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Cholesterol distribution in plasma membranes of β1 integrin-expressing and β1 integrin-deficient fibroblasts

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Abstract

The effect of integrin receptors on the level and transmembrane localization of cholesterol molecules was investigated in β1 integrin-expressing (β1) and β1 integrin-deficient (β1 null) cells. We found that the content of specific raft components—cholesterol, sphingomyelin, and caveolin—was increased in integrin-expressing cells. Integrin presence affected as well the transmembrane distribution of cholesterol—a higher percent was found in the plasma membrane outer monolayer of β1 compared to β1 null cells. Sphingomyelin depletion reduced the presence of cholesterol in the outer membrane monolayer of both cell lines, but the differences in cholesterol asymmetry, observed between β1 and β1 null cells before sphingomyelinase treatment were preserved. These findings implied that integrin receptors affected the non-random transmembrane distribution of cholesterol. Finally, a higher percent of detergent-resistant membranes was obtained from β1 integrin-expressing cells, suggesting that the presence of these receptors in the membranes influenced the formation and/or stabilization of lipid raft domains.

Keywords: Integrin receptors; Cholesterol; Raft domains; Sphingomyelin; Caveolin; Detergent-resistant membranes; Cholesterol asymmetry; Plasma membranes; Transmembrane lipid distribution; Sphingomyelinase; Cyclodextrin; Integrin-expressing cells; Integrin-deficient cells; Fluorescence quenching

Under normal conditions plasma membrane lipids are asymmetrically distributed between the two membrane monolayers: the aminophospholipids, phosphatidylethano-lamine and phosphatidylserine, are concentrated predominantly in the inner membrane leaflet, whereas the choline-containing phosphatidylcholine and sphingomyelin are sequestered mostly in the extracellular leaflet [1–3]. Phospholipid asymmetry between the membrane monolayers is assumed to influence the intramembrane distribution of cholesterol—an essential lipid component of mammalian plasma membranes [4,5]. This sterol molecule is also asymmetrically distributed between the two membrane leaflets [6] and its membrane translocation has been studied for a variety of cells [7].

Cholesterol, together with sphingomyelin is recognized as an essential component of specific membrane domains referred to as lipid raft domains [8]. According to the raft hypothesis certain naturally occurring lipids such as cholesterol and sphingomyelin aggregate in the plane of the membrane due to intermolecular interactions, thus forming raft domains [9,10]. So far the composition and size of lipid rafts, how are they formed, and what exactly are their functions remain unclear. Recent studies have implicated lipid rafts in signal transduction events initiated by adhesion of cells to extracellular

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matrix [11,12]. This process is mediated by integrins, which are a family of transmembrane receptors participating in cell adhesion to extracellular matrix [13]. It has been suggested that integrins may regulate different cellular processes by influencing the positioning of lipid rafts on plasma membranes [12,14]. In addition, integrin receptors were reported to be associated with the membrane raft domains, where cholesterol is one of the two major lipid components [15]. Another protein constituent of the raft domains is caveolin [16], which appears to play a major role in carrying cholesterol between ER and caveolae membranes [17]. While the association of integrins and caveolin with rafts is well documented, the relationship between these proteins and specific lipid raft components is largely unknown.

In this study, we have used β1 integrin-expressing (β1) cells and β1 integrin-deficient (β1 null) cells derived from embryonic stem cells [18] in an attempt to clarify whether the presence of membrane-associated integrin receptors affected the level and distribution of cholesterol molecules within the plasma membranes. The elucidation of this problem is of particular importance for understanding of the mechanisms of raft formation and the role of certain protein components involved in signaling events.

Materials and methods

Materials

Antibodies to human β1 integrins included rat mAb 9EG7 (Pharmingen), mouse mAb 12G10 described by Mould et al. [19], mAb K20 (Immunotech), and rabbit antibody Rab 4080 described by Tran et al. [20]. Antibodies against vinculin and caveolin, bovine serum albumin, 2′,7′-dichlorofluorescein and all other chemicals (unless otherwise stated) were purchased from Sigma and BD Transduction. Fluorescent lipid analogs like 7-nitrobenz-2-oxa-1,3-diazol-4-yl-cholesterol (NBD-cholesterol)[1] and 1-oleoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*- glycero-3-phosphoethanolamine (NBD-PE) were obtained from Avanti Polar Lipids (Alabaster AL). Culture medium, antibiotic/antimycotic solution, and serum were obtained from Gibco-BRL Life Technologies.

Cell culture

The GD25 β1-null fibroblast cell line was a generous gift from Reinhard Fässler, Max Planck Institute, Germany. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml fungizone. GD25 β1-null fibroblasts expressing human β1 integrins were obtained as described [21]. Briefly, plasmid DNA encoding wild-type human β1 integrin (kindly provided by Erkki Ruoslahti, The Burnham Institute, CA) was co-transfected together with the puromycin selection vector pHA262pur (generously provided by Dr. Hein te Riele, Netherlands Cancer Institute) by electroporation. Mixed population of stable-transfectant cells expressing β1 integrin was established by selection in medium containing 10 μg/ml puromycin and by four consecutive fluorescence-activated cell sortings with anti-human β1 integrin antibody K20 performed over a 3-month period. Control cells were transfected with pHA262pur vector only and mixed population of stable-transfectant cells was established after puromycin selection.

Immunofluorescence

Cells for immunofluorescence analysis were plated on fibronectin (5 μg/ml) pre-coated glass coverslips (12 mm, Carolina Biological Supply Company) and cultured. Samples were fixed with 4% paraformaldehyde in PBS containing 5% sucrose for 20 min and permeabilized with 0.5% Triton X-100 in PBS for 3 min. Primary antibodies were used at 10 μg/ml and visualized with secondary CY3- or FITC-conjugated antibody. Stained samples were mounted in GEL/MOUNT (Biomeda Corp.) containing 1 mg/ml 1,4-phenylendiamine (Fluka) to reduce photobleaching. Immunofluorescent images were obtained with a Zeiss Axiophot microscope equipped with a Photometrics CH350 cooled CCD camera. Digital images were obtained using MetaMorph 3.5 software (Universal Imaging).

Immunoblotting

Overnight cultures of β1 integrin-expressing and β1 integrin-null cells were solubilized on ice in RIPA buffer (150 mM NaCl, 2 mM EDTA, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 10% glycerol, and 50 mM Hepes, pH 7.5) containing a cocktail of protease inhibitors Complete (Boehringer–Mannheim). Cell lysates were mixed with equal volume of reducing SDS–PAGE sample buffer and resolved on 4–12% gradient gels (Novex). After electrotransfer to nitrocellulose membranes (Novex), the membranes were blocked (5% non-fat dry milk in 150 mM NaCl, 50 mM Tris–HCl, and 0.1% Tween 20, pH 7.4) and probed with the indicated antibodies, followed by the appropriate secondary horseradish peroxidase-conjugated antibodies. Immunoblots were visualized using the ECL system and Hyperfilm X-ray film (Amersham).

[1] *Abbreviations used:* NBD-cholesterol, 7-nitrobenz-2-oxa-1,3-diazol-4-yl-cholesterol; NBD-PE, 1-oleoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphoethanolamine; DRM, detergent resistant membrane.

Isolation of plasma membrane fractions

The plasma membrane fraction was obtained according to the procedure described by Evans [22] with modifications [23] including differential centrifugation of β1 and β1 null cells. The post-nuclear supernatant was loaded on a discontinuous sucrose gradient and centrifuged at 100,000*g* for 2.5 h. The plasma membrane fraction was collected at a density of approximately 45% (w/v), suspended in ice-cold 100 mM Tris buffer, pH 7.5, and used immediately for lipid analysis.

Preparation of detergent resistant membrane fractions

Detergent resistant membrane (DRM) fractions were isolated as described elsewhere [24] with slight modifications. In brief, plasma membranes were resuspended in buffer consisting of 0.25 M sucrose, 150 mM NaCl, 1 mM EDTA and 100 mM Tris–HCl buffer (pH 7.6) containing 1% Triton X-100 at 4 °C and dispersed by 10 passages through a 21 gauge needle. The detergent-treated membrane suspension obtained after this procedure was diluted with an equal volume of 80 %(w/v) sucrose, put in a centrifuge tube, and covered by successive layers of 30% (w/v) sucrose (4 ml) and 5% (w/v) sucrose (3.5 ml). The density gradients thus obtained were centrifuged for 18 h at 38,000*g* in a Sorvall OTD-50 ultracentrifuge. The DRM fraction was collected at the 5/30% (w/v) sucrose interface.

Labeling with fluorescent analogs and dithionite assay

Unilamellar vesicles were prepared by sonication under an inert atmosphere of nitrogen. Vesicles contained 1-palmitoyl-2-oleoyl-*sn*-glycerophosphocholine (POPC) and 5 mol % of the corresponding NBD-lipid fluorescent analog. Fluorescent labeling was performed as described by Kok et al. [25] with modifications [26]. Fibroblasts were incubated with labeled vesicles (ULV) at 20 °C for different periods of time as indicated. After incubation the cells were washed three times with Hepes-buffered saline (HBS) to remove the non-inserted lipid analogs and the fluorescence was measured. The distribution of NBD-cholesterol within the plasma membranes was estimated by the sodium dithionite quenching assay [27]. The cells were incubated in HBS and dithionite was added to a final concentration of 50 mM. Fluorescence intensity was measured after 4 min of incubation at 20 °C.

Fluorescence measurements

Fluorescence measurements were performed on a Jobin Yvone JY 3D spectrophotometer, using 10-nm slit widths, as described by McIntyre and Sleight [27]. The fluorescence of NBD-labeled lipid analogs was measured at a wavelength of 530 nm (excitation wavelength 470 nm). To estimate the amount of NBD-lipid accessible to dithionite quenching the following equation was used:

$$\text{percent NBD-cholesterol accessible to quenching} = [1 - [(F_r - F_{ap})/(F_o - F_{ap})]] \times 100,$$

where F_r is the fluorescence intensity after treatment with sodium dithionite, F_{ap} is the apparent fluorescence of the cells with no NBD-lipid, and F_o is the fluorescence of the cells containing NBD-lipid before sodium dithionite treatment. The value of fluorescence intensity (F_o) was decreased upon dithionite addition due to quenching of the fluorescence of NBD-cholesterol present in the outer membrane leaflet. The fluorescence intensity (F_r) was measured 4 min after dithionite addition when there was no more reduction of this value. The addition of 2% Triton X-100 induced further decrease of the fluorescence intensity since the NBD-cholesterol localized in the inner membrane monolayer became accessible to dithionite quenching.

Back exchange

The internalization of lipid fluorescent analogs into the inner membrane leaflet was assessed by back exchange to serum albumin as described by Pomorski et al. [28] with modifications. Exchangeable fluorescent lipids located in the outer membrane leaflet can be removed, allowing quantitative determination of the lipid internalized into the inner monolayer, i.e., the lipid fraction which could not be back exchanged. In short, cells were incubated twice with fatty acid-free BSA on ice for 10 min, followed by extensive washing with HBS and centrifugation at 12,000*g*. The pellets were solubilized in 2% Triton X-100 and the amount of internalized lipid was determined by comparing the fluorescent intensity before and after back exchange to albumin.

Sphingomyelin degradation by exogenous sphingomyelinase [29]

Degradation of sphingomyelin was performed using 0.5 U/ml sphingomyelinase added to the incubation medium for 1 h at 37 °C. Cell permeability to trypan blue was not altered as a result of sphingomyelinase treatment of cells. Total lipids were extracted from the cell dishes and analyzed as described below.

Lipid extraction and phospholipid analysis

Lipid extraction was performed with chloroform/methanol according to the method of Bligh and Dyer [30]. The organic phase obtained after extraction was concentrated and analyzed by thin layer chromatogra-

phy. The phospholipid fractions were separated on silica gel G 60 plates in a solvent system containing chloroform/methanol/2-propanol/triethylamine/0.25% KCl (30:9:25:18:6 v/v) [31]. The location of the separate fractions was determined either by spraying the plates with 2′,7′-dichlorofluorescein and visualization under UV light or by iodine staining. The spots were scraped and quantified by determination of the inorganic phosphorