The involvement of lipid rafts in the regulation of integrin function

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Summary
Integrin activity on cells such as T lymphocytes is tightly controlled. Here we demonstrate a key role for lipid rafts in regulating integrin function. Without stimulation integrin LFA-1 is excluded from lipid rafts, but following activation LFA-1 is mobilised to the lipid raft compartment. An LFA-1 construct from which the I domain has been deleted mimics activated integrin and is constitutively found in lipid rafts. This correlation between integrin activation and raft localisation extends to a second integrin, α4β1, and the clustering of α4β1 is also raft dependent. Both LFA-1 and α4β1-mediated adhesion is dependent upon intact lipid rafts providing proof of the functional relevance of the lipid raft localisation. Finally we find that non-raft integrins are excluded from the rafts by cytoskeletal constraints. The presence of integrin in lipid rafts under stimulating conditions that activate these receptors strongly indicates that the rafts have a key role in positively regulating integrin activity.

Key words: Integrin, Lipid rafts, Cytoskeleton

Introduction
Integrins are αβ heterodimeric transmembrane receptors that mediate cell adhesion (Harris et al., 2000; Plow et al., 2000) and they can exist in several functional states. An increase in ligand binding activity can be brought about either by conformational changes that lead to higher affinity forms of the integrin or by lateral association of integrins into clusters on the plasma membrane, which increases ligand binding avidity by providing multiple contact sites (Stewart and Hogg, 1996; van Kooyk and Figdor, 2000). The cytoplasmic domains of integrins associate with the cytoskeleton (Giancotti and Ruoslahti, 1999), and these interactions alter during the course of adhesion. Cytoskeletal interactions can regulate the clustering of integrins, such as lymphocyte function-associated antigen 1 (LFA-1) (Stewart et al., 1998; van Kooyk et al., 1999).

Ligand binding to integrins results in signals being transmitted into the cell for which the target pathways are being identified, particularly in mesenchymal cells (Fashena and Thomas, 2000). It is becoming more certain that integrins on leukocytes can also signal and one outcome of integrin-mediated signalling is the altered activity of other integrins, a process termed ‘integrin cross talk’ (Blystone et al., 1994; Porter and Hogg, 1997). Thus one subset of integrins can operate to regulate either positively (Chan et al., 2000; Leitinger and Hogg, 2000a; Pacifi et al., 1994; Porter and Hogg, 1997; Weerasinghe et al., 1998) or negatively (Blystone et al., 1994; Diaz-Gonzalez et al., 1996; Porter and Hogg, 1997) a second set of integrins on the same cell membrane. The molecular mechanism of integrin crosstalk is currently not well understood and, at present, only two kinases have been reported to play a role (Blystone et al., 1999; Pacifi et al., 1994).

The integrin I domain contained in the α subunit is the principal ligand binding site of those integrins, including LFA-1, which possess it (reviewed by Leitinger and Hogg, 2000b). When activation of such integrins occurs, the conformation and positioning of the I domain alters. Recently, we have removed the I domain from LFA-1 and expressed the resulting integrin (ΔI-LFA-1) in Jurkat T cells (Leitinger and Hogg, 2000a). ΔI-LFA-1 is unable to bind ligand ICAM-1, but has features of an active integrin in that it exhibits LFA-1 activation-dependent mAb epitopes. A key feature of T cells expressing ΔI-LFA-1, compared with T cells expressing wild-type (wt) LFA-1, is that the β1 integrins, α4β1 and α5β1, show increased binding activity to ligands VCAM-1 and fibronectin. This crosstalk between integrins is associated with increased clustering of the β1 integrins and is dependent on an intact cytoskeleton.

