The Hepatitis C Envelope 2 Protein Inhibits LFA-1-Transduced Protein Kinase C Signaling for T-Lymphocyte Migration

YURI VOLKOV,* AIDEEN LONG, ‡ MICHAEL FREELEY, ‡ LUCY GOLDEN–MASON, § CLIONA O’FARRELLY, § ANNE MURPHY,* and DERMOT KELLEHER*

*Dublin Molecular Medicine Centre and Department of Clinical Medicine, Trinity Centre College, James’s Street, Dublin; ‡Department of Biochemistry, Royal College of Surgeons in Ireland, Dublin; and §Education and Research Centre, St. Vincent’s Hospital, Dublin, Ireland

Background & Aims: The ability of viruses to escape the host immune response represents a globally important problem related to a wide variety of pathogens. Hepatitis C is one of the major causes of liver disease worldwide. Clearance rates of this virus are low, and this condition normally involves a chronic inflammatory process. This raises a possibility that the virus may have developed mechanisms enabling it to evade T-cell-mediated immune surveillance. The aim of this study was to investigate the effect of the hepatitis C envelope protein E2 on LFA-1-stimulated T-cell migration and macrophage inflammatory protein (MIP-1α, MIP-1β) secretion.

Methods: T cells were stimulated through the leukocyte function-associated molecule-1 (LFA-1) receptor by incubating with either intracellular adhesion molecule 1 (ICAM-1)-Fc fusion protein or anti-LFA-1 immobilized on 8-well chamber slides. Subcellular localization of protein kinase C (PKC)-β, CD81, and LFA-1 was determined by immunofluorescence analysis. Lipid raft formation was assessed using the Cellomics Kineticscan reader. MIP-1α and MIP-1β levels were detected by enzyme-linked immunosorbent assay.

Results: We report that the hepatitis C envelope protein E2 can dramatically inhibit T-lymphocyte motility and chemokine release induced via LFA-1 integrin ligation. We have demonstrated a novel T-lymphocyte-directed viral inhibitory mechanism involving the PKC-β enzyme as a definitive intracellular target. E2-CD81 interaction stimulates translocation of PKC-β to lipid rafts, thereby preventing its association with the centrosome and microtubule cytoskeleton, which is crucial to the process of T-cell migration.

Conclusions: These studies identify a mechanism whereby the hepatitis C virus can evade the host immune response by inhibition of T-cell migration.

One of the most widespread viral infections worldwide, hepatitis C is characterized by the development of chronic hepatitis in the majority of infected cases with both portal and parenchymal inflammation.1,2 The progression is characterized by incipient fibrosis in the portal tracts with the ultimate outcome of cirrhosis.3 Although initial reports suggested that progression to cirrhosis was relatively rapid in hepatitis C, analysis of the 1977 Irish cohort of anti-D-infected women revealed that inflammation was relatively mild almost 20 years postinfection.4 Subsequent studies by Poynard et al have also confirmed the slow rate of progression of hepatitis C,5 which is characterized by a mild inflammatory infiltrate in the majority of patients.4 A striking feature of hepatitis C infection is its high rate of chronic infection. In those with persistent infection, there is a broad spectrum of disease; some will remain asymptomatic, whereas others will have severe disease resulting in cirrhosis, hepatocellular carcinoma, and premature death.

A number of bacterial and viral infections are accompanied by an inefficient immune response resulting in the failure of the host organism to clear the invading pathogens. In an effective immune system, lymphocytes can migrate between the blood, tissue, and lymphatics, providing surveillance for exogenous antigens and responding to infection. The actual movement of lymphocytes across the endothelium (diapedesis) in response to a chemokine signal is mediated in part by the integrin family of adhesion molecules. In particular, the interaction of the leukocyte integrin leukocyte function-associated molecule-1 (LFA-1) with its ligand, intracellular adhesion molecule 1 (ICAM-1), on endothelial cells is critical for this process.6

CD81 has been identified as one of the major receptors for hepatitis C, binding to the hepatitis C envelope protein (E2); however, this receptor is not necessary for infection, as is evident by studies in a knockout mouse model.8 CD81 is a member of the tetraspanin family of cell receptors. These molecules frequently associate with both epithelial and leukocyte integrin molecules,9,10 and

Abbreviations used in this paper: ICAM-1, intracellular adhesion molecule 1; LFA-1, leukocyte function-associated molecule-1; MIP, macrophage inflammatory protein; NK, natural killer; PBTL, peripheral blood T lymphocytes; PKC, protein kinase C; TCR, T-cell receptor.

