Inhibition of caveolar uptake, SV40 infection, and β1-integrin signaling by a nonnatural glycosphingolipid stereoisomer

Raman Deep Singh,[1] Eileen L. Holicky,[1] Zhi-jie Cheng,[1] Seong-Youl Kim,[1] Christine L. Wheatley,[1] David L. Marks,[1] Robert Bittman,[2] and Richard E. Pagano[1]

[1]Department of Biochemistry and Molecular Biology, Thoracic Diseases Research Unit, Mayo Clinic College of Medicine, Rochester, MN 55905
[2]Department of Chemistry and Biochemistry, Queens College, The City University of New York, Flushing, NY 11367

Caveolar endocytosis is an important mechanism for the uptake of certain pathogens and toxins and also plays a role in the internalization of some plasma membrane (PM) lipids and proteins. However, the regulation of caveolar endocytosis is not well understood. We previously demonstrated that caveolar endocytosis and β1-integrin signaling are stimulated by exogenous glycosphingolipids (GSLs). In this study, we show that a synthetic GSL with nonnatural stereochemistry, β-D-lactosyl-*N*-octanoyl-L-*threo*-sphingosine, (1) selectively inhibits caveolar endocytosis and SV40 virus infection, (2) blocks the clustering of lipids and proteins into GSLs and cholesterol-enriched microdomains (rafts) at the PM, and (3) inhibits β1-integrin activation and downstream signaling. Finally, we show that small interfering RNA knockdown of β1 integrin in human skin fibroblasts blocks caveolar endocytosis and the stimulation of signaling by a GSL with natural stereochemistry. These experiments identify a new compound that can interfere with biological processes by inhibiting microdomain formation and also identify β1 integrin as a potential mediator of signaling by GSLs.

Introduction

Several clathrin-independent mechanisms of endocytosis have been described and are the focus of much attention among cell biologists (Nichols and Lippincott-Schwartz, 2001; Johannes and Lamaze, 2002). Of particular interest is caveolar endocytosis, a dynamin-dependent, cholesterol-sensitive uptake mechanism (Mineo and Anderson, 2001; Pelkmans and Helenius, 2002; Kirkham and Parton, 2005; Cheng et al., 2006a). Caveolar cargo varies with cell type and includes fluorophore-modified albumins, SV40 virus, cholera toxin B (CtxB) subunit, and fluorescent glycosphingolipid (GSL) analogues. Internalization of certain cell surface integrins also occurs via caveolae once the integrins are activated (e.g., by cross-linking; Upla et al., 2004; Sharma et al., 2005). Regulation of caveolar endocytosis is only beginning to be understood; uptake by this mechanism is stimulated when cells are treated with phosphatase inhibitors (e.g., okadaic acid) or when certain caveolar cargo binds to its receptor (Parton et al., 1994; Tagawa et al., 2005). The stimulation of caveolar endocytosis is accompanied by the increased activation of src and phosphorylation of caveolin-1 and dynamin, suggesting that stimulation occurs via increased kinase activities. Indeed, a recent screen of the human kinome identified a total of 80 different kinases that are somehow involved in the uptake of SV40 virus, a caveolar marker (Pelkmans et al., 2005). We recently showed that caveolar uptake is also stimulated when cells are briefly incubated with either natural (e.g., bovine brain lactosylceramide [LacCer] or GM_1 ganglioside) or synthetic (e.g., β-D-lactosyl-*N*-octanoyl-sphingosine) GSLs (Sharma et al., 2004; Kirkham et al., 2005). In each case, the sphingosine moiety was the natural D-*erythro* (D-*e*; also referred to as 2*S*,3*R*) stereoisomer. In this study, we tested a nonnatural stereoisomer of LacCer, β-D-lactosyl-*N*-octanoyl-L-*threo*-sphingosine (L-*t*-LacCer or 2*S*,3*S*), and found unexpectedly that this analogue selectively inhibited the internalization of multiple caveolar markers, including the SV40 virus, rather than stimulating their uptake. We further show that this inhibition was caused by the disruption of plasma membrane (PM) microdomains enriched in GSLs and cholesterol and by an inhibition of integrin activation and signaling.

Correspondence to Richard E. Pagano: pagano.richard@mayo.edu

Abbreviations used in this paper: Ab, antibody; β1-stim Ab, stimulatory β1-integrin Ab; CtxB, cholera toxin B; GPI, glycosyl-phosphatidylinositol; GSL, glycosphingolipid; HSF, human skin fibroblast; IL-2R, interleukin-2 receptor β subunit; LacCer, lactosylceramide; PM, plasma membrane; Tfn, transferrin.

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Results and discussion

To examine the effect of LacCer stereoisomers on mechanisms of endocytosis in human skin fibroblasts (HSFs), various fluorescent endocytic markers were used. These probes were previously characterized as relatively specific markers of uptake via caveolae- (anti–β1-integrin [β1-Fab] and fluorescent albumin), clathrin- (transferrin [Tfn]), and RhoA (interleukin-2 receptor β subunit [IL-2R])-regulated mechanisms using dominant-negative proteins and biochemical inhibitors (Lamaze et al., 2001; Sabharanjak et al., 2002; Sharma et al., 2004, 2005; Cheng et al., 2006b). We used 1 mg/ml of fluorescent dextran as a probe for Cdc42-regulated endocytosis, as this concentration of dextran has been shown to selectively label endosomes derived from this pathway (Sabharanjak et al., 2002; Cheng et al., 2006b). An Fab fragment of an antibody (Ab) against GFP was used to follow the endocytosis of glycosylphosphatidylinositol (GPI)–GFP via this pathway. Endocytosis of GPI-GFP was perturbed by the expression of dominant-negative Cdc42 (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200609149/DC1). In addition, endocytosed GPI-GFP closely colocalized with endocytosed dextran (Fig. S1 B). These results indicate that dextran and GPI-GFP are internalized via the Cdc42-regulated pathway in HSFs as they are in CHO cells (Sabharanjak et al., 2002).