It is increasingly recognised that the lipid bilayer of the plasma membrane is composed of different subdomains and the cholesterol- and sphingolipid-rich microdomains known as lipid rafts have attracted much recent interest (Brown and London, 2000; Cherukuri et al., 2001; Simons and Ikonen, 1997). These lipid domains are platforms for cellular signalling, particularly as defined for T cells and other leukocytes (Guo et al., 2000; Janes et al., 1999; Montixi et al., 1998; Viola et al., 1999; Xavier et al., 1998). Proteins with glycosyl phosphatidylinositol (GPI)-anchors and many dually acylated cytoplasmic proteins are enriched in the lipid rafts (Brown and London, 2000). Although the rafts are generally deficient in transmembrane proteins, several such proteins are raft associated potentially through receptor oligomerisation (Cherukuri et al., 2001). Early studies suggested that integrins were not localised to lipid rafts (Fra et al., 1994), but recently integrins have been found to be raft associated (Green et al., 1999; Krauss and Altevogt, 1999; Skubitz et al., 2000).
However, the relevance of this association with regard to function remains to be understood.

We demonstrate here a correlation between LFA-1 activity and lipid raft localisation. In addition, the presence of active LFA-1 in lipid rafts promotes the movement of α4β1 integrin to the rafts. Furthermore, adhesion mediated by LFA-1 or α4β1/α5β1 and the increased clustering of activated α4β1 are all dependent on intact lipid rafts. Finally we show that inactive integrins, LFA-1 and α4β1, are tethered away from lipid rafts by cytoskeletal restraints.

Materials and Methods
Monoclonal antibodies (mAb) and other reagents
Anti-α4 mAb 7.2R (CD49d) and anti-DAF mAb 67 (CD55) were prepared by the ICRF Antibody Production Service. The β2-integrin activating mAb KIM 185 was a gift from M. Robinson, (Celltech, Slough, UK). Anti-LFA-1 mAb G25.2 (CD11a) was purchased from Becton Dickinson (Oxford, UK) and anti-human transferrin receptor mAb (CD71) from Roche Diagnostics (Lewes, UK). Cytochalasin D and methyl-β-cyclodextrin, cholesterol and fatty acid free BSA were from Sigma (Poole, UK). Latrunculin A was a gift from R. Treisman, ICRF.

Cell lines and cell culture
The generation of the human T lymphoma Jurkat cell lines stably expressing wt LFA-1 or ΔI-LFA-1 has been described (Leitinger and Hogg, 2000a). Cells were maintained in RPMI 1640 medium containing 10% FCS (Life Technologies, Paisley, UK) supplemented with 250 μg/ml Zeocin (Invitrogen, Leek, The Netherlands). Human T cells were prepared and cultured as previously (Porter and Hogg, 2000a). Cells were maintained in RPMI 1640 medium expressing wt LFA-1 or ΔI-LFA-1 (D) with a human immunoglobulin G1 (IgG1) Fc sequence, was performed as described (Leitinger and Hogg, 2000a). Cell adhesion to ICAM-1Fc, a chimeric protein containing the five extracellular domains of human ICAM-1 fused to a human immunoglobulin G1 (IgG1) Fc sequence, was performed as described (Leitinger and Hogg, 2000a). Cell adhesion to fibronectin was performed using flat bottom tissue culture 96-well plates (Microtest³M, Falcon, Becton Dickinson, Oxford, UK) coated with fibronectin at 2 μg/ml.