© 2006 by the American Gastroenterological Association
0016-5085/06/$32.00
we hypothesized that E2 binding to CD81 could have the potential to modulate leukocyte integrin function. In earlier studies, it has been demonstrated that the binding of E2 to CD81 provided a costimulatory signal for human lymphocytes activated through the T-cell receptor.\textsuperscript{11} In addition, E2 protein engagement of CD81 has been reported to inhibit the function of natural killer (NK) cells by blocking tyrosine phosphorylation with consequent effects on NK-cell cytokine production and cytotoxic granule release.\textsuperscript{12} Furthermore, anti-CD81 has been shown to modulate LFA-1 (leukocyte \( \alpha L\beta 2 \) integrin) signalling in B cells and thymocytes.\textsuperscript{13,14} In previous studies on LFA-1 function, we demonstrated that cross-linking of LFA-1 signals for induction of lymphocyte migration through a protein kinase C (PKC)-\( \beta \)-dependent pathway.\textsuperscript{15} Second, activation of LFA-1 induces secretion of chemokines such as macrophage inflammatory protein (MIP)-1\( \alpha \) and MIP-1\( \beta \), which have the potential to significantly enhance local immune responses through induction of directed cell migration to specific tissue sites,\textsuperscript{16} although secretion of chemokines may involve multiple pathways.\textsuperscript{17} Here, we addressed the hypothesis that the hepatitis C virus has developed a specific instrument directed against the most central processes mediating T-lymphocyte migration and recruitment into the affected tissue. We demonstrate that hepatitis C E2 binding to CD81 stimulates the recruitment of PKC-\( \beta \) to cell membrane lipid raft domains. The sequestration of PKC-\( \beta \) in lipid rafts inhibits crucial intracellular mechanisms of directed T-cell locomotion, resulting in a dramatic reduction of LFA-1-mediated lymphocyte motility.

\section*{Materials and Methods}

\textbf{T-Cell Activation and Motility}

In T-cell locomotory studies, we used a previously characterized migration-triggering model system,\textsuperscript{15} which requires preactivation either via CD3 receptors or by the use of phorbol esters. Peripheral blood T lymphocytes (PBTL) separated by RosetteStep (StemCell Technologies, Vancouver, BC, Canada) and Ficoll-Hypaque sedimentation were treated with hepatitis C virus (HCV) recombinant proteins, including envelope protein (E2), C22 (core peptide), and C33 (a peptide from the NS5 coding region) (kind gifts of Dr Sergio Abrignani, Chiron Corporation, Emeryville, CA) at 1 \( \mu \)g/mL for 24 hours prior to or following activation with 25 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO) for 72 hours. HCV recombinant proteins were prepared in Chinese hamster ovary (CHO) cells and were at a purity of >98\%. Cell viability after the peptide treatment and activation was >98\%. Cells were cultured in RPMI (Gibco, Invitrogen, Life Technologies) containing 10\% fetal calf serum (FCS). T-cell motility studies were carried out in LabTek 8-well Permanox chamber slides (Nalge; Nunc International, Naperville, IL), precoated with human recombinant ICAM-1/Fc fusion protein overnight at 2 \( \mu \)g/mL (R&D Systems, Minneapolis, MN) or 0.1 mg/mL fibronectin (Sigma). Alternatively, wells were coated with goat anti-mouse immunoglobulins (DAKO, Carpinteria, CA), followed by either anti-LFA-1 monoclonal antibody (motility-inducing clone SPV-L7; Monosan, Sandbo, Uden, The Netherlands) or a motility-inducing monoclonal antibody to CD44 (D2.1) as described.\textsuperscript{15,16} T cells at a concentration of 1 \( \times 10^5 \) were incubated in the wells of precoated chamber slides at 37°C, in 5\% \( \mathrm{CO}_2 \) for 2 hours prior to assessing the motile behavior as described previously.\textsuperscript{18}

To confirm that the inhibitory effect of E2 on T-cell migration was mediated via its interaction with CD81, a peptide previously shown to inhibit CD81-E2 interaction was used.\textsuperscript{19} This peptide, CSPQYWTGPAC, when phage displayed could competitively inhibit the ability of E2 to bind native human CD81-expressing MOLT-4 cells. A scrambled version of this peptide was used as a control. Peptides were prepared by the Organic Synthesis Core, Institute of Biopharmaceutical Sciences, Royal College of Surgeons in Ireland, Dublin, and were of >98\% purity.