Cells were coincubated with the endocytic probes and 5 μM L-*t*-LacCer or the corresponding natural (D-*e*) stereoisomer for 30 min at 10°C (see Materials and methods). Biochemical analysis indicated that approximately equal amounts of either LacCer stereoisomer became cell associated under these incubation conditions (~3 pmol/10^6 cells). The samples were then briefly warmed to 37°C to allow endocytosis to occur, and the amount of internalization was quantified by image analysis. Pretreatment of cells with L-*t*-LacCer inhibited the uptake of caveolar markers (labeled albumin or anti–β1-integrin Fab fragment) by ~70% relative to untreated control cells, whereas there was a slight stimulation (~10–20%) of Cdc42-regulated (dextran and GPI-GFP) and RhoA-regulated (IL-2R) endocytosis; little effect was seen on the clathrin-dependent internalization of Tfn (Fig. 1, A and B). In contrast, when β-D-lactosyl-*N*-octanoyl-D-*erythro*-sphingosine (D-*e*-LacCer) was used, internalization via caveolae was increased 60–100% relative to untreated controls as previously reported (Sharma et al., 2004). Quantitative analysis of surface caveolae by electron microscopy (Sharma et al., 2004) after treatment of cells with the LacCer stereoisomers showed that the L-*t* isomer increased the number of caveolae present at the PM, which is consistent with the inhibition of internalization by this route, whereas the D-*e* isomer reduced the number of surface-connected caveolae, presumably reflecting an increase in internalization of

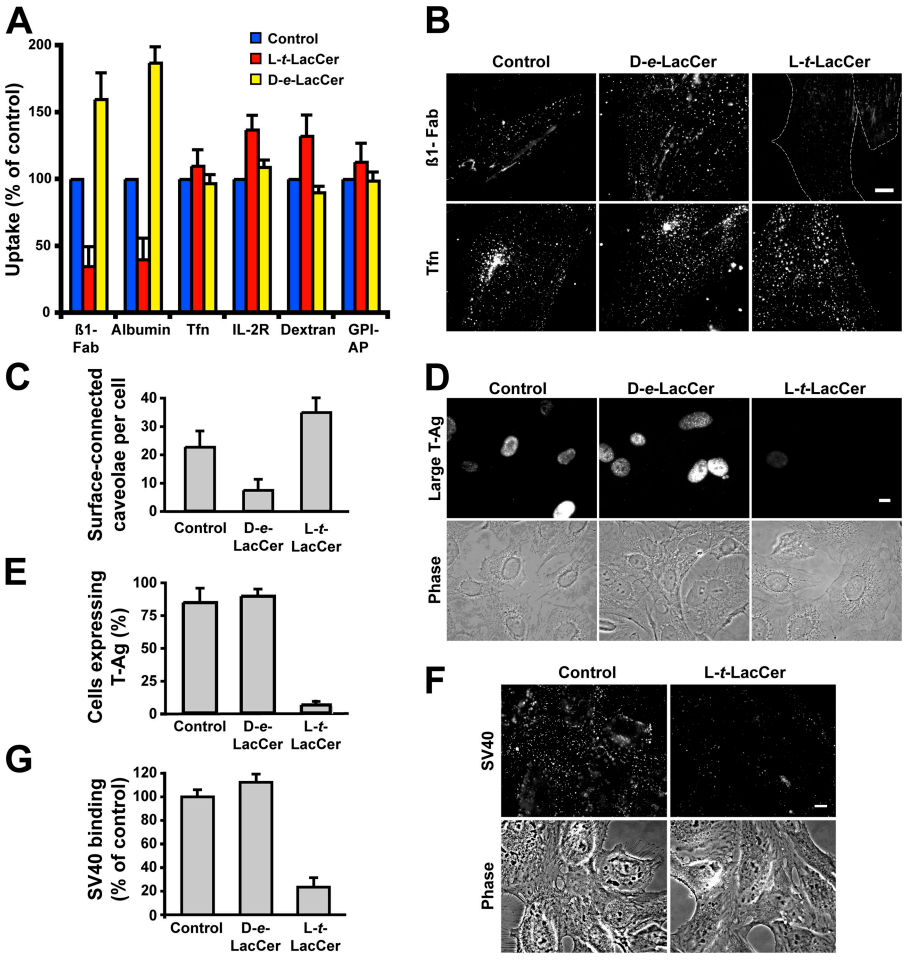

Figure 1. **L-*t*-LacCer inhibits caveolar endocytosis and SV40 infection.** (A and B) HSFs were incubated ± LacCer stereoisomers for 30 min at 10°C, and endocytosis of various markers after 5 min at 37°C was observed (see Materials and methods). Endocytosis was quantified by image analysis ($n \geq 30$ cells/marker) and expressed as a percentage of controls without lipid addition. Note that L-*t*-LacCer inhibited the uptake of caveolar markers (anti–β1-integrin Fab and albumin) but not markers for other endocytic mechanisms. Dotted lines in B outline two cells in the field. (C) HSFs were incubated ± LacCer stereoisomers as in A, warmed for 30 s at 37°C, processed for EM, and the number of surface-connected caveolae was determined ($n \geq 15$ cells/condition; Sharma et al., 2004). (D and E) CV1 cells were incubated ± LacCer stereoisomers as in A and were coincubated for 1 h at 10°C with SV40 virus (MOI = 15). Samples were washed, incubated for 14 h, and stained for the large T antigen (Tsai et al., 2003). Values in E are means ± SD (error bars; $n \geq 50$ cells/condition; three independent experiments). (F and G) CV1 cells were treated with SV40 for 1 h as in D and E, and SV40 binding to live cells was detected using anti-SV40 VP1 mAb (Chen and Norkin, 1999). Values in G are means ± SD ($n \geq 20$ cells from two independent experiments). Bars, 10 μm.

caveolae-derived vesicles (Fig. 1 C; Sharma et al., 2004). The results in Fig. 1 show that pretreatment of cells with the nonnatural LacCer stereoisomer blocks caveolar endocytosis while not markedly affecting other mechanisms of internalization.

The effect of L-*t*-LacCer treatment on SV40 infection in monkey CV1 cells was examined next because endocytosis of this virus has been extensively characterized in this cell type and is shown to occur via caveolae (Anderson et al., 1996; Stang et al., 1997; Pelkmans et al., 2001). Pretreatment of cells with L-*t*-LacCer dramatically reduced viral infection as monitored by the expression of the large T antigen after 14 h at 37°C, whereas D-*e*-LacCer had no effect relative to untreated control samples (Fig. 1, D and E). Because the inhibition of SV40 infection by L-*t*-LacCer could result from effects on virus binding as well as endocytosis, we also studied the effect of L-*t*-LacCer on SV40 binding. We found that the L-*t*-LacCer treatment inhibited SV40 binding by ~80% (Fig. 1, F and G), as detected using a mAb to the SV40 major capsid protein VP1 (Chen and Norkin, 1999). Thus, the effect of the nonnatural LacCer isomer on SV40 infection was probably mainly caused by its inhibition of SV40 binding to the cell surface.

We next addressed potential mechanisms by which L-*t*-LacCer might selectively inhibit caveolar endocytosis as well as the binding of SV40 to the cell surface. One possibility is that L-*t*-LacCer might disrupt PM microdomains, which are local regions of the PM enriched in GSLs and cholesterol that may act as organizing centers for particular proteins (for reviews see London and Brown, 2000; Rajendran and Simons, 2005). To test this possibility, cells were incubated with AlexaFluor594-CtxB at 10°C to label GM_1 ganglioside at the PM followed by an anti-CtxB IgG. This treatment caused the formation of numerous micrometer-size clusters of CtxB at the PM, which were not present in the absence of anti-CtxB IgG (Fig. 2 A, left vs. middle). Importantly, when cells were pretreated with L-*t*-LacCer for 30 min at 10°C before incubation with the labeled CtxB and cross-linking anti-CtxB IgG, clustering into PM domains was prevented (Fig. 2 A, right). No inhibition of domain formation was observed using D-*e*-LacCer; rather, the D-*e* isomer induced the formation of large domains enriched in GM_1 ganglioside and cholesterol in the absence of the cross-linking IgG (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200609149/DC1; Sharma et al., 2005).

We then incubated CV1 cells at low temperature with SV40 virus and found that this treatment induced the formation of PM domains enriched in GM_1 ganglioside and cholesterol (Fig. 2, B and C; left vs. middle), which is remarkably similar to the clustered microdomains induced by CtxB cross-linking (Fig. 2 A). Interestingly, the pretreatment of CV1 cells with L-*t*-LacCer prevented the SV40 induction of these PM domains (Fig. 2, B and C; right). These results provide a potential explanation for the reduction in SV40 binding to CV1 cells because GM_1 ganglioside is a receptor for SV40 (Tsai et al., 2003), and clustering of GM_1 may be required for maximal SV40 binding.

Together, these experiments demonstrate that the induction of PM domains by various treatments (cross-linking Abs or SV40) is prevented by L-*t*-LacCer. This modulation of PM domain organization may disrupt the distribution of potential cargo in microdomains and, thus, inhibit their endocytosis via caveolae. The concept that microdomain clustering is important for caveolar endocytosis is supported by previous observations. First, unlike BODIPY–D-*e*-LacCer, which associates with microdomains and is internalized via caveolae, the L-*t* isomer of BODIPY-LacCer does not partition into microdomains at the PM and is not selectively endocytosed via caveolae (Singh et al., 2006). Second, agents that stimulate the clustering of GSL-enriched microdomains at the PM (e.g., exogenous GSLs and β1-integrin cross-linking Abs) also stimulate caveolar endocytosis (Sharma et al., 2004, 2005).

A second mechanism by which L-*t*-LacCer might inhibit caveolar internalization is by disrupting transmembrane signaling events required for endocytosis. We focused on signaling through β1 integrin because this integrin is internalized via caveolae in HSFs and other cell types (Upla et al., 2004; Sharma et al., 2005) and because an early event after integrin activation is signaling through src, a kinase whose activity is required for caveolar endocytosis (Mineo and Anderson, 2001; Arias-Salgado et al., 2003; Sharma et al., 2005). We first examined the activation of β1 integrin in HSFs after cross-linking with a stimulatory β1-integrin Ab (β1-stim Ab) using the HUTS-4 Ab, which only binds to β1 integrins in their activated conformation (Luque et al., 1996). Treatment with the stimulatory Ab dramatically increased HUTS binding (Fig. 3, A and B), whereas

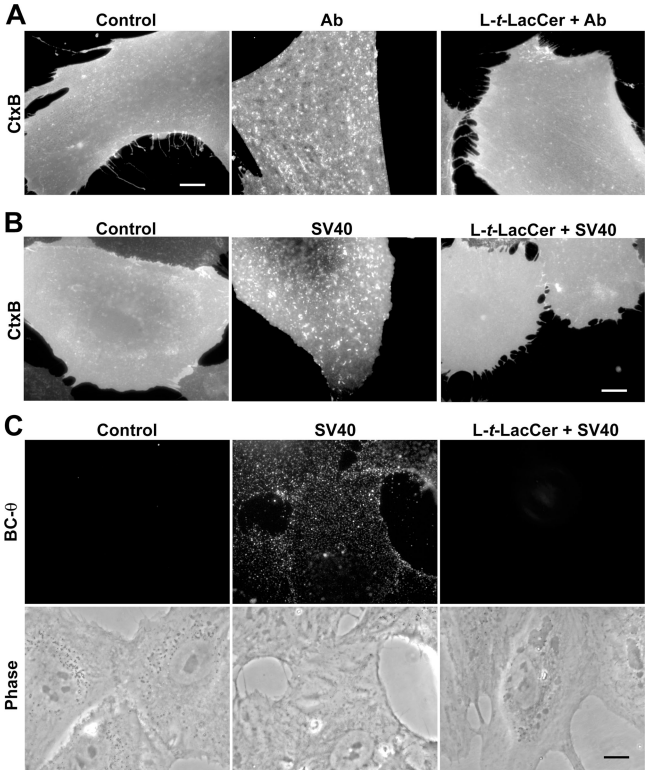

Figure 2. **L-*t*-LacCer inhibits the formation of PM domains.** (A–C) PM domains enriched in GM_1 ganglioside (A and B) or cholesterol (C) were visualized using fluorescent CtxB or BC-Θ, respectively (see Materials and methods). Clustering of these probes was induced using an anti-CtxB Ab or SV40 virus. Note the inhibition of microdomain clustering when cells were pretreated with L-*t*-LacCer compared with untreated control samples. A, HSFs; B and C, CV1 cells. Bars, 10 μm.

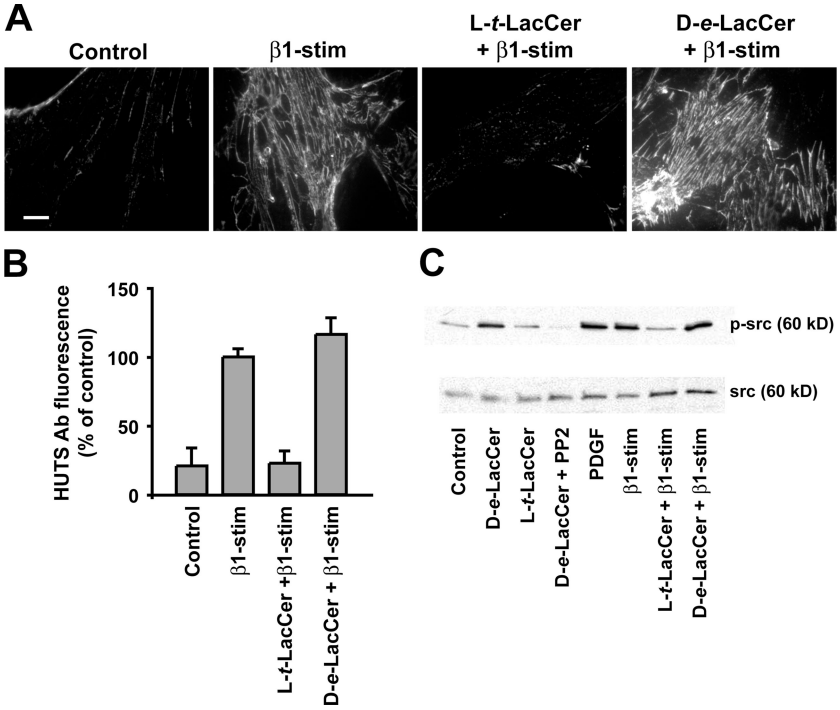

Figure 3. **Activation of β1 integrin and stimulation of src kinase is inhibited by L-*t*-LacCer.** (A and B) HSFs were serum starved for 2–3 h and were either untreated (control) or incubated with a stimulating β1-integrin IgG (β1-stim) or with the indicated LacCer isomers for 30 min at 10°C. For treatments with the LacCer isomers, samples were further incubated with β1-stim Ab and the indicated LacCer for 30 min at 10°C. All samples were then warmed for 30 s at 37°C, fixed, and stained with HUTS-4 Ab that recognizes β1 integrin in its active conformation (Luque et al., 1996). Note the absence of β1-integrin activation in cells pretreated with L-*t*-LacCer. Quantitation in B was performed by image analysis ($n \geq 10$ cells/condition; three independent experiments). Error bars represent SD. Bar, 10 μm. (C) HSFs were treated with D-e-LacCer (±PP2), L-*t*-LacCer, PDGF, or with β1 stim Ab ± LacCer isomers (see Materials and methods). Samples were warmed for 30 s at 37°C, lysed, and the cell lysates were blotted for src and phospho-*Src* (Y416).

pretreatment with L-*t*-LacCer before incubation with β1-stim Ab reduced HUTS-4 binding to levels seen in untreated control cells. In contrast, when D-*e*-LacCer was used, the Ab-induced activation of β1 integrin was not inhibited. When L-*t*-LacCer was incubated with HSFs in the absence of β1-stim Ab, no increase in HUTS binding was seen (Fig. S3, A and B; available at http://www.jcb.org/cgi/content/full/jcb.200609149/DC1). This is in contrast to D-*e*-LacCer, which activated β1 integrin in the absence of the β1-stim Ab to a similar extent as when the Ab was used alone (Fig. S3, A and B; Sharma et al., 2005).

Because β1 integrins have been shown to activate src kinases and src phosphorylation is required for caveolar endocytosis (Arias-Salgado et al., 2003; Sharma et al., 2004), we also examined the effect of L-*t*-LacCer treatments on this process. Cells were incubated for 30 min at 10°C ± the LacCer stereoisomers and/or the β1-stim Ab followed by a 30-s incubation at 37°C. Cell lysates were then immunoblotted for src and phospho-(Y416) src. Src kinase was activated (phosphorylated at Y416) by treatment with D-*e*-LacCer to a level similar to that seen with the stimulating Ab, and this activation could be blocked by the src inhibitor PP2 (Fig. 3 C). In contrast, little or no src activation was observed upon treatment with L-*t*-LacCer. Furthermore, pretreatment of cells with L-*t*-LacCer inhibited src phosphorylation induced by the β1-stim Ab, whereas pretreatment with D-*e*-LacCer did not substantially affect src phosphorylation in response to β1-stim Ab (Fig. 3 C).