Fluorescence microscopy and treatment of cells
Raft patching
Lipid raft aggregation or patching was performed according to Janes et al. (Janes et al., 1999). Aliquots of 1×10⁶ cells (in 100 μl) were labelled in RPMI 1640 medium with 10 μg/ml TRITC-conjugated cholera toxin B (List Biological Laboratories, Quadratech, Epsom, UK), which binds to the ganglioside GM1 on the cell surface, for 30 minutes on ice. After three washes, cells were incubated with rabbit anti-cholera toxin IgG (Sigma; 1/150 in PBS with 0.2% BSA) for 30 minutes on ice, followed by a 20 minute incubation at 37°C. After three washes, cells were fixed in 1% paraformaldehyde for 30 minutes on ice and stained with anti-integrin mAbs 7.2R or G25.2 (at 10 μg/ml), anti-human transferrin receptor mAb (at 20 μg/ml) or anti-DAF mAb 67 (at 10 μg/ml), followed by Alexa 488-conjugated goat anti-mouse IgG, (Molecular Probes, Eugene, OR) at 10 μg/ml for 30 minutes on ice. After three washes, cells were attached to poly-L-lysine-coated 13 mm round glass coverslips, fixed in 3% formaldehyde in PBS, and mounted onto slides in Mowiol (Calbiochem, Nottingham, UK) dissolved in Citifluor antifade solution (UKC Chemical Laboratory, Canterbury, UK).

Methyl-β-cyclodextrin treatment
Cells were preincubated with 10 nM (final concentration) methyl-β-cyclodextrin (MβCD) in RPMI 1640 for 30 minutes at 37°C. Aliquots of 1×10⁶ cells were then rapidly chilled and incubated with MβCD 7.2R at 10 μg/ml for 30 minutes on ice, then washed three times in PBS. To prevent antibody-induced clusters, cells were fixed in 1% paraformaldehyde in PBS for 20 minutes on ice before a second incubation with Alexa 488-conjugated goat anti-mouse IgG, as described above. Viability of the cells was tested with trypan blue exclusion. No significant cell death occurred due to cholesterol extraction.

Incubation with integrin activating agonists
Cells were incubated with a final concentration of either 0.5 mM Mn²⁺ or 100 nM phorbol 12,13-dibutyrate (PdBu) in 20 mM Hepes, 140 mM NaCl, 2 mg/ml glucose, pH 7.4 for 30 minutes at 37°C. Aliquots of 1×10⁶ cells were then rapidly chilled and incubated with 10 μg/ml TRITC-conjugated cholera toxin B and processed as described above (see Raft patching).

Confluent microscopy
Fluorescence was analysed using a Zeiss LSM 510 confocal laser scanning microscope equipped with a 63×, numerical aperture 1.4 objective. Single channel fluorescence was analysed with an argon laser (wavelength 488 nm). For double channel fluorescence imaging a second helium neon laser (wavelength 543 nm) was used. Cell surface distribution was evaluated taking horizontal optical sections at 0.35 μm vertical steps throughout the whole height of representative cells or at mid section through the cells. Images of optical sections (512×512 pixels) were digitally recorded. The resulting images were processed using Adobe (Mountain View, CA) PhotoShop software.

Manipulation of plasma membrane cholesterol content using methyl-β-cyclodextrin
All treatments were performed in RPMI with 0.1% fatty-acid-free BSA. Cholesterol depletion and replenishment: cells (at 4×10⁶/ml) were incubated in either RPMI 1640 (untreated), 10 mM MβCD, or 5 mM MβCD plus 5 mM MβCD-cholesterol in RPMI 1640 for 15 minutes at 37°C. Cholesterol repletion of cholesterol-depleted cells: after MβCD incubation as above, cells were washed in RPMI 1640 and incubated with MβCD-cholesterol inclusion complexes at 0.5 mM cholesterol for 1 hour at 37°C. After the various treatments, cells were directly used for the adhesion assay, whereby 50 μl aliquots of cells (at 4×10⁶/ml) were added to 50 μl of 2× stimuli.

Preparation of methyl-β-cyclodextrin-cholesterol inclusion complexes
MβCD-cholesterol complexes were prepared as described (Klein et al., 1995). Briefly, a solution of 25 mg cholesterol, dissolved in 333 μl of methanol/chloroform (2:1, v/v) was added drop-wise to a stirred solution of 833 mg MβCD in 9 ml PBS on a water bath (80°C). The mixture was stirred until a clear solution resulted. The MβCD-cholesterol complexes were then lyophilised and stored at room temperature.

Results
The association of ΔI-LFA-1 but not wt LFA-1 with lipid rafts
We have previously removed the I domain from LFA-1 and expressed the resulting I minus LFA-1 (ΔI-LFA-1) in the Jurkat
Recruitment of activated integrin to lipid rafts

T-cell line J-β2.7, which lacks endogenous expression of LFA-1 (Weber et al., 1997). ΔI-LFA-1 is unable to bind ligand ICAM-1, but has the features of a constitutively active integrin including the expression of many epitopes associated with LFA-1 activation (Leitinger and Hogg, 2000a). By contrast, without prior stimulation, wt LFA-1 does not express these activation epitopes and has no ligand binding activity. Therefore, wt LFA-1 is here considered to be ‘inactive’. Comparison of inactive wt LFA-1 with ΔI-LFA-1, which resembles active integrin, thus offers the opportunity to define differences between these two forms of integrin. Krauss and Altevogt have recently reported that LFA-1 is associated with lipid rafts on the plasma membrane of murine thymocytes (Krauss and Altevogt, 1999). We therefore asked whether LFA-1 was similarly localised on human T cells and whether the state of integrin activation made any difference to its distribution.

**Fig. 1.** Integrin LFA-1 has higher affinity for lipid raft patches on J-β2.7 cells expressing ΔI-LFA-1 than on J-β2.7 cells expressing wt LFA-1. Cells were incubated with TRITC-conjugated Ctx-B, then crosslinked with rabbit anti-Ctx-B antibody. Cells were fixed and stained with mAbs against LFA-1 (A), DAF (B), or TfR (C), followed by Alexa 488-conjugated goat anti-mouse IgG. Single optical sections taken at mid-height of the cells are shown. Top panels, ΔI-LFA-1-expressing cells; bottom panels, wt LFA-1-expressing cells. Cell surface proteins, green; Ctx-B, red. Data are representative of six experiments (A), and three experiments (B,C). Bars, 10 μm.

**Fig. 2.** Colocalisation of LFA-1, DAF and TfR with lipid raft patches. Experimental details are as described in Fig. 1. Patches of the different cell surface markers were scored into three categories: good (>80% overlap); medium (partial but clearly overlapping regions); and none (random distribution or segregation of staining). The percentages of cells falling into each category are expressed as means±s.d. Data are representative of six experiments for LFA-1 (A), and three experiments for each of DAF and TfR (B,C). Black bars, J-β2.7 cells expressing ΔI-LFA-1; white bars, J-β2.7 cells expressing wt LFA-1.
To this end we employed the method used by Janes et al. (Janes et al., 1999), who visualised lipid rafts on Jurkat T-cell membranes using fluorescence microscopy. Although lipid rafts are not usually visible by light microscopy, it is possible to detect aggregated lipid rafts as distinct patches by clustering of raft markers with antibodies or other reagents (Harder et al., 1998). Thus upon coalescence of the lipid rafts into larger domains, other raft-associated proteins will colocalise with these patches. Non raft-associated proteins do not colocalise with the raft patches because of the immiscibility of the different lipid phases. Using confocal microscopy we detected the lipid rafts by crosslinking the raft enriched glycosphingolipid GM1 through binding to the cholera toxin (Ctx) B subunit and patching with anti-Ctx antibodies. The distribution of ΔI-LFA-1 largely overlapped with the patched Ctx staining showing a preferential association with the lipid rafts (Fig. 1A, top). By contrast, wt LFA-1 appeared less associated with the lipid rafts as staining did not colocalise with the Ctx patches (Fig. 1A, bottom). GPI-linked proteins are preferentially associated with lipid rafts (Brown and London, 2000; Brown and Rose, 1992). Therefore, a useful positive control for the localisation and identification of the lipid rafts was the GPI-linked decay accelerating factor (DAF; CD55) protein (Fig. 1B). Transferrin receptor (TIR; CD71) does not associate with the lipid rafts (Harder et al., 1998; Harder and Simons, 1999; Janes et al., 1999) and served as a negative control (Fig. 1C).

