweber et al., 2009). It is exclusively expressed in endothelium and localized to the intercellular border. Like other cadherins, VE-cadherin contains five homologous extracellular domain repeats, (EC1–5) (Dejana et al., 2008; Vestweber, 2008; Vincent et al., 2004). The extracellular domains mediate homophilic binding of VE-cadherin between adjacent cells and stabilize the adherens junction. It has been well demonstrated that the EC1 domain is the core element required for the homophilic interaction of VE-cadherin (Dejana et al., 2008; Vestweber, 2008; Vincent et al., 2004).

Despite its crucial importance in the process of TEM, the membrane composition and selection mechanism for molecules residing in the LBRC is unknown. Here, we have approached this issue by studying PECAM, the best known component of the LBRC. We hoped that understanding how PECAM entered the LBRC would provide clues to how other components enter. Previous studies have demonstrated that mutation of tyrosine 663 in the PECAM cytoplasmic tail to phenylalanine interferes with the efficient movement of PECAM between the endothelial cell border and the LBRC (Dasgupta et al., 2009). However, standard signaling functions of PECAM were not altered (Dasgupta et al., 2009). This suggests the possibility that, similar to some other

RESEARCH ARTICLE

Segregation of VE-cadherin from the LBRC depends on the ectodomain sequence required for homophilic adhesion

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cytoplasmic tail tyrosine motifs (Lipardi et al., 2002; Rohrer et al., 1996), there is a recognition signal on the cytoplasmic tag of PECAM for inclusion into the LBRC.

Here, we have tested this hypothesis by expressing chimeric molecules containing the cytoplasmic tail of PECAM and the extracellular domains of other transmembrane proteins. Although the cytoplasmic domain of PECAM enhanced the ability of chimeric molecules to get to the lateral border, it was not necessary to bring exogenous molecules into the LBRC. Contrary to our hypothesis, we found that entrance into the LBRC was a default pathway for molecules present on the endothelial cell borders. Moreover, even endogenous molecules that enter the LBRC move with it as the LBRC traffics to surround transmigrating leukocytes. By contrast, VE-cadherin was excluded, apparently by homophilic interactions with other VE-cadherin molecules at the cell surface.

**RESULTS**

PECAM does not use a positive sorting signal to enter the LBRC

If the cytoplasmic tail of PECAM contained a sorting motif to direct it to the LBRC, we hypothesized that, when expressed on a molecule not normally found in the LBRC, it could direct that molecule into the LBRC. To test this hypothesis, we constructed a chimera that contained the transmembrane and cytoplasmic domains of PECAM fused to the extracellular domain of Tac (also known as interleukin 2 receptor α chain or CD25), a protein not expressed by endothelial cells (Fig. 1).

In order to compare endothelial cells consistently expressing the same levels of chimera, we made stable transfectants in immortalized human umbilical vein endothelial cells (iHUVECs), which faithfully recapitulate the known characteristics of primary endothelial cells (Ancuta et al., 2003; Yang et al., 2005; Yang et al., 2006). Both iHUVECs and HUVECs displayed the same levels of chimera, we made stable transfectants in

**A Wild type**

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**B Constructs**

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<th>Flag–VE–CAD</th>
<th>Flag–ΔN</th>
<th>Flag–ΔRVDAE</th>
<th>Flag–ΔEC1</th>
<th>Flag–VE–CAD/PECAM</th>
<th>Flag–ΔN/PECAM</th>
<th>Flag–ΔRVDAE/PECAM</th>
<th>Flag–ΔEC1/PECAM</th>
<th>D1,2-Tac</th>
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**Construct Name**

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**Fig. 1. Schematic diagram of constructs.**

Tac/PECAM, chimera contains extracellular and transmembrane domain of Tac and cytoplasmic tail of PECAM. FLAG–VE–CAD, full-length wild-type VE-cadherin with integrated FLAG peptide at the N-terminus. FLAG–ΔN, truncated FLAG–VE–CAD with deletion of residues 1–46 on the extracellular domain of VE-cadherin. FLAG–ΔRVDAE, truncated FLAG–VE–CAD with deletion of residues 1–51 (including the RVDAE motif, residues 47–51) on the extracellular domain. FLAG–ΔEC1, truncated FLAG–VE–CAD with deletion of residues 1–104 (the full length of the EC1 domain) from the extracellular domain. FLAG–VE–CAD/PECAM, FLAG–ΔN/PECAM, FLAG–ΔRVDAE/PECAM and FLAG–ΔEC1/PECAM are the corresponding chimeras with replacement of the C-terminal tail from PECAM. D1,2–Tac or D1,2–Tac/PECAM, full-length Tac or Tac/PECAM with IgG Domain 1 and 2 (D1,2) from the extracellular part of PECAM on the N-terminus.
only localize partially to junctions, but also enter the LBRC (Fig. 2D), consistent with the hypothesis that the cytoplasmic tail of PECAM contains a sorting signal for entry. Surprisingly, even though Tac was expressed diffusely over the cell (Fig. 2C), it was still found in the LBRC (Fig. 2D).