Several features of our studies suggest that the LacCer stereoisomers may regulate caveolar endocytosis via modulation of β1-integrin signaling. First, both D-*e*-LacCer and β1 integrin stimulate src activation and caveolar endocytosis to a similar degree (Fig. 3; Sharma et al., 2004, 2005). Second, treatment with L-*t*-LacCer inhibits β1 integrin–mediated src signaling as well as caveolar endocytosis (Figs. 1 A and 3 C). To further examine the role of β1 integrin in the regulation of caveolar endocytosis in HSFs, we used an siRNA approach to deplete this integrin and studied the uptake of various markers. We first validated the use of three different β1-integrin siRNAs and found that these reduced β1-integrin levels in HeLa cells ∼80% relative to untreated cells (Fig. S3 C). We then used electroporation to transfect HSFs with a β1-integrin siRNA and found that the levels of β1 integrin were reduced by ∼75% in the transfected cells as assessed by immunofluorescence (Fig. S3 D).

We then examined the effect of β1-integrin knockdown on the uptake of multiple endocytic markers. Endocytosis of the caveolar markers albumin and BODIPY-LacCer was dramatically reduced in cells transfected with β1-integrin siRNA relative to that in nontransfected cells. In contrast, no effect of β1-integrin siRNA treatment was seen on clathrin (Tfn), Cdc42 (dextran and GPI-GFP), or RhoA (IL-2R) internalization (Fig. 4, A and B). Control experiments showed that clathrin heavy chain siRNA treatment resulted in a strong inhibition of Tfn uptake but had little effect on albumin or BODIPY-LacCer internalization (Fig. 4 B). Finally, we examined the effect of β1-integrin knockdown on src phosphorylation in HSFs treated with the LacCer stereoisomers using immunofluorescence. Although the phosphorylation of src was increased in D-*e*-LacCer–treated cells (Fig. 3 C), this stimulation was reduced by 65–75% in individual cells in which β1 integrin had been depleted (Fig. 4 C). As expected, no further reduction of phospho-src was observed in cells treated with L-*t*-LacCer and depleted of β1 integrin (not depicted) because src phosphorylation was inhibited by the L-*t* stereoisomer (Fig. 3 C).

The inhibition of caveolar endocytosis by β1-integrin knockdown in HSFs suggests a key role for β1 integrin in the regulation of this endocytic process by GSLs. For example, natural GSLs such as D-*e*-LacCer that stimulate caveolar endocytosis

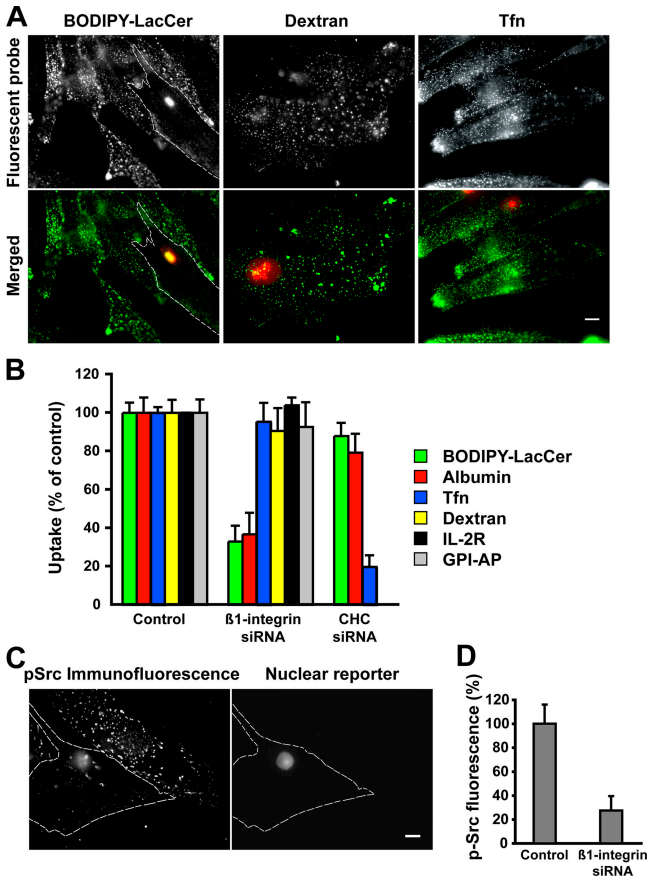

Figure 4. **Knockdown of β1 integrin inhibits caveolar endocytosis and phosphorylation of src in HSFs.** (A and B) Cells were cotransfected with β1-integrin siRNA (±nuclear reporter; see Materials and methods). After 48 h, cells were incubated with BODIPY-LacCer, fluorescent albumin, Tfn, dextran, anti–IL-2R, or anti–GFP-Fab (see Materials and methods), and endocytosis was examined after 3 min at 37°C. (A) Note the inhibition of BODIPY-LacCer (but not Tfn or dextran) internalization. (B) Results were quantified by image analysis. Additional experiments using a siRNA for the clathrin heavy chain (CHC) demonstrated the inhibition of Tfn uptake, with no effect on albumin or BODIPY-LacCer internalization. (C and D) β1 integrin was depleted in HSFs by transfection as in A. The cells were then incubated with D-*e*-LacCer for 30 min at 10°C, washed, and warmed for 30 s at 37°C. Immunofluorescence was performed using anti-pSrc (Y416) mAb and was quantified by image analysis relative to adjacent nontransfected cells ($n \geq 10$ cells per condition in two independent experiments; D). Note the inhibition of p-Src staining in cells showing nuclear reporter fluorescence. Error bars represent SD. Bars, 10 μm.