To provide a more extensive analysis of the relative overlap of the patched Ctx with the different membrane markers, the fluorescent images of 30-40 cells per experiment were scored into three different categories: good, medium or no colocalisation (see legend to Fig. 2). The analysis demonstrated that there was a greater tendency for ΔI-LFA-1, than for wt LFA-1, to be associated with lipid rafts (Fig. 2A). As expected, the GPI-linked DAF protein had a similar distribution in both types of LFA-1 expressing cells, being largely raft localised (Fig. 2B). Conversely, the TIR was excluded from the same membrane structures in both cell lines (Fig. 2C). Therefore the two forms of LFA-1 have different affinities for the lipid rafts, and ΔI-LFA-1 was more strongly raft associated than wt LFA-1.

The association of Mn2+ and phorbol ester-activated LFA-1 with lipid rafts

As inactive wt LFA-1 is excluded from the lipid rafts, whereas ΔI-LFA-1, which resembles active integrin is associated with the rafts, it was predicted that activation of wt LFA-1 with agonists would mobilise this integrin from the non-raft compartment to the raft compartment. To test this hypothesis, the wt LFA-1-expressing Jurkat T cells were exposed to either 0.5 mM Mn2+, which activates integrin by conformationally altering the integrin ectodomain, or to 100 nM phorbol ester...
Recruitment of activated integrin to lipid rafts

PdBu, which activates integrin through an intracellular signalling pathway (Stewart and Hogg, 1996). As expected, both agonists caused an increase in binding of Jurkat T-cell-expressed wt LFA-1 to immobilised ICAM-1 (Fig. 3A).

Next, the effect of these treatments on the colocalisation of LFA-1 with the lipid raft patches was examined. Wild-type LFA-1 on untreated T cells was generally excluded from the rafts as was observed in Fig. 1 (Fig. 3B, unstim). However, following exposure of wt LFA-1-expressing cells to either Mn²⁺ or PdBu, LFA-1 was largely relocated to the raft compartment of the membrane (Fig. 3B,C). To provide a quantitative analysis of the degree of overlap between the LFA-1 signal and the patched Ctx signal, we calculated colocalisation of the two signals using NIH Image software. Table 1 shows that, relative to unstimulated cells, the overlap between the LFA-1 signal and the lipid raft signal increased on Mn²⁺ and PdBu-stimulated cells. These findings correlate well with those shown in Fig. 3C and thus validate our semi-quantitative analysis. The correlation between raft association and ligand binding activity is strong evidence that the mobilisation of LFA-1 into the lipid raft compartment is a key component in the regulation of the adhesive activity of this integrin. In addition, the association with the lipid rafts of both ΔI-LFA-1 and agonist-activated LFA-1 further confirms that ΔI-LFA-1 does mimic the active ligand binding form of LFA-1.

ΔI-LFA-1 crosstalk to α4β1 integrin

Certain integrins can ‘crosstalk’ to other classes of integrin on the same cells and either induce or suppress their ligand binding activity (Porter and Hogg, 1998). A characteristic of the ΔI-LFA-1-expressing T cells, compared with cells expressing wt LFA-1, is the constitutively elevated ligand binding activity of the β1 integrins, α4β1 and α5β1. Therefore we next asked whether the distribution of α4β1 was influenced by the membrane localisation of LFA-1 and, specifically, whether there was any association of α4β1 with lipid rafts. Examination of overlap between Ctx membrane patches and α4β1 showed that there was good colocalisation on ΔI-LFA-1.
1-expressing Jurkat cells, but not on wt LFA-1-expressing cells (Fig. 4A,B). Therefore, expression of ΔI-LFA-1 caused α4β1 association with the lipid rafts, whereas on wt LFA-1-expressing cells, neither LFA-1 nor α4 integrins were predominantly raft associated.