\textbf{Analysis of T-Cell Migratory Characteristics Following Lipid Raft Modification}

Evaluation of T-cell migratory phenotype in experiments with lipid raft disruptor methyl-\( \beta \)-cyclodextrin (MCD; purchased from Sigma) was carried out on the Cellomics Kineticscan reader (Cellomics, Inc, Pittsburgh, PA). Cells were treated with E2 peptide as described above and subsequently incubated for 60 minutes in flat-bottomed, 96-well plates on immobilized rICAM-1 in the presence of MCD at 0–5 mmol/L concentration range as shown on Figure 8. Cells were fixed and stained with a mixture of acridine orange and Höechst 33342 to visualize the nuclei and analyzed on the Kineticscan reader at 20\( \times \) magnification. Shape factor based on the ratio of major and minor object diameter (as per Cellomics application software) was used to evaluate cell polarization because it proved to be more sensitive than the more traditional circularity index determined as the object perimeter to area ratio. Five microscopic fields were scanned in each well, and all the experimental conditions were set up in triplicate.

\textbf{Evaluation of MIP-1\( \alpha \) and MIP-1\( \beta \) Secretion}

PBTL at a concentration of 1 \( \times 10^6 \) cells/mL were treated with HCV recombinant proteins E2, C22, and C33 (all at 1 \( \mu \)g/mL) for 4 hours either before or immediately after preactivation with PMA or anti-CD3 as described previously. Cells were incubated for 24 hours in 12-well plates (Nunclon; Nalgene-Nunc International, Naperville, IL) coated either with anti-LFA-1 as described above, antitransferrin receptor (TFR; clone T9, DAKO, Glostrup, Denmark), or a control irrelevant antibody to mouse HLA anti-IE (ATCC). MIP-1\( \alpha \) and MIP-1\( \beta \) levels in the supernatants were measured by enzyme-linked immunosorbent assay (ELISA; R&D Systems).
Aliquots of supernatants were also stored for subsequent evaluation of chemotactic activity.

**Chemotaxis**

Directed migration of T cells was evaluated in modified Boyden chamber system. Chemoattractant activity was assessed in the supernatants generated from PBTL incubated on anti-LFA-1, anti-CD44 (D2.1), or fibronectin-coated plates as described above. Cells used for the migration assays were PBTL activated with OKT3 for 2 hours at 37°C in 5% CO₂ at cell density of 1 × 10⁶/mL. The distance the cells migrated (μm) from the leading edge (maximum number of cells visible) into the supernatant-saturated filters through 3 μm-pore nitrocellulose disks (minimum number of cells visible) was measured using a light microscope (Leica-DMLB).

**Immunostaining**

T cells in the chamber slides, activated and incubated as described in the T-Cell Activation and Motility section (above) with or without E2 or other hepatitis C proteins were used for PKC immunostaining. At the end of the incubation periods, slides with attached cells were separated from the upper chamber material, washed twice with warm phosphate-buffered saline (PBS), and fixed/permeabilized in −20°C acetone for 10 minutes. Nonspecific binding sites were blocked for 30 minutes in 5% normal goat serum (DAKO), cells were washed twice with PBS, and then incubated at room temperature in 1:100 dilution of antibody solutions. After washing, slides were incubated in AlexaFluor-488 or 568 goat anti-rabbit (1/1000) (Molecular Probes, Eugene, OR) for 15 minutes at ambient temperature. For analysis of PKC-β and PKC-δ.
lipid raft localization, cells were costained with cholera-toxin FITC (Sigma). Slides were finally washed with PBS, drained, and mounted with DAKO fluorescent mounting medium. Immunostaining for colocalization of LFA-1 and CD81 was carried out in live activated T cells, incubated on immobilized recombinant ICAM-1/Fc. Slides were blocked with goat anti-mouse immunoglobulins (DAKO), followed by anti-LFA-1 antibodies (clone MHM-24) (DAKO) and goat anti-mouse AlexaFluor568 (Molecular Probes). Immunostaining for CD81 was performed using a rabbit polyclonal anti-CD81 antibody (clone H 121) (Santa Cruz) and goat anti-rabbit Alexa Fluor488 (Molecular Probes). After fixation, slides were mounted with fluorescent mounting medium (DAKO). Fluorescent microscopy was performed on a Perkin Elmer LCI confocal workstation with a Kr/Ar laser attached to a Nikon TE2000-U inverted microscope under Ultraview (Perkin Elmer) and Volocity-2 (Improvision Inc.) image acquisition and processing software, respectively. Flow cytometry studies to analyze the expression of the LFA-1 activation epitope were performed on the Beckton Dickinson FACS analyzer using mAb24 antibody clone (Monosan).