may do so by increasing β1-integrin clustering, whereas L-*t*-LacCer may inhibit endocytosis via caveolae by preventing this clustering. Further studies are needed to determine (1) the applicability of our findings to other cell types, (2) the role of other integrin components (e.g., various αβ heterodimers) in modulating caveolar uptake, and (3) whether the regulation of caveolar endocytosis is restricted to integrins that are internalized via caveolae or also includes integrins that are endocytosed via the clathrin pathway (e.g., αVβ5; Memmo and McKeown-Longo, 1998).

Our data support a general model in which exogenously supplied D-*e*-LacCer or other sphingolipids with the natural D-*e* stereochemistry promote the coalescence of PM microdomains, leading to the clustering and activation of transmembrane proteins that can initiate a signaling cascade required for caveolar endocytosis. In HSFs, β1 integrin appears to be a key molecule involved in transducing this signal across the PM bilayer; however, other signaling proteins that partition into lipid microdomains may be similarly affected by exogenous lipids in HSFs or other cell types. Our results also suggest a potential mechanism whereby certain tumor cells that shed gangliosides can alter the properties of nearby cells (Birkle et al., 2003; Guerrera and Ladisch, 2003). Most importantly, the results of the current study document a dominant-negative lipid, L-*t*-LacCer, which selectively inhibits caveolar endocytosis of multiple markers by interfering with microdomain clustering and β1-integrin signaling. Of additional interest is our finding that the nonnatural stereoisomer of LacCer dramatically inhibited SV40 infection, most likely by disrupting PM domains that are required for optimal binding of the virus to the cell surface. Finally, we suggest that the disruption of membrane microdomains by L-*t*-LacCer may represent a new approach for the treatment of certain diseases and infectious agents that use lipid rafts or raft proteins as targets (Simons and Ehehalt, 2002).

Materials and methods

Cell culture

Normal HSFs (Coriell Institute for Medical Research), HeLa, and CV1 cells (American Type Culture Collection) were used as described previously (Pelkmans et al., 2001; Singh et al., 2003). The SV40 construct (pUC-SVH388-2) was obtained from the American Type Culture Collection, transfected into CV1 cells, and the virus was harvested after maximum cytopathic effect. The virus was titered and used at an MOI of 15.

Lipids, fluorescent probes, and miscellaneous reagents

D-*e*-LacCer was purchased from Avanti Polar Lipids, Inc., and L-*t*-LacCer was synthesized by N. Gretskaya (Shemyakin Institute, Moscow, Russia). LacCer isomers were complexed to defatted BSA and incubated with cells at a final concentration of 5 μM (Sharma et al., 2005). Fluorescent AlexaFluor594- and -647–labeled CtxB, Tfn, dextran (10 kD), Ab labeling kits, and fluorescent secondary Abs were obtained from Invitrogen. An anti–β1 integrin (IgG1) from BD Biosciences was used as a stimulatory Ab (β1-stim Ab) and for the preparation of an Fab fragment using a kit from Pierce Chemical Co. Abs were fluorescently labeled with an AlexaFluor dye as described previously (Sharma et al., 2005). An AlexaFluor594-Fab fragment against GFP was prepared similarly from an anti-GFP Ab (Invitrogen). The HUTS-4 β1-integrin Ab was purchased from Chemicon. A phycoerythrin-labeled IL-2R Ab was obtained from BD Biosciences. *Src* and phospho-*Src* Abs for Western blotting were obtained from Cell Signaling Technology. Antiphospho-src (Y416) mAb for immunofluorescence studies was purchased from Upstate Biotechnology. CtxB, large T antigen Abs, and tyrosine kinase inhibitor (PP2) were purchased from Calbiochem. The biotinylated derivative (BC-Θ) of perfringolysin O (Θ toxin) was obtained from Y. Ohno-Iwashita (Tokyo Metropolitan Institute, Tokyo, Japan) and used as described previously (Waheed et al., 2001) using AlexaFluor594-streptavidin (Invitrogen) to visualize its distribution on the cell surface. A mAb (pab597) against SV40 VP1 was provided by L. Norkin (University of Massachusetts, Amherst, MA) and used as described previously (Chen and Norkin, 1999). All other reagents were purchased from Sigma-Aldrich.

Constructs, transfections, and knockdown experiments

DNA constructs encoding GPI-GFP, dominant-negative Cdc42, and IL-2R were gifts from J. Lippincott-Schwartz (National Institutes of Health, Bethesda, MD), D. Billadeau (Mayo Foundation, Rochester, MN) and A. Dautry-Varsat (Institute Pasteur, Paris, France), respectively. pDsRed2-nuc and pECFP-nuc were purchased from CLONTECH Laboratories, Inc. Transfection of DNA constructs was performed using a Nucleofector II apparatus (Amaxa Biosystems).

siRNAs for β1 integrin (Stealth Select RNAi; oligonucleotide IDs HSS105559, HSS105560, and HSS105561) and clathrin heavy chain

(ON-TARGETplus SMARTpool L-004001-00-0010) were purchased from Invitrogen and Dharmacon, respectively. Knockdown in HeLa cells was performed using LipofectAMINE 2000 (Invitrogen). For knockdown in HSFs, cells were cotransfected with the siRNA of interest and pDsRed2-Nuc (experiments using BODIPY-LacCer, albumin, or dextran), pECFP-Nuc (experiments using AlexaFluor594-Tfn), or no reporter (experiments using GPI-GFP or IL-2R) using the Nucleofector II apparatus. Transfections were performed using 360 pmol siRNA and 1 μg of reporter DNA. Experiments were performed 48 h after transfection.

Endocytosis assays

For endocytosis of BODIPY-LacCer or AlexaFluor-labeled anti–β1-integrin Fab or Tfn, HSFs were preincubated with the fluorescent marker for 30 min at 10°C, washed, and further incubated for 3 or 5 min at 37°C. For AlexaFluor-labeled albumin and dextran, cells were incubated for 3 or 5 min at 37°C without preincubation. Endocytosis of IL-2R was performed as described previously (Cheng et al., 2006b). For GPI-anchored protein uptake, HSFs were first transfected with GPI-GFP for 48 h and then were incubated with AlexaFluor594-labeled anti–GFP-Fab for 30 min at 10°C, washed, and incubated for 5 min at 37°C. All samples were then either acid stripped or back exchanged to remove cell surface fluorescence before fluorescence microscopy (Singh et al., 2003).

PM binding of SV40 virus

CV1 cells were incubated ± LacCer stereoisomers for 30 min at 10°C and coincubated for 1 h at 10°C with SV40 virus (MOI = 15). Samples were then washed and stained for SV40 binding at the PM using anti-SV40 VP1 mAb for 30 min at 10°C . Cells were washed, incubated with AlexaFluor594-labeled anti–mouse secondary Ab, and observed by fluorescence microscopy at 10°C.

Visualization of PM microdomains

For the visualization of GM_1 ganglioside microdomains at the PM, HSFs and CV1 cells were incubated for 30 min at 10°C ± L-*t*-LacCer and further incubated (for 30 min at 10°C) with fluorescent CtxB. Samples were washed and incubated with anti-CtxB IgG (30 min for HSFs) or SV40 virus (1 h for CV1 cells) at 10°C. Cells were then washed and observed under the fluorescence microscope (IX70; Olympus) at 10°C. For the visualization of cholesterol microdomains, CV1 cells were incubated (for 30 min at 10°C) ± L-*t*-LacCer followed by SV40 virus for 1 h at 10°C. Cells were then washed and incubated with biotinylated BC-Θ followed by AlexaFluor594-streptavidin.

Src phosphorylation

HSFs were serum starved for 2–3 h and treated with D-*e*-LacCer, L-*t*-LacCer, or 50 μg/ml PDGF for 30 min at 10°C in HBSS. In one experiment, cells were pretreated with 15 μM PP2 for 30 min at 37°C before incubation with D-*e*-LacCer. In another experiment, cells were either untreated or treated with the LacCer isomers for 30 min at 10°C followed by incubation with β1-stim Ab for 30 min at 10°C. All samples were then washed and warmed for 30 s at 37°C before cell lysis. Lysates were then immunoblotted for *Src* and phospho-*Src* (Y416).

Miscellaneous methods

Fluorescence microscopy was performed using a fluorescence microscope (IX70; Olympus) equipped with 60× 1.4 NA and 100× 1.35 NA objectives and a CCD camera (C4742; Hamamatsu). For quantitative studies, all photomicrographs in a given experiment were exposed and processed identically for a given fluorophore and were analyzed using the MetaMorph image processing program (version 6.2; Universal Imaging Corp.). Quantitative results are expressed as means ± SDs. Images were prepared for individual figures using Photoshop CS (Adobe).

Online supplemental material

Fig. S1 shows the characterization of endocytosis of GPI-GFP via the Cdc42–regulated pathway in HSFs. Fig. S2 illustrates microdomains containing cholesterol, GM_1 ganglioside, and β1 integrin in HSFs treated with D-*e*-LacCer versus L-*t*-LacCer. Fig. S3 shows the effects of D-*e*- and L-*t*-LacCer on β1-integrin activation and the characterization of β1-integrin knockdown in HeLa cells and HSFs. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200609149/DC1.

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References

Anderson, H.A., Y. Chen, and L.C. Norkin. 1996. Bound simian virus 40 translocates to caveolin-enriched membrane domains, and its entry is inhibited by drugs that selectively disrupt caveolae. *Mol. Biol. Cell*. 7:1825–1834.

Arias-Salgado, E.G., S. Lizano, S. Sarkar, J.S. Brugge, M.H. Ginsberg, and S.J. Shattil. 2003. Src kinase activation by direct interaction with the integrin β cytoplasmic domain. *Proc. Natl. Acad. Sci. USA*. 100:13298–13302.

Birkle, S., G. Zeng, L. Gao, R.K. Yu, and J. Aubry. 2003. Role of tumor-associated gangliosides in cancer progression. *Biochimie*. 85:455–463.

Chen, Y., and L.C. Norkin. 1999. Extracellular simian virus 40 transmits a signal that promotes virus enclosure within caveolae. *Exp. Cell Res.* 246:83–90.

Cheng, Z.J., R.D. Singh, D.L. Marks, and R.E. Pagano. 2006a. Membrane microdomains, caveolae, and caveolar endocytosis of sphingolipids. *Mol. Membr. Biol.* 23:101–110.

Cheng, Z.J., R.D. Singh, D.K. Sharma, E.L. Holicky, K. Hanada, D.L. Marks, and R.E. Pagano. 2006b. Distinct mechanisms of clathrin-independent endocytosis have unique sphingolipid requirements. *Mol. Biol. Cell*. 17:3197–3210.

Guerrera, M., and S. Ladisch. 2003. N-butyldeoxynojirimycin inhibits murine melanoma cell ganglioside metabolism and delays tumor onset. *Cancer Lett.* 201:31–40.

Johannes, L., and C. Lamaze. 2002. Clathrin-dependent or not: is it still the question? *Traffic*. 3:443–451.