One possibility was that ΔI-LFA-1 was controlling the behaviour of α4β1 through physically associating with it on the membrane. The use of double laser confocal microscopy (but not Ctx crosslinking conditions) showed that there was no significant colocalisation of α4β1 and LFA-1 on Jurkat cells expressing ΔI-LFA-1 or wt LFA-1 (data not shown). Thus ΔI-LFA-1 and α4β1 are located within different lipid rafts that then cocluster with patched Ctx. This emphasises the indirect effect of ΔI-LFA-1 on crosstalk to α4β1 integrin.

Depletion of cellular cholesterol inhibits clustering of α4β1 on cells expressing ΔI-LFA-1

To test whether the presence of integrins in lipid rafts was relevant for integrin-mediated adhesion, the rafts were disrupted using methyl-β-cycloexdrin (MβCD), which depletes the essential cholesterol component of lipid rafts and has been used to disrupt the rafts in Jurkat cells (Harder and Kuhn, 2000; Janes et al., 1999). Jurkat cells were activated by agonists that act either through an intracellular signalling pathway (PdBu) or by engaging the integrin ectodomain (Mn2+), and adhered to fibronectin (Fig. 5A). Adhesion was dependent on α4β1 and α5β1 (data not shown). Following treatment with 10 mM MβCD, adhesion was reduced to background levels. Evidence that MβCD was causing cholesterol depletion and not some other effect was demonstrated by the lack of effect on adhesion when cells were treated with 5 mM MβCD plus 5 mM MβCD-cholesterol conjugates. This latter treatment exposed the cells to the same concentration of MβCD as when cholesterol was depleted but provided the cells with cholesterol in the form of MβCD-cholesterol conjugates, which facilitate the incorporation of exogenous cholesterol into membranes (Klein et al., 1995). Finally Jurkat T cells were treated first with 10 mM MβCD and then repleted with MβCD-cholesterol conjugates at 0.5 mM cholesterol. This treatment completely restored adhesion for Mn2+-treated cells and partially restored adhesion for PdBu-treated cells, demonstrating that cholesterol depletion was reversible.

We next tested whether the adhesion of primary human T cells was also dependent upon intact lipid rafts. In these experiments the ability of T-cell LFA-1 to bind to ICAM-1 was assessed, as for the Jurkat cells, following stimulation with agonists PdBu or Mn2+ as well as the β2-integrin-activating mAb KIM 185 (Fig. 5B). In all cases, treatment with 10 mM MβCD inhibited adhesion; this was due to cholesterol depletion as treatment with 5 mM MβCD plus 5 mM MβCD-cholesterol conjugates maintained adhesion at control levels. Repletion experiments with MβCD-cholesterol conjugates following MβCD treatment also restored LFA-1 adhesion to ICAM-1. Therefore, for both LFA-1 and α4β1 (and α5β1), which are the integrins that are the focus of this study, there is dependence on intact lipid rafts for adhesion. It is of interest that this dependence on rafts is independent of the means of integrin activation.

Depletion of cellular cholesterol inhibits clustering of α4β1 on cells expressing ΔI-LFA-1

Integrin-mediated adhesion requires intact lipid rafts

We have previously demonstrated enhanced α4β1 clustering on ΔI-LFA-1-expressing Jurkat cells (Leitinger and Hogg, 2000a) and in this study we show an association of active α4 integrin with the lipid rafts. Therefore, we asked whether the clustered form of α4β1 was dependent upon lipid raft components. Following treatment of Jurkat cells with 10 mM MβCD, the clustered distribution of α4β1 on the ΔI-LFA-1-expressing cells (Fig. 6A) was reduced to background levels (Fig. 6, compare B with C). The MβCD treatment had no effect on the distribution of α4 integrin in wt LFA-1-expressing cells (Fig. 6D). These results indicate that cholesterol, which is required for lipid raft integrity, is also necessary for the
formation of α4β1 integrin clusters on ΔI-LFA-1-expressing J-β2.7 cells. Thus, for α4β1 a link exists between integrin clustering and lipid rafts.