Although immuno-EM is the gold standard for identifying LBRC components, it only examines a small fraction of the cell borders. To determine whether our results were representative of the entire cell population, we exploited the fact that antibodies are unable to enter the LBRC at 4°C. We previously used an immunoprecipitation assay to examine the distribution of phosphorylated PECAM between the surface and LBRC (Dasgupta and Muller, 2008). In the current study, we applied a similar rationale to develop a sequential immunoprecipitation assay (see Materials and Methods) to determine whether a membrane protein was in the LBRC. Using this assay we can readily distinguish between the surface and LBRC pools of PECAM (Fig. 3A). Consistent with our previous observation, about a third of total PECAM is in the LBRC (Dasgupta and Muller, 2008; Mamdouh et al., 2003). By contrast, VE-cadherin, which has been shown previously to be absent from the LBRC (Mamdouh et al., 2009; Sullivan et al., 2013), was almost undetectable in the LBRC (Fig. 3A).

This assay cannot distinguish between pools protected in the LBRC and internal pools (i.e. proteins found in endosomes or late-stage biosynthetic precursors) that might be more abundant for exogenously expressed proteins. To control for this, we treated cell monolayers with NHS-sulfo-LC-biotin, a membrane-impermeant biotinylation reagent. Our previous work has shown that the LBRC membrane is continuous with the plasma membrane and accessible to small compounds, but not Fab fragments of antibody, at 4°C (Mamdouh et al., 2003). Therefore, the surface and LBRC pools but not the internal pools of the target protein should be biotinylated and detected (Fig. 3B). This modification ensures that we detect the protein that is only on the surface and LBRC but not in the internal pools. This method measures the distribution at steady state, not at a time after incubation with antibody. Furthermore, given that the labeling is performed in the absence of antibodies, this verifies that the compartmentalization of membrane is not influenced by interaction with the antibodies. Modification with biotin does not affect the outcome; the distribution of VE-cadherin and PECAM are similar in both assays (compare Fig. 3A,B). The trace amount of VE-cadherin sometimes detectable in the LBRC fraction might represent residual surface molecules that escaped binding by the antibody in the first round, because no more protected VE-cadherin was seen when we used biotin to restrict labeling to surface and LBRC (Fig. 3B).

Using this assay, we examined the ability of Tac/PECAM and Tac to enter into the LBRC. As shown by immuno-EM, both Tac/PECAM (Fig. 3C) and Tac (Fig. 3D) are clearly detectable in the LBRC. The presence of PECAM in the LBRC was used as a control to ensure that the LBRC was properly formed in these two (Fig. 3C,D) and all other transfectants used in this assay. Apparently, molecules of Tac that were present at the cell borders were capable of entering. This was not consistent with our original hypothesis, and instead suggests that any protein that can get to the endothelial cell border can potentially enter the LBRC.

**Exogenous LBRC molecules traffic along with endogenous molecules**

Tac/PECAM and Tac were found in compartments resembling the LBRC, but do these compartments function like part of the
LBRC? Furthermore, although it is assumed that all proteins that localize to the LBRC traffic together, there has never been any direct demonstration of this, because we do not have antibodies against any of the known LBRC constituents, except PECAM, that do not block LBRC function and transmigration. The presence of exogenous Tac in the LBRC gave us the opportunity to test this. The targeted recycling assay (Mamdouh et al., 2003) tracks membrane coming from the LBRC to surround a leukocyte during TEM. When we performed this assay on Tac/PECAM and Tac, neither construct was enriched around the migrating leukocyte (Fig. 4E). We speculated that the constructs were recycled with the LBRC, but did not remain enriched around the leukocyte because they had no cognate binding partner on the leukocyte and are outcompeted by molecules that do. To provide a functional interaction with a transmigrating leukocyte, we modified the Tac/PECAM and Tac constructs to each contain IgG Domain 1 and 2 (D1,2) from the extracellular part of PECAM, which is known to facilitate homophilic interactions, to make chimeras D1,2-Tac/PECAM and D1,2-Tac, and we then established transfectants (Fig. 4A). As expected, both constructs were largely localized to the junction because they now bore the homophilic interaction domain of PECAM (Fig. 4B). By utilizing the sequential immunoprecipitation assay, we also observed that both constructs entered the LBRC and that the distribution of PECAM and VE-cadherin between the surface and LBRC fraction. VE-cadherin or PECAM were biotinylated, labeled, separated and detected by using HRP–streptavidin. (C) Tac/PECAM is in the LBRC fraction. VE-cadherin, Tac/PECAM and PECAM in the transfectant were analyzed by the method described in B. (D) Tac is in the LBRC fraction. VE-cadherin, Tac and PECAM in the transfectant were analyzed by the method described in B. Quantitative data show the mean±s.d.; **P<0.01 versus PECAM in the LBRC fraction.

Fig. 3. The sequential immunoprecipitation assay shows that both Tac/PECAM and Tac are in the LBRC. (A) PECAM but not VE-cadherin is in the LBRC. VE-cadherin or PECAM without biotinylation on the surface or LBRC was labeled, separated by sequential immunoprecipitation and detected by using specific antibodies. Left, western blots; right, densitometric analysis. The abundance of protein in the surface:LBRC fraction is presented as a percentage of the total amount of protein. EC, endothelial cell. (B) Biotinylation does not change the distribution of PECAM and VE-cadherin between the surface and LBRC fraction. VE-cadherin or PECAM were biotinylated, labeled, separated and detected by using HRP–streptavidin. (C) Tac/PECAM is in the LBRC fraction. VE-cadherin, Tac/PECAM and PECAM in the transfectant were analyzed by the method described in B. (D) Tac is in the LBRC fraction. VE-cadherin, Tac and PECAM in the transfectant were analyzed by the method described in B. Quantitative data show the mean±s.d.; **P<0.01 versus PECAM in the LBRC fraction.
junctions, PECAM, D1,2-Tac/PECAM and D1,2-Tac were diffusely distributed on the cell surface (supplementary material Fig. S3). Upon crosslinking of PECAM using a monoclonal antibody (mAb) that binds to domain 5 but not D1,2, the native PECAM on the cell surface formed many concentrated foci (supplementary material Fig. S3B,D). However, D1,2-Tac (supplementary material Fig. S3B) and D1,2-Tac/PECAM (supplementary material Fig. S3D) did not move into these foci, but remained diffusely localized. Therefore, we conclude that targeted recycling of Tac from the LBRC to the site of
transmigration represents its intrinsic tendency to move with the LBRC.

**VE-cadherin exclusion from the LBRC does not require its cytoplasmic tail**

Our observation that even exogenous proteins gain access to the LBRC suggests that entrance into the LBRC is a default pathway. Because VE-cadherin is the only junctional transmembrane molecule known to be excluded from the LBRC, we investigated the mechanism behind this exclusion. VE-cadherin is connected to the actin cytoskeleton through the p120- and β-catenins that bind to specific phosphorylated tyrosine residues (Y658 and Y731, respectively) on its cytoplasmic tail (Potter et al., 2005).

To examine the role of the cytoplasmic tail in the exclusion of VE-cadherin from the LBRC, we established cell lines expressing N-terminally FLAG-tagged full-length VE-cadherin (FLAG–VE-CAD) and VE-cadherin with the cytoplasmic tail of PECAM (FLAG–VE-CAD/PECAM). The presence of the FLAG epitope enables us to distinguish them from endogenous VE-cadherin. Flow cytometry (Fig. 5A) and western blotting (Fig. 5B) showed that both constructs were expressed on the surface of endothelial cells at approximately the same level. Immunofluorescence staining showed that both proteins localized to the junction (Fig. 5C). Expression of FLAG–VE-CAD or FLAG–VE-CAD/PECAM did not influence PECAM expression (supplementary material Fig. S2E) or TEM (supplementary material Fig. S2F). Our use of only a single FLAG sequence in these constructs might have helped prevent changes in VE-cadherin adhesion.

We then examined both constructs by using anti-FLAG antibodies in immuno-EM studies (Fig. 5D). As expected, FLAG–VE-CAD was only observed at the junction and not found in typical LBRC vesicular structures. FLAG–VE-CAD/PECAM had the same distribution despite the PECAM cytoplasmic tail it contained. We further utilized the sequential immunoprecipitation assay to verify the electron microscopy findings. Biotinylation interfered with the ability of the anti-FLAG antibody to recognize the epitope, presumably due to the two lysine residues in the FLAG sequence. Our attempts to immunoprecipitate FLAG-tagged proteins and detect them in western blots with the commercial anti-FLAG antibodies were unsuccessful. Therefore, we used the anti-VE-cadherin antibody for both native VE-cadherin and its corresponding FLAG-tagged constructs (Figs 5, 7). Note that anti-VE-cadherin antibody cannot distinguish native VE-cadherin from the corresponding full-length or truncated FLAG–VE-CAD constructs owing to the similarities in their molecular masses (Fig. 5E; Fig. 7A,C,E). The anti-VE-cadherin antibody can distinguish native VE-cadherin from all FLAG–VE-CAD/PECAM constructs (Fig. 5F; Fig. 7B,D,F). Both FLAG–VE-CAD (Fig. 5E) and FLAG–VE-CAD/PECAM were not detected in the LBRC (Fig. 5E,F). This observation is consistent with our immuno-EM findings. Taken together, these findings suggest that the cytoplasmic domain of VE-cadherin is not necessary for its exclusion from the LBRC.

**Homophilic interactions of VE-cadherin extracellular domain 1 prevent its entry into the LBRC**

Having ruled out the possibility that the cytoplasmic tail of VE-cadherin is crucial to its exclusion from the LBRC, we next investigated the role of the extracellular domain. Because the N-terminal cadherin domain, EC1, particularly the pentapeptide motif RVDAE within EC1, of VE-cadherin has been reported to facilitate intercellular homophilic interactions (Dejana et al., 2008; Heupel et al., 2009; Komarova et al., 2012; Vestweber, 2008; Vincent et al., 2004), we first focused on this region. We prepared a series of constructs in which we sequentially deleted portions of the VE-cadherin EC1 in the FLAG–VE-CAD and FLAG–VE-CAD/PECAM chimeras. More specifically, we made constructs with deletion of the entire EC1 (FLAG–ΔEC1 and FLAG–ΔEC1/PECAM lacking residues 1–104 of VE-cadherin), deletion of up to and including the RVDAE motif (FLAG–ΔRVDAE and FLAG–ΔRVDAE/PECAM, lacking residues 1–51 of VE-cadherin) and deletion up to the RVDAE, leaving these amino acids intact (FLAG–ΔN and FLAG–ΔN/PECAM, lacking residues 1–46 of VE-cadherin).