**Cell Microinjection**

To investigate the effects of hepatitis C viral E2 protein on PKC-β-dependent T-cell motility, we utilized a PKC-β-deficient cell clone K-4 derived from a parental HUT-78 lymphoma line (constitutively activated T-cell phenotype not requiring inside-out signal for integrin activation) by the method described previously. K-4 cells were micro-

![Figure 2. Quantitative evaluation of the number of PBTL developing a polarized migratory phenotype on anti-CD44 (A) or fibronectin (B). Cells were exposed to recombinant viral proteins at different stages of activation: preactivation, proteins added before PMA; postactivation, proteins added following PBTL activation with PMA; E2 alone, cells were treated with viral proteins only and no PMA prior to exposure to immobilized anti-CD44 or fibronectin. When cells were incubated with a peptide that blocks CD81-E2 interactions (IP) prior to E2 addition, the number of cells demonstrating migratory phenotype significantly increased (P < .05 in comparison with E2 added at preactivation stage) but remained significantly lower than the numbers expressing migratory phenotype in the absence of E2 (P < .05). Reversal to the migratory phenotype was not observed when a scrambled, control peptide (CP) was used.](image)

![Figure 3. Hepatitis C protein E2 inhibits the secretion of MIP-1α and MIP-1β in response to anti-LFA-1. Secretion of MIP-1α (A) and MIP-1β (B) by PBTL activated via TCR/CD3 cross-linking and subsequently exposed to immobilized motility-triggering LFA-1 mAbs. Cells were incubated with recombinant viral proteins at different stages of activation: preactivation, proteins added before anti-CD3; postactivation, proteins added following PBTL activation with anti-CD3; protein alone, cells were treated with viral proteins only and no anti-CD3 prior to exposure to immobilized LFA-1 mAbs. Columns: open, cells activated with anti-CD3 mAbs alone but not stimulated with anti-LFA-1; solid, cells not treated with any of recombinant proteins; hatched, cells exposed to E2 envelope viral protein; crosshatched, C22 control protein; checkered, C33 control protein. R (second column from left), resting cells exposed to immobilized LFA-1 mAbs. Asterisks in A and B indicate statistically significant reduction in cytokine secretion in relation to the respective values from activated cells not exposed to any viral proteins (P < .05).](image)
injected with plasmid expressing PKC-\beta\textsubscript{H9252}/EGFP (Clontech, Palo Alto, CA). Cells in control wells were microinjected with “empty” EGFP vector (Clontech). Cells were allowed to recover for a further 5 hours at 37°C in the incubator and analyzed for PKC-\beta\textsubscript{H9252}-EGFP expression, polarization, and motility characteristics on the immobilized LFA-1 antibodies. In the absence of HCV E2, on average, 80% of the cells expressing PKC-\beta\textsubscript{H9252}-EGFP developed a polarized locomotory phenotype upon triggering via LFA-1. None of the cells expressing EGFP alone showed a significant degree of polarization, and the cells remained static over ∼3 hours of subsequent continuous observation.

Tissue Specimens and Extraction of Protein

Normal liver wedge biopsy specimens (50–100 mg, n = 14) were obtained from donor organs at time of liver transplantation. HCV-infected (n = 11), cirrhotic control liver (ALD; n = 9), and primary biliary cirrhosis (PBC; n = 11) specimens were obtained at time of liver transplantation for end-stage liver disease. Protein was extracted from ∼100 mg powdered tissue. ELISA antibody pairs for the detection of MIP-1\alpha and MIP-1\beta levels in protein were obtained from R&D Systems as described.\textsuperscript{20}

Figure 4. Exposure of T cells to E2 protein at an early stage of activation suppresses chemoattractant secretion and chemotaxis. Migration of anti-TCR/CD3 activated PBTL toward supernatants derived from T cells exposed to E2 protein at different stages of activation. Anti-TFR, migration of PBTL towards supernatants derived from cells exposed to antitransferrin receptor mAbs; anti-LFA-1, migration of PBTL towards supernatants derived from cells exposed to motility-inducing LFA-1 mAbs. Supernatants were obtained from the T lymphocytes incubated with recombinant E2 protein at different stages of activation: \textit{preactivation}, protein added before anti-CD3; \textit{postactivation}, protein added following activation with anti-CD3. \textit{R}, migration toward supernatants derived from the resting cells (no activation or protein addition). \textit{Asterisk} indicates statistically significant reduction in comparison with distances migrated toward supernatants derived from T cells not exposed to E2 protein (\(P < .05\)).

Figure 5. Addition of the E2 protein at the stage of preactivation reduces the transmigration ability of T lymphocytes towards supernatants derived upon stimulation with either anti-LFA-1, CD44 cross-linking, or fibronectin. Migration of PMA-activated PBTL toward supernatants derived from T cells exposed to E2 protein at different stages of activation (modified Boyden chamber assay). Anti-LFA-1, migration of PBTL towards supernatants derived from cells exposed to motility-inducing LFA-1 mAbs; anti-CD44, migration of PBTL towards supernatants derived from cells exposed to anti-CD44 mAbs; fibronectin, migration towards supernatants derived from cells exposed to purified fibronectin; TFR, migration towards supernatants derived from cells exposed to antitransferrin receptor mAb. Supernatants were obtained from the T lymphocytes incubated with recombinant E2 or control C22 protein at different stages of activation: \textit{preactivation}, protein added before PMA; \textit{postactivation}, protein added following activation with PMA. \textit{Open column}, migration toward supernatants derived from the resting cells (no activation or protein addition).
Results

Viral Envelope Protein E2 Inhibits T-Cell Locomotion

Activated T-lymphocytes triggered via LFA-1/ICAM-1 receptor/ligand interaction rapidly polarize and acquire active motile behavior. The locomotion-associated phenotype induced by LFA-1 signalling fully develops when lymphocytes have been stimulated either through the T-cell receptor (TCR) or with phorbol esters (PMA), both of which are known to induce the inside-out signal for activation of the leukocyte integrins (Figure 1A and 1B). Treatment of peripheral blood lymphocytes with hepatitis C C22 and C33 proteins at the stage of preactivation did not have any significant impact on either the locomotion-associated phenotype or cell migration (Figure 1C, E, and I). However, the presence of the hepatitis C envelope protein E2 during preactivation dramatically modified the response to subsequent LFA-1 signalling through interactions with ICAM-1/Fc with a complete loss of the locomotion-associated phenotype (Figure 1G and I). Treatment with E2 protein following the inside-out signal for LFA-1 activation did not significantly modulate the development of locomotion-associated phenotype (Figure 1J). A peptide, previously shown to block E2 binding to CD81, reversed the inhibitory effect of E2 on T-cell polarization (Figure 1J), demonstrating that E2 was at least partially mediating its effect via interaction with CD81. The data,

Figure 7. Inhibition of LFA-1-dependent T-cell migration by E2 is mediated by PKC-β. T cells shown in all the panels were exposed to motility-triggering anti-LFA-1 mAbs in various experimental conditions given below. (A) Resting (nonactivated) cells immunostained for PKC-β (left panel). Resting cells display unpolarized phenotype and diffuse cytoplasmic PKC-β staining pattern. (B) T cells exposed to control C33 protein prior to PMA activation retain a locomotory phenotype and display dramatic redistribution of PKC-β to the centrosome and trailing projections. (C) E2 protein pretreated cells (before PMA activation) are not polarized, fail to develop a locomotory phenotype when exposed to anti-LFA-1, and display a juxtamembranous expression pattern of PKC-β. Middle and right panels in A–C represent 3-D projections of individual cells indicated by arrows in respective left panels and, hence, show distribution of protein kinase C β in the designated vertical or horizontal plane as indicated by the arrow. (D) To confirm a definitive role for PKC-β, we used the PKC-β-deficient cell clone K-4. K-4 cells microinjected with “empty” EGFP vector and exposed to motility-triggering anti-LFA-1 mAbs fail to undergo significant polarization. (E) K-4 cells injected with PKC-β-EGFP plasmid and treated with C33 protein polarize and display net body translocation (F) illustrated by green and tinted yellow of starting and end images of the same cell over a 30-minute interval. (G) K-4 cells pretreated with E2 prior to injection with PKC-β-EGFP plasmid fail to polarize and migrate on anti-LFA-1. (D–G) Provide images of representative cells out of 50–100 successfully transfected.
however, cannot exclude the possibility that there is another receptor mediating the remainder of the E2 effects.

Activated T cells display a similar migratory phenotype when stimulated through CD44 or β1 integrin. However, the presence of E2 during preactivation significantly decreased the induction of a locomotory phenotype by fibronectin or anti-CD44 (Figure 2). Again, this inhibition could be reversed by blocking E2-CD81 interaction using the inhibitory peptide but not the “scrambled” control peptide variant.

**Hepatitis C Virus Suppresses Chemokine Secretion In Vitro and In Vivo**

The secretion of chemokines MIP-1α and MIP-1β was subsequently analyzed in lymphocytes stimulated through the TCR in response to cross-linking of the LFA-1 receptor (Figure 3A and B). Cross-linking of TCR alone produces a low level of MIP-1α and MIP-1β (Figure 3A and B, open bars). Pretreatment with E2 protein resulted in significant attenuation of both MIP-1α \( (P = .03) \) and MIP-1β \( (P = .04) \) production in response to anti-LFA-1, although this inhibition was incomplete. Similar results were seen when cells were stimulated with anti-CD44 or fibronectin (data not shown). Pretreatment of lymphocytes with control hepatitis C peptides C22 and C33 did not inhibit the secretion of these chemokines. The effect of E2-mediated reduction in chemokine secretion was confirmed using a modified Boyden chamber approach22 (Figure 4). Addition of the E2 protein at the stage of preactivation dramatically reduced the transmigration ability of T lymphocytes in response to LFA-1 cross-linking. A similar effect of E2 on anti-CD44 and fibronectin-stimulated transmigration was also registered (Figure 5). The observed in vitro phenomenon of reduction in chemokine levels was further investigated on ex vivo hepatic tissue from patients suffering from hepatitis C. The data obtained from the patients demonstrated that MIP-1α chemokine concentrations in liver biopsy tissue of individuals with hepatitis C were significantly lower than in primary biliary cirrhosis or alcoholic liver disease. However, MIP-1β levels were not reduced (Figure 6).

**Inhibition of T-Cell Migration by E2 Targets a Key Lymphocyte Motility Mechanism**

To investigate the specific mechanisms of T-cell motility inhibition by hepatitis C envelope protein, we
analyzed the intracellular distribution and functional involvement in this process of the PKC-β enzyme, representing a central cytoskeleton-targeted system controlling active locomotion in T cells. The exposure of peripheral blood lymphocytes to E2 protein at the stage of preactivation dramatically changes the PKC-β translocation pattern (Figure 7C) and results in a complete loss of the typical motile phenotype upon LFA-1 ligation (Figure 7A–C). In E2-treated T cells, PKC-β is no longer associated with the centrosome and displays juxtamembrane staining pattern (Figure 7C). This is in sharp contrast with PKC-β distribution at the centrosome and along the trailing projections observed in control C33-treated cells (Figure 7B) and with the diffuse intracytoplasmic PKC-β staining in resting T cells (Figure 7A).

To obtain direct evidence of E2-mediated targeting of PKC-β in living cells, we utilized the PKC-β-deficient clone of HUT78 lymphoma cell line K-4, which is nonresponsive to a locomotion-triggering signal through LFA-1 cross-linking (Figure 7D). K-4 cells transfected with GFP-PKC-β migrated in response to LFA-1 ligation and demonstrated PKC-β within the trailing process (Figure 7E and F). Redistribution of PKC-β was accompanied by the net body translocation (Figure 7F). In K-4 cells treated with E2, characteristic PKC-β redistribution and migration did not occur (Figure 7G).