The association between the cytoskeleton, integrins and lipid rafts

Other studies have shown that interactions of proteins with lipid raft components can be regulated or stabilised by the cytoskeleton (Holowka et al., 2000; Oliferenko et al., 1999). Cytochalasin D is well known to abolish LFA-1-mediated adhesion (Lub et al., 1997; Stewart and Hogg, 1996) and the cytoskeleton is implicated in integrin crosstalk as cytochalasin D prevented the increased ligand binding activity of α4β1 in the ΔI-LFA-1-expressing Jurkat T cells (Leitinger and Hogg, 2000a). To understand more about the connection between the cytoskeleton, integrins and the lipid rafts we investigated the association of LFA-1 and α4β1 with Ctx crosslinked lipid rafts in Jurkat cells in which the cytoskeleton had been disrupted. The first observation of the cytochalasin D-treated lipid rafts was that the rafts formed exceedingly large patches or ‘caps’ in the cells treated in this manner (Fig. 7A). Second, treatment with cytochalasin D caused both ΔI-LFA-1 and wt LFA-1 to associate with the rafts in an equivalent and extensive fashion. A similar observation was made for α4β1, in that, on both ΔI- LFA-1- and wt LFA-1-expressing Jurkat T cells treated with cytochalasin D, the α4 integrin was associated mainly with the lipid rafts (Fig. 7B). As expected, the distribution of the GPI-linked DAF protein also coincided with the lipid raft patches (Fig. 7C), while the distribution of the non-raft-associated TfR was unaffected by cytochalasin D and remained outside the raft membrane compartment (Fig. 7D). These findings strongly imply that ‘inactive’ LFA-1 and α4β1 are restrained by cytoskeleton tethers so as to be excluded from the lipid rafts and that release of the constraint allows the integrins to move into the lipid rafts.

The effect of cytochalasin D on the cytoskeleton is to cap the barbed ends of F-actin filaments and prevent their lengthening (Cooper, 1987). To test the effects on the lipid rafts of an actin binding drug with a different mode of action, we investigated latrunculin A, which blocks polymerisation of monomeric G actin to F actin (Coue et al., 1987). Similar to the results shown in Fig. 7, both cytochalasin D and latrunculin A caused large patches of Ctx crosslinked lipid rafts and coassociation of integrin α4β1 from both ΔI-LFA-1- (Fig. 8A) and wt LFA-1-expressing (Fig. 8B) cells. Latrunculin A also had similar effects on the distribution of LFA-1 on both cell lines (data not shown). This further contributes to the evidence that following release from cytoskeletal constraint, integrin is mobilised to lipid rafts.

Paradoxically, the effects of cytochalasin D and latrunculin A, which disrupt both LFA-1 and α4β1 function, cause more lipid raft association of these integrins. However, the fact that cytochalasin D and latrunculin A caused raft ‘capping’ suggests that the cytoskeleton must have an additional role in the normal stabilisation of the lipid raft structure. These results