We first tested the ability of FLAG–VE-CAD (full length and the construct without RVDAE motif) to engage in homophilic adhesion. L cells were transfected with FLAG–VE-CAD, FLAG–ΔRVDAE or empty pBABE-puro (as control). Flow cytometry showed that the expression of FLAG–VE-CAD and FLAG–ΔRVDAE was comparable among the transfectants (supplementary material Fig. S4A). In the classic aggregation assays, which first defined cadherin function (Takeichi, 1990), the ability of a molecule to support adhesion is assessed by expressing it in cells and determining whether its expression imparts on these cells the ability to aggregate (Albelda et al., 1991; Bronner-Fraser, 1985; Muller et al., 1992; Takeichi, 1990). As seen in supplementary material Fig. S4B, expression of FLAG–VE-cadherin in L cells imparts on them the ability to aggregate. However, deletion of the RVDAE motif of VE-cadherin significantly impaired this ability and only background levels of aggregation were seen (supplementary material Fig. S4B). Experiments using mixtures of fluorescently labeled and unlabeled transfected and non-transfected cells confirmed that FLAG–VE-cadherin aggregation was homophilic, whereas the small numbers of aggregates seen with FLAG–ΔRVDAE transfecteds were mixtures of transfected and non-transfected cells (data not shown).

Next, endothelial cell transfectants expressing each of these truncated constructs were created and validated using flow cytometry (Fig. 6A) and immunofluorescence (Fig. 6B). All chimeras were expressed on the cell surface, all at roughly double the level of endogenous VE-cadherin, and all were mostly localized to intercellular junctions. Our anti-VE-cadherin mAb, hec1, recognizes the EC4 domain (Corada et al., 2001) and thus, its ability to bind to the truncated constructs is not affected. Similar to other constructs used, truncated FLAG–VE-CAD and FLAG–VE-CAD/PECAM constructs did not interfere with PECAM expression (supplementary material Fig. S2G) and leukocyte TEM (supplementary material Fig. S2H).

To examine the ability of these constructs to enter the LBRC, we performed the immuno-EM studies by labeling cells with horseradish peroxidase (HRP)-conjugated anti-FLAG antibodies (Fig. 6C). All constructs that lacked the RVDAE motif were readily detected in LBRC-like structures (black arrows), including truncated native VE-cadherin, whereas the constructs with the RVDAE motif (FLAG–ΔN and FLAG–ΔN/PECAM) were only detected at the junction whether they bore the cytoplasmic tail of VE-cadherin or PECAM, even though unstained LBRC-like structures were observed in these samples (white arrows).

To confirm these findings, we performed the sequential immunoprecipitation assay as described above. As seen in Fig. 7, all the constructs that lack the EC1 domain or RVDAE...
motif had significant proportions detected in the LBRC fractions, consistent with LBRC localization (Fig. 7C–F). Densitometric analysis from FLAG–EC1/PECAM and FLAG–RVDAE/PECAM showed that they had the same distribution pattern as PECAM – about a third of chimera is in the LBRC (Fig. 7D,F).

For FLAG–ΔEC1 and FLAG–ΔRVDAE, because the close molecular mass does not allow us to distinguish endogenous VE-cadherin from exogenous FLAG constructs, the densitometric analysis actually compares the portion of FLAG proteins in the LBRC to the combination of FLAG proteins and native VE-cadherin on the surface. Because native VE-cadherin is only on the surface, the percentage of FLAG proteins in the LBRC fraction is therefore an underestimate. Densitometric analysis is consistent with this idea in that their percentage in the LBRC is lower compared to PECAM (Fig. 7C,E) but clearly demonstrates the presence of VE-cadherin lacking the RVDAE motif in the LBRC. The two truncation constructs that still contain the RVDAE motif were excluded from the LBRC (Fig. 7A,B), a finding consistent with our immuno-EM results (Fig. 6C).

To further verify these findings, we tested whether interrupting homophilic interactions of native VE-cadherin would allow it to enter the LBRC. It has been reported that the blocking peptide...
(RVDAE) but not the scrambled peptide (ADVRE) can interrupt the homophilic interaction of VE-cadherin in other *in vitro* models (Heupel et al., 2009; Komarova et al., 2012). We measured the distribution of VE-cadherin in wild-type endothelial cells treated with blocking peptide or scrambled peptide. Because we had to maintain monolayer integrity, we had to use submaximal blocking conditions. Nevertheless, blocking the RVDAE motif did cause significant amounts of VE-cadherin to

Fig. 6. Extracellular domain 1 of VE-cadherin and its RVDAE motif prevent entry of the molecule into the LBRC. Expression and distribution of truncated FLAG–VE-CAD (FLAG–ΔN, FLAG–ΔRVDAE and FLAG–ΔEC1) and FLAG–VE-CAD/PECAM (FLAG–ΔN/PECAM, FLAG–ΔRVDAE/PECAM and FLAG–ΔEC1/PECAM) were analyzed by (A) flow cytometry and (B) immunofluorescence. (A) Cells were stained with anti (α)-FLAG or anti-VE-cadherin antibodies. Non-filled red curve, isotype control (CT); filled blue curve, labeled cells. (B) Cells were stained with anti-FLAG (red in merge) and anti-VE-cadherin (green in merge). Scale bar: 10 μm. (C) FLAG–VE-CAD and FLAG–VE-CAD/PECAM without RVDAE motif or EC1 domain can enter into the LBRC. Cells were treated with the HRP-conjugated anti-FLAG antibody at 37°C and processed for immuno-EM. Black arrows, LBRC-interconnected vesicles visualized by diaminobenzidene staining. White arrows indicate what are likely to be unstained LBRC vesicles. Scale bar: 1 μm. Data are representative at least nine images of each sample. For FLAG–ΔN, FLAG–ΔRVDAE and FLAG–ΔEC1 cells, 180.9, 150.1 and 158.5 μm of the membrane at junctions was examined, respectively. For FLAG–ΔN/PECAM, FLAG–ΔRVDAE/PECAM and FLAG–ΔEC1/PECAM cells, 192.2, 168.1 and 172.4 μm of membrane at junctions was examined, respectively.
enter the LBRC (Fig. 7G–I). We also observed that under these conditions the blocking peptide but not scrambled peptide increases monolayer permeability (supplementary material Fig. S4C). Taken together these findings suggest that VE-cadherin is excluded from the LBRC by means of the RVDAE motif in EC1.

**DISCUSSION**

We hypothesized the cytoplasmic tail of PECAM carried a recognition motif for selective sorting into the LBRC. Our findings indicate that the transmembrane and cytoplasmic tail of PECAM can at least partially concentrate a protein at the LBRC.
endothelial cell border. However, ultrastructural studies showed that Tac, a molecule not expressed on endothelium, could enter into the LBRC when exogenously expressed, even though it did not show any junctional enrichment (Fig. 2). This finding suggested that even though the transmembrane and cytoplasmic tail of PECAM might have some role in directing molecules to the endothelial cell border, it is not required to bring the molecule into the LBRC. All of the known membrane constituents of the LBRC are molecules concentrated at the endothelial cell borders. Therefore, these molecules are quantitatively more likely to enter the LBRC. However, our data suggest that any molecule that finds itself at the endothelial border might enter the LBRC unless it is actively excluded. These results might explain why we did not find any conserved sequence motifs on the cytoplasmic tails of other known LBRC proteins (PVR, CD99 and JAM-A).

There are clearly additional factors that influence the efficiency by which proteins might get into the LBRC. One is obvious – proteins concentrated at the cell borders are more likely to get in because of their proximity. PECAM is more concentrated at the endothelial borders than Tac/PECAM because homophilic interactions with molecules on the surface of the apposing cell keep it there by diffusion trapping (Sun et al., 2000). Tac/PECAM might be more concentrated at the endothelial borders than Tac by virtue of its interaction with native PECAM through the cytoplasmic tail. Differences in the rate at which membrane proteins transit through the LBRC could also potentially affect their distribution on the surface versus LBRC. In a previous study, we showed that substitution of phenylalanine for tyrosine at position 663 in the cytoplasmic tail of PECAM decreases the efficiency of trafficking of PECAM into and out of the LBRC (and hence of targeted recycling required for leukocyte transmigration), but does not totally eliminate it (Dasgupta et al., 2009). During the constitutive recycling assays performed in our previous study, endothelial cells were exposed to antibody for 1 hour, which was sufficient time to saturate native PECAM, but might not have been enough time for a slowly recycling mutant. In the present study, we show the distribution of membrane proteins at steady state, so potential differences in recycling rate do not affect our conclusions.

Because our data failed to demonstrate a positive sorting mechanism to bring membrane proteins into the LBRC, we next directed our attention to understanding how they might be excluded from the LBRC. VE-cadherin is the only known transmembrane protein at the endothelial border that is not in the LBRC. A variety of previous studies suggest the cytoplasmic tail of VE-cadherin is involved in regulation of endocytosis and downstream signaling by virtue of its interaction with native PECAM through the N-terminus displayed the same targeted recycling pattern as VE-cadherin. Comparing the cytoplasmic tail of VE-cadherin with that of PECAM did not allow the chimera to enter the LBRC (Fig. 5). Thus, the cytoplasmic tail of VE-cadherin is not required for the exclusion of VE-cadherin from the LBRC. Furthermore, all of the VE-cadherin constructs in this study contained the transmembrane domain of VE-cadherin, whether or not they were able to enter the LBRC. Therefore, the transmembrane domain is not sufficient for sorting into the LBRC. However, we cannot formally rule out that the cytoplasmic tail of VE-cadherin might play an additional role in exclusion from the LBRC.

We next turned our attention to the EC1 domain of VE-cadherin, which mediates homophilic interactions (Ahrens et al., 2003; Corada et al., 2001; Hewat et al., 2007; Legrand et al., 2001). The RVDAE motif of the EC1 domain, a pentapeptide that is relatively specific for VE-cadherin, has recently been identified as the core element for the homophilic interaction of VE-cadherin. Eliminating the RVDAE motif or blocking it with the inhibitory peptide significantly inhibits VE-cadherin trans-interaction, as shown in the current study (supplementary material Fig. S4) and in previous reports (Heupel et al., 2009; Komarova et al., 2012). Our findings suggest that the EC1 domain and its RVDAE motif regulate the subcellular localization of VE-cadherin and block it from entering the LBRC (Figs 6, 7). It was somewhat surprising that VE-cadherin without its cytoplasmic domain bound tightly enough by its homophilic interaction domain to exclude it from the LBRC. However, native VE-cadherin was able to enter the LBRC when homoepic VE-cadherin interactions were blocked by incubation with RVDAE peptide (Fig. 7G–I). These data demonstrate that exclusion of VE-cadherin from the LBRC depends on the integrity of its homophilic interaction domain and they strongly suggest that homophilic adhesions (in trans) keep VE-cadherin out of the LBRC.