Inhibition of LFA-1-Dependent Migration by E2 Can Be Rescued by Lipid Raft Modification

We further addressed the mechanism of the inhibition of T-cell migration by E2 by exploring the possibility of specific compartmentalization of PKC-β by the viral protein. In the studies conducted to determine the cytoskeletal modifications induced by E2, we detected “patchiness” of actin distribution, potentially indicative of enhanced lipid raft formation (data not shown). To evaluate this process further, we utilized the Cellomics Kineticscan, which permits the objective digital acquisition of data on shape change on large numbers of individual cells within populations. In experiments carried out utilizing this technology, it was found that the typical elongated locomotory T-cell phenotype could be rescued in the E2-treated (nonmigratory) cells by addition of the lipid raft disrupter MCD in a dose-dependent manner (Figure 8A). Moreover, co-staining performed in these cells using cholera toxin as a marker for lipid raft formation revealed that a significant amount of PKC-β was indeed colocalized with lipid rafts at the plasma membrane in the cells exposed to E2 and not treated with MCD (Figure 8B). The disruption of the E2-induced lipid rafts resulted in “liberation” of PKC-β with consequent translocation to the microtubule cytoskeleton and initiation of cell migration (Figure 8C).

CD81 Colocalizes With LFA-1 in the Early But Not the Late Stage of Lymphocyte Adhesion and Migration

Many tetraspanins have previously been shown to colocalize with integrin molecules. The effects of E2 protein on the distribution of CD81 and colocalization of CD81 and LFA-1 were further studied by live cell confocal microscopy (Figure 9). Both LFA-1 and CD81 were widely distributed on the cell membrane without significant colocalization (Figure 9A). On pretreatment with envelope protein, a significant portion of CD81 was
found to colocalize with LFA-1, although a substantial amount of this receptor remains in locations remote from LFA-1 (Figure 9E). In the migratory lymphocytes pre-treated with C33 control protein, it is clear that CD81 and LFA-1 do not colocalize (Figure 9D). LFA-1 is found predominantly clustered around the “neck” of the migrating cell posterior to the nucleus, as previously described, whereas CD81 is frequently seen at the tip of the trailing process (Figure 9D, arrows).

However, in the cells undergoing the early stages of initial adhesion, spreading, and polarization (Figure 9B and C), there were detectable areas of transient LFA-1/CD81 colocalization. Ligation of CD81 by E2 does not affect the activation of the LFA-1 molecule itself because pretreatment of the cells with E2 at the stage of preactivation did not inhibit the appearance of the LFA-1 activation epitope recognized by the mAb24 antibody (data not shown). E2-mediated inhibition of the migration signal delivered through β1 integrin or CD44, in addition to LFA-1, suggests that the target of this viral protein is downstream of the cell surface adhesion molecule, ie, PKC-β (Figure 8) and that the inhibitory signal is transmitted by CD81 itself. E2-CD81 interaction does not on its own affect the integrity of the microtubule or vimentin cytoskeleton, although it did induce a “patchy” membrane distribution of actin, consistent with lipid raft formation (data not shown).

**Discussion**

We demonstrate here that human T lymphocytes exposed to the hepatitis C virus recombinant envelope protein have a severely impaired ability to polarize and migrate in response to the triggering stimulus generated as a result of LFA-1 interaction with its natural ligand ICAM-1. The inhibiting effect on T-cell locomotion represents a phenomenon specific to the viral protein E2 because lymphocyte incubation with other recombinant peptides, including core protein C22 and C33 from the NS3-coding region, did not produce similar results. The observed effect was dramatic, leading to a complete loss of LFA-1-mediated, T-cell polarization and developed exclusively if the E2 protein was applied to the cells at the stage preceding the integrin activation step, crucial for establishing T-cell locomotory behavior. This might represent a fundamental mechanism enabling the virus to escape T-lymphocyte attack at initial infection. On the other hand, the ability of the viral E2 protein to suppress both cell migration and the secretion of T-cell-derived chemotactic factors suggests that the virus might also utilize a second line of defense by blocking further lymphocyte recruitment into already established inflammatory sites within the liver.