Fig. 7. Cytochalasin D treatment affects colocalisation with lipid raft patches of LFA-1, α4β1 and DAF, but has no effect on the distribution of TfR. Cells were preincubated with 5 μM cytochalasin D, then subjected to lipid raft patching and cell surface protein staining, as in Fig. 1: LFA-1 (A); α4β1 (B); DAF (C); TfR (D). For each A-D section, top panels, ΔI-LFA-1-expressing cells; bottom panels, wt LFA-1-expressing cells. Cell surface proteins, green; Ctx-B, red. Single optical sections taken at mid-height of the cells are shown. Data are representative of three experiments. Bar, 10 μm.
but preferential association with the active form of an integrin (2001; Thorne et al., 2000) have been identified in lipid rafts, as well as other classes of integrin such as LFA-1 and Mac-1 (Krauss and Altevogt, 1999; Skubitz et al., 1999). This finding is in keeping with the fact that activated integrins LFA-1 are preferentially found in lipid rafts. Activated LFA-1 is mimicked by LFA-1 in which the I domain is deleted (ΔI-LFA-1, Cherukuri et al., 2001). As α4β1 on ΔI-LFA-1-expressing cells is clustered through crosstalk, and the integrin activating regimes used in this study also enhance integrin clustering (Stewart et al., 1998), it is an attractive possibility that integrin oligomerisation might trigger the association with the rafts. How integrins are held within the lipid raft compartment remains to be resolved. Neither subunit of the integrin αβ heterodimer is modified by palmitoylation, a characteristic of many raft transmembrane proteins (Brown and London, 2000). It is conceivable that raft localisation is dependent on complex formation with other membrane proteins. Integrins can complex with multimembrane spanning proteins such as integrin-associated protein (IAP) and members of the transmembrane 4 superfamily (TM4SF) (reviewed by Porter and Hogg, 1998), and there is increasing evidence that these complexes associate with the lipid rafts (Claas et al., 2001; Green et al., 1999). β2 integrins can form complexes with the TM4SF proteins CD82 (Shibagaki et al., 1999) and CD63 (Skubitz et al., 2000) and, in the case of CD63, the β2 integrin/CD63 complex was isolated from the raft membrane fraction. However, it is clear that integrin/TM4SF complexes exist outside the raft compartment (Claas et al., 2001). There is also increasing evidence that ligand-induced oligomerisation provides the stimulus for raft association (reviewed by Cherukuri et al., 2001). As α4β1 on ΔI-LFA-1-expressing cells is clustered through crosstalk, and the integrin activating regimes used in this study also enhance integrin clustering (Stewart et al., 1998), it is an attractive possibility that integrin oligomerisation might trigger the association with the rafts.

The fact that inactive wt LFA-1 and α4β1 are largely excluded from the raft fraction implies that the activation process involves movement between the two types of membrane compartment. Activation of B cells by agonists such as phorbol esters causes LFA-1 mobility in the cell membrane (Kucik et al., 1996). Previous reports suggest that this mobility comes about as a result of the untethering of LFA-1 from the cytoskeleton (Lub et al., 1997; Stewart et al., 1998). Our results add to this information by demonstrating that, following activation, a proportion of the T cell’s LFA-1 and α4β1 moves to the lipid raft compartment of the membrane. Moreover, following cytochalasin D or latrunculin A treatment, the majority of inactive wt LFA-1 and α4β1 becomes associated with lipid rafts. These findings provide strong evidence that LFA-1 and α4β1 are restrained by cytoskeletal tethers in a manner that causes exclusion from the lipid rafts and that, following response to activating agonists, a proportion of integrins are untethered. This finding is in keeping with studies of the interactions of FcεRI and CD44 with lipid rafts, which are also regulated by the actin cytoskeleton (Holowka et al., 2000; Oliferenko et al., 1999). In the case of CD44, a similar result to the one presented here was obtained, in that disruption
of the actin cytoskeleton dramatically increased the proportion of CD44 that was isolated from the lipid raft fraction (Oli?erenko et al., 1999).

There has been little information about the mechanism of integrin crosstalk. Here we show that the presence of active LFA-1 in lipid rafts has consequential effects on α4β1, causing it to move into the rafts. The conjecture is that Δι-LFA-1, which resembles high affinity integrin, can directly signal the release of α4β1 from the cytoskeleton. In this way, the mechanism that induces Δι-LFA-1 to associate with lipid rafts will act on other integrins on the same cell surface, and cause their association with lipid rafts and thus contribute to their activation.


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