This begs the question of how the VE-cadherin mutants lacking RVDAE are able to be concentrated at the cell junctions. We do not know, but suspect that interactions of the VE-cadherin cytoplasmic tail (or the PECAM cytoplasmic tail for the chimeric molecules) with elements of the cytoskeleton favor its association at the junctional plasmalemma, much as Tac/PECAM chimeras are concentrated at the cell borders. Furthermore, homophilic interactions in cis (Dejana and Giampietro, 2012; Hewat et al., 2007) might stabilize them at the plasmalemma. In our studies, native VE-cadherin was present for these mutated molecules to interact with. However, the interactions in cis in the absence of the trans homoepic interactions are apparently not enough to prevent internalization into the LBRC. This also raises the question of how PECAM, CD99 and JAM-A, all of which normally engage in homophilic interactions with apposing cells, are not excluded from the LBRC? Again, we do not know. However, these molecules do not engage in extensive cis interactions and their homophilic trans interactions are relatively weak. Therefore, disengaging them would be much easier than for VE-cadherin.

In addition, we were able to demonstrate, using our Tac and Tac/PECAM chimeras, that all LBRC molecules participate in targeted recycling and move in concert to surround the transmigrating leukocyte. Owing to the technical constraints, this could not be demonstrated previously. Tac and Tac/PECAM carrying the PECAM homophilic interaction domains (D1,2) at the N-terminus displayed the same targeted recycling pattern as PECAM. Whereas the D1,2 Tac/PECAM chimera could have been drawn there by association with the cytoplasmic tail of endogenous PECAM, the D1,2 Tac chimera has no association and the PECAM extracellular domains attached to it would not be expected to be in a position to physically associate with endogenous PECAM, because they would be too close to the membrane, adjacent to endogenous PECAM domains 5 and 6. Furthermore, control experiments show no physical interaction of the chimeras with native PECAM (supplementary material Fig. S3). It is important to reiterate that in these experiments, chimeric or FLAG-tagged molecules were introduced into endothelial cells containing normal levels of VE-cadherin, PECAM, etc., so that the integrity of the junctions and of the LBRC would be maintained and we would observe only an effect on the partitioning of exogenous molecules into the LBRC (and be able to compare this with the localization of endogenous
molecules as internal controls), rather than a substitution of these molecules for the normal components. Indeed, TEM was not affected by these manipulations.

While the membrane of the LBRC moves to surround the leukocyte as it transmigrates, VE-cadherin, which maintains barrier function at the adherens junction, leaves the site of transmigration (Allport et al., 1997; Allport et al., 2000; Shaw et al., 2001). VE-cadherin is excluded from the LBRC. This difference in protein distribution might have a direct impact on endothelial cell function during TEM. VE-cadherin might be internalized into clathrin-coated vesicles (Xiao et al., 2005) and removed from the site of TEM as recycling LBRC membrane moves in, or it might be pushed aside in the plane of the membrane (Allport et al., 1997; Allport et al., 2000; Shaw et al., 2001). Both the influx of LBRC and efflux of VE-cadherin are required for TEM to occur. How their movements are coordinated is the subject of ongoing investigation.

**MATERIALS AND METHODS**

**Antibodies, peptides and endothelial cells**

Monoclonal antibodies (mAbs), hec1 (anti-VE-cadherin), hec2 (anti-CD99), hec7 (anti-PECAM) and IB4 (anti-CD18) were generated as described previously (Ali et al., 1997; Muller et al., 1989; Schenkel et al., 2002). mAb 7G7B6 (anti-Tac) (hybridoma provided by Dr Andrew Kowalczyk; Emory University, Atlanta, GA) and polyclonal Ab 301 (anti-cytoplasmic tail of PECAM) were generated in-house. Non-blocking anti-PECAM mAb P1.1 was provided by Dr Peter Newman (Medical College of Wisconsin, Milwaukee, WI). Anti-FLAG antibody M2 was from Sigma-Aldrich and anti-Tac antibody 2R12 was from Thermo Scientific. Fab were cut from P1.1 or 7G7B6 by using immobilized papan (Thermo Scientific). Antibodies for immunofluorescence were coupled to Alexa Fluor 488 and Alexa Fluor 546 (Invitrogen). The blocking peptide RVDAE and scrambled peptide ADVRE were obtained from Dr Yulia Komarova (University of Illinois at Chicago, Chicago, IL).

iHUVECs were made by transduction of HUVECs with LSNX-16E6E7, an amphotrophic retrovirus encoding the oncoproteins E6 and E7 of human papillomavirus type 16 (provided by Dr David Klumpp; Emory University, Atlanta, GA) and polyclonal Ab 301 (anti-cytoplasmic tail of PECAM) were generated in-house. Non-blocking anti-PECAM and its corresponding truncated constructs, the extracellular and transmembrane domain of VE-cadherin were linked to the cytoplasmic tail of PECAM through BamHI. For FLAG proteins, an expression signal containing signal peptide and propeptide of VE-cadherin, plus a FLAG peptide, was generated by PCR and linked to the N-terminal region of the extracellular domain through XbaI. For D1.2 Tac/PECAM and D1.2 Tac, the signal peptide and IgG-like domain 1 and 2 of PECAM was linked to the extracellular domain of Tac/PECAM or Tac through KpnI. Retrovirus carrying these constructs was transduced into iHUVECs or L cells. Stable cell lines were established under puromycin selection.

**Flow cytometry**

Cells were removed from the plate with Hanks’ Balanced Salt Solution (HBSS) containing 10 mM EDTA. The cell suspension was incubated with the appropriate primary antibody and secondary antibody (FITC goat anti-mouse-IgG, DAKO) at 10 μg/ml. Cell fluorescence was assayed on a BD FACSCanto II flow cytometer (BD Biosciences) and data were analyzed with FlowJo (Tree Star, Inc.).

**Immunofluorescence microscopy**

Cells were fixed in 2% paraformaldehyde for 10 minutes, blocked with 5% BSA (Sigma-Aldrich) for 30 minutes and incubated with the appropriate direct-conjugated antibody at 10 μg/ml for 1 hour at room temperature. Images were acquired by using a restoration workstation (Delta Vision 3D; Applied Precision) equipped with an inverted 40x objective lens. Images were analyzed using ImageJ software.

**Immunoelectron microscopy**

hec7, 7G7B6 and M2 antibodies were conjugated to horseradish peroxidase (HRP). Monolayers of cells on Thermolax coverslips (Thermo Fisher Scientific) were incubated with the appropriate HRP-conjugated antibody for 1 hour at 37°C and fixed with 4% glutaraldehyde in 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences). Cells were washed and exposed to a mixture of 10 mg/ml diaminobenzidine (Sigma-Aldrich) and 0.03% hydrogen peroxide in PBS for 20 minutes. Samples were embedded. En face sections (70 nm thick) were examined on a Tecnai Spirit electron microscope (FEI Company).

**Human leukocyte isolation, TEM assay and targeted recycling assay**

Peripheral blood mononucleated cells (PBMCs) were harvested as described previously (Muller and Lusczynska, 2008) and resuspended in iHUVEC medium (Invitrogen) containing 0.1% human serum albumin (Gibofls Biologicals Inc.). TEM assays were performed as described previously (Muller and Lusczynska, 2008). Recycling of the LBRC protein during TEM was performed as described previously (Mamdouh et al., 2003).

**Western blotting**

Cells were lysed with RIPA buffer (0.4% NP-40, 100 mM NaCl, 10 mM sodium phosphate, 50 mM NaF, 1 mM EDTA, 1 mM EGTA) with 1 mM orthovanadate and protease inhibitor cocktail (Sigma). The lysate was run on an SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane. The blots were then blocked, probed with primary and HRP-conjugated secondary antibodies, and developed.

**Sequential immunoprecipitation assay (isolation of molecules from the cell surface and LBRC)**

To avoid interference from proteins in the Golgi or post-Golgi compartments, proteins on the cell surface and LBRC were selectively biotinylated. Endothelial cells were incubated with 2 mM NHS-sulfo-LC-biotin (Pierce) for 30 minutes on ice. At this temperature there is no movement of LBRC, and large molecules, like the Fab fragments of antibodies, cannot enter the LBRC, but protons and biotin can (Mamdouh et al., 2003). The reaction was quenched using 100 mM glycine. To selectively label specific surface molecules, antibodies were added at 4°C for 1 hour to saturate the binding. Antibodies were washed out and cells were lysed. The lysate was incubated with Protein-G–Sepharose (Invitrogen) for several rounds to exhaustively immunoprecipitate the antibody-labeled surface protein. The samples were then incubated with antibody and Protein-G–Sepharose to immunoprecipitate the remaining specific protein in the lysate (fraction from LBRC, which had been inaccessible to antibody in the intact cells owing to its location). Samples of the surface and LBRC fractions were analyzed by western blotting using HRP-conjugated streptavidin. The density of protein bands was analyzed with ImageJ. For experiments with blocking or scrambled peptide, endothelial cells were pre-treated with peptide at 100 μM for 1 hour at 37°C.

**Aggregation assay**

The aggregation assay was performed as described previously (Albelda et al., 1991; Bronner-Fraser, 1985; Muller et al., 1992). L cells carrying
vacant pBABE-puro, FLAG–VE-CAD or FLAG–ΔRVDae were used for the assay. Briefly, confluent cells were detached with 10 mM EDTA and then resuspended in HBSS containing 2 mM CaCl2 and 1 mM MgCl2 to a final cell density of 1×10^6 cells/mL. Cell suspension was shaken at 90 rpm, 37°C.

**Patching assay**

The patching assay was used to determine whether D1,2 Tac or D1,2-Tac/PECAM would be directly affected by the movement of native PECAM. Endothelial cell transfectants, D1,2 Tac/PECAM and D1,2 Tac were seced at density of 2000 cells/cm². PECAM on the surface was labeled with P1.1 (which binds to the 5th Ig-like domain on PECAM) and thus will not interact with D1,2 Tac/PECAM and D1,2 Tac) for 30 minutes at 4°C. To crosslink PECAM, cells were incubated with Rhodamine-conjugated goat anti-mouse-IgG (Jackson ImmunoResearch) for 10 minutes at 37°C, washed and fixed. For non-crosslinked control, after the labeling with P1.1, cells were fixed first and then incubated with Rhodamine-conjugated goat anti-mouse-IgG for 10 minutes at room temperature. Both crosslinked and non-crosslinked groups were then incubated with Alexa-Fluor-488-conjugated 7G7B6 to label D1,2 Tac/PECAM or D1,2-Tac. Images of samples were acquired and analyzed as described above.