During active migration, T lymphocytes utilize a complex machinery of the dynamically restructuring cytoskeleton governed by intracellular phosphorylation cascades largely involving the enzymes of the PKC family. In previous studies, we have documented that cross-linking of LFA-1 in T lymphocytes resulted in the translocation of PKC-β to a site adjacent to the centrosome and along the trailing processes of these cells and also demonstrated the crucial impact of the PKC-β enzyme on the microtubule functioning in motile T cells. Translocation of PKC-β to the microtubule-rich uropods thereby represents a hallmark of activated T-lymphocyte locomotion induced not only by LFA-1 but also by molecules such as CD44 and VLA-4. The loss of this event in E2-treated T cells clearly indicates that the hepatitis C virus is exerting its impact on a significant system underlying lymphocyte motility. Further evidence was received from the results of the studies in the PKC-β-deficient cell clone K-4, which were unable to respond to LFA-1 cross-linking. Locomotory behavior in response to stimulation through LFA-1 was restored on expression of PKC-βI but not when these cells were pre-exposed to E2 protein. The mechanism of PKC-β targeting was further elucidated when it was determined that the isoenzyme colocalized with the marker of lipid rafts cholera toxin-FITC when cells were treated with E2. Lipid rafts are defined as detergent-resistant membrane microdomains of specific lipid and protein composition in which lipid-modified signalling molecules accumulate. Accumulation of signalling molecules in lipid rafts may be a part of normal signalling processes through the TCR. However, the stimulation of PKC-β translocation to lipid rafts following treatment of the cells with E2 resulted in a “trapping” of the isoenzyme in this domain and a failure of its ability to associate with the centrosome and microtubule cytoskeleton, a process necessary for cellular migration following stimulation through LFA-1. This association of PKC-β with lipid rafts following E2-mediated signalling through CD81 could be blocked using the inhibitor of lipid raft formation, MCD, thus reversing the attenuation of migration by E2. Although it has been shown that CD81 itself exists in lipid raft-like domains, the signal delivered by ligation of CD81 by the viral E2 protein, which is driving PKC-β to the lipid rafts, represents a novel manipulation of a signalling pathway directly affecting cellular function in the context of hepatitis C. Intriguingly, other viruses including the human immunodeficiency virus (HIV) and measles virus may also target...
T-cell function through modulation of lipid rafts, albeit using different mechanisms.

Our studies indicate that interaction of the CD81 receptor with hepatitis C envelope protein has profound and significant effects on the process of PKC-β translocation to the cytoskeleton with consequent effect on LFA-1-dependent signaling for lymphocyte migration. These effects may be mediated via transient LFA-1/CD81 interactions following CD81 receptor ligation with E2. Studies in other systems have revealed that tetraspanin molecules, indeed, frequently associate with integrins. For example, the tetraspanin molecule CD151 is known to associate with the integrin α6β4 in regulating the spatial organization of hemidesmosomes. CD81 has been demonstrated to associate with α3β1 in a functionally relevant fashion in neural cells, and it has also been shown that tetraspanins recruit PKC isoforms to their cytoplasmic domains, bringing them into proximity with integrin molecules. However, in the current study, the association of CD81 with the LFA-1 molecule appears to be transient and limited and does not appear to play a major role in the inhibition of lymphocyte migration. We cannot exclude the possibility that transient association might have some modulatory effects on other LFA-1-mediated functions.

In the current study in which the signal is delivered through the LFA-1 molecule, at a time point separate from the TCR stimulation, the effects of E2 are found to mediate a decrease in lymphocyte motility and a reduction in the release of chemokines, both of which may have direct functional relevance to the pathogenesis of hepatitis C. Because inflammation is mild in many patients with hepatitis C, it is conceivable that the impact of interaction between the E2 envelope protein and CD81 may result in reduced accumulation of functionally relevant cells at the site in which their activity is needed for effective host defense against the virus. This thesis is supported by the finding that MIP-1α levels within the liver were significantly reduced in patients with hepatitis C, although signaling for chemokine release is likely to be multifactorial. Genetic data indicating a role for CCR-5 receptors in the outcome of hepatitis C infection further strengthens the hypothesis that lymphocyte migration patterns may be critical to pathogenesis of this disease. Hence, we suggest that the hepatitis C virus may impact on the host immune response by specifically altering the lymphocyte recruitment to the infected liver. Although this may potentially result in less severe disease, it may also interfere with the capacity of the host to eliminate this virus.

References
18. Kelleher D, Murphy A, Feighery C, Casey EB. Leukocyte function-associated antigen 1 (LFA-1) and CD44 are signalling molecules...