Kirkham, M., and R.G. Parton. 2005. Clathrin-independent endocytosis: new insights into caveolae and non-caveolar lipid raft carriers. *Biochim. Biophys. Acta*. 1746:349–363.

Kirkham, M., A. Fujita, R. Chadda, S.J. Nixon, T.V. Kurzchalia, D.K. Sharma, R.E. Pagano, J.F. Hancock, S. Mayor, and R.G. Parton. 2005. Ultrastructural identification of uncoated caveolin-independent early endocytic vehicles. *J. Cell Biol.* 168:465–476.

Lamaze, C., A. Dujeancourt, T. Baba, C.G. Lo, A. Benmerah, and A. Dautry-Varsat. 2001. Interleukin 2 receptors and detergent-resistant membrane domains define a clathrin-independent endocytic pathway. *Mol. Cell*. 7:661–671.

London, E., and D.A. Brown. 2000. Insolubility of lipids in triton X-100: physical origin and relationship to sphingolipid/cholesterol membrane domains (rafts). *Biochim. Biophys. Acta*. 1508:182–195.

Luque, A., M. Gomez, W. Puzon, Y. Takada, F. Sanchez-Madrid, and C. Cabanas. 1996. Activated conformations of very late activation integrins detected by a group of antibodies (HUTS) specific for a novel regulatory region (355-425) of the common beta 1 chain. *J. Biol. Chem.* 271:11067–11075.

Memmo, L.M., and P. McKeown-Longo. 1998. The alphavbeta5 integrin functions as an endocytic receptor for vitronectin. *J. Cell Sci.* 111:425–433.

Mineo, C., and R.G. Anderson. 2001. Potocytosis. Robert Feulgen Lecture. *Histochem. Cell Biol.* 116:109–118.

Nichols, B.J., and J. Lippincott-Schwartz. 2001. Endocytosis without clathrin coats. *Trends Cell Biol.* 11:406–412.

Parton, R.G., B. Joggerst, and K. Simons. 1994. Regulated internalization of caveolae. *J. Cell Biol.* 127:1199–1215.

Pelkmans, L., and A. Helenius. 2002. Endocytosis via caveolae. *Traffic*. 3:311–320.

Pelkmans, L., J. Kartenbeck, and A. Helenius. 2001. Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. *Nat. Cell Biol.* 3:473–483.

Pelkmans, L., E. Fava, H. Grabner, M. Hannus, B. Habermann, E. Krausz, and M. Zerial. 2005. Genome-wide analysis of human kinases in clathrin- and caveolae/raft-mediated endocytosis. *Nature*. 436:78–86.

Rajendran, L., and K. Simons. 2005. Lipid rafts and membrane dynamics. *J. Cell Sci.* 118:1099–1102.

Sabharanjak, S., P. Sharma, R.G. Parton, and S. Mayor. 2002. GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. *Dev. Cell*. 2:411–423.

Sharma, D.K., J.C. Brown, A. Choudhury, T.E. Peterson, E. Holicky, D.L. Marks, R. Simari, R.G. Parton, and R.E. Pagano. 2004. Selective stimulation of caveolar endocytosis by glycosphingolipids and cholesterol. *Mol. Biol. Cell*. 15:3114–3122.

Sharma, D.K., J.C. Brown, Z. Cheng, E.L. Holicky, D.L. Marks, and R.E. Pagano. 2005. The glycosphingolipid, lactosylceramide, regulates beta1-integrin clustering and endocytosis. *Cancer Res.* 65:8233–8241.

Simons, K., and R. Ehehalt. 2002. Cholesterol, lipid rafts, and disease. *J. Clin. Invest.* 110:597–603.

Singh, R.D., V. Puri, J.T. Valiyaveettil, D.L. Marks, R. Bittman, and R.E. Pagano. 2003. Selective caveolin-1-dependent endocytosis of glycosphingolipids. *Mol. Biol. Cell*. 14:3254–3265.

Singh, R.D., Y. Liu, C.L. Wheatley, E.L. Holicky, A. Makino, D.L. Marks, T. Kobayashi, G. Subramaniam, R. Bittman, and R.E. Pagano. 2006. Caveolar endocytosis and microdomain association of a glycosphingolipid analog is dependent on its sphingosine stereochemistry. *J. Biol. Chem.* 281:30660–30668.

Stang, E., J. Kartenbeck, and R.G. Parton. 1997. Major histocompatibility complex class I molecules mediate association of SV40 with caveolae. *Mol. Biol. Cell*. 8:47–57.

Tagawa, A., A. Mezzacasa, A. Hayer, A. Longatti, L. Pelkmans, and A. Helenius. 2005. Assembly and trafficking of caveolar domains in the cell: caveolae as stable, cargo-triggered, vesicular transporters. *J. Cell Biol.* 170:769–779.

Tsai, B., J.M. Gilbert, T. Stehle, W. Lencer, T.L. Benjamin, and T.A. Rapoport. 2003. Gangliosides are receptors for murine polyoma virus and SV40. *EMBO J.* 22:4346–4355.

Upla, P., V. Marjomaki, P. Kankaanpaa, J. Ivaska, T. Hyypia, F.G. Van Der Goot, and J. Heino. 2004. Clustering induces a lateral redistribution of $\alpha 2\beta 1$ integrin from membrane rafts to caveolae and subsequent protein kinase C-dependent internalization. *Mol. Biol. Cell*. 15:625–636.

Waheed, A.A., Y. Shimada, H.F. Heijnen, M. Nakamura, M. Inomata, M. Hayashi, S. Iwashita, J.W. Slot, and Y. Ohno-Iwashita. 2001. Selective binding of perfringolysin O derivative to cholesterol-rich membrane microdomains (rafts). *Proc. Natl. Acad. Sci. USA*. 98:4926–4931.