**Permeability assay**

Experiments were performed as described previously (Winger et al., 2014), with some modifications. iHUVECs were pretreated with either blocking or scrambled peptide at 37˚C for 1 hour. Peptide was washed away and cells were incubated with FITC–dextran in culture medium for 1 hour. The relative fluorescence intensity (RFU) was measured by using a FilterMax F5 microplate reader (Molecular Devices).

**Statistics**

Experiments with quantification were performed at least three times. The Student’s t-test (or one-way ANOVA in aggregation assays) was used to evaluate statistical significance, and data in the figures are presented as the mean±s.d.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

G.F. designed and conducted experiments, analyzed data and wrote the manuscript. D.P.S. participated in experimental design and data analysis and wrote the manuscript. F.H. contributed to data acquisition and analysis. W.A.M. designed experiments, analyzed data and wrote the manuscript.

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**Supplementary material**

Supplementary material available online at http://jcs.biologists.org/lookup/suppl?doi=10.1242/jcs.159053/-/DC1

**References**


Legends for supplementary materials:

Figure S1  **iHUVEC accurately reproduce the essential features of HUVEC.**

(A) Expression of junctional proteins, PECAM, VE-Cad and CD99, on iHUVEC and HUVEC were detected by flow cytometry. Non-filled red curve: isotype control. Filled blue curve: labeled cells. (B) Leukocyte TEM on iHUVEC is PECAM-dependent. Both HUVEC and iHUVEC showed the same TEM percentage. TEM on both cells was blocked by PECAM blockade Ab (hec7) at the same level. (C) Immuno-EM assay shows the same morphology of the LBRC in iHUVEC as in HUVEC (Mamdouh et al., 2003). BLACK arrows: stained LBRC vesicles. Scale bars: 1 μm. (D) Targeted recycling of the LBRC membrane to the TEM site in both iHUVEC and HUVEC. Recycled LBRC was stained and visualized by fluorescence as described (left column). Monocytes were stained with anti CD11 Ab (IB4, middle column). Merged images (right column) show that LBRC (white arrow) in both iHUVEC and HUVEC recycles to the site of TEM. Scale bar: 1 μm.

Figure S2  **Expression of PECAM and TEM in EC transfectants.**

(A and B) Tac/PECAM and Tac. (C and D) D1,2-Tac/PECAM and D1,2-Tac. (E and F) FLAG-VE-CAD and FLAG-VE-CAD/PECAM. (G and H) truncated FLAG-VE-CAD and FLAG-VE-CAD/PECAM. Flow cytometry data (A, C, E, G) showed there was no alter of PECAM in transfectants. Non-filled red curve: isotype control. Filled blue curve: labeled cells. TEM data (B, D, F, H) showed the same TEM percentage in transfectants with or without PECAM blockage Ab (hec7) as that in wild type.

Figure S3  **D1,2-Tac and D1,2-Tac/PECAM do not bind in cis to native PECAM.**

Left column: top panel, PECAM on surface. Blue bar: selected region. Bottom panel: density plot of selected region. Middle column: top panel, D1,2-Tac or D1,2-Tac/PECAM on cell surface. Blue bar: selected region. Bottom panel: density plot of selected region. Right column: Merged images of PECAM (red) and D1,2-Tac or D1,2-Tac/PECAM (green). (A) D1,2-Tac cells without the crosslink (XL) of PECAM. Both PECAM and D1,2-Tac are diffusely distributed. Density plots showed that they have similar distribution patterns. (B) D1,2-Tac cells with the crosslink of PECAM. After the crosslink, PECAM aggregated into many concentrated
foci. Density plot also showed concentration of PECAM at these points. The distribution of D1,2-Tac remained essentially unchanged. (C) D1,2-Tac/PECAM cells without the crosslink of PECAM. Both PECAM and D1,2-Tac/PECAM evenly distributed on cell surface as seen in both immunofluorescence staining and density plots. (D) D1,2-Tac/PECAM cells with the crosslink of PECAM. After the crosslink of PECAM, the staining of PECAM showed many concentrated foci, while the staining pattern of D1,2-Tac/PECAM remained unchanged. Data are representative of hundreds of cell images for each condition.

Figure S4  **Removal of RVDAE motif from VE-cadherin abolishes homophilic adhesion.**

(A) Expression of FLAG-VE-CAD and FLAG-ΔRVDAE on L cells transfectants was analyzed by flow cytometry. Non-filled red curve: isotype control. Filled blue curve: labeled cells. FLAG-VE-CAD and FLAG-ΔRVDAE have similar expression level. Signal in vector control (BABE, L cells transduced with vacant pBABE-puro) is similar to isotype control. (B) Expression of FLAG-VE-CAD in L cells imparts on them the ability to aggregate. FLAG-VE-CAD displayed significant higher level of aggregation than FLAG-ΔRVDAE or vector control (BABE). Data shown are the percentage of total cells in aggregates. (C) Blocking peptide (B.P.) but not Scrambled peptide (S.P.) results higher permeability in EC. **: P < 0.01.
Figure S3

A  
PECAM  |  D1,2-Tac  |  Merged  
Non XL

B  
PECAM  |  D1,2-Tac  |  Merged  
XL

C  
PECAM  |  D1,2-Tac/PECAM  |  Merged  
Non XL

D  
PECAM  |  D1,2-Tac/PECAM  |  Merged  
XL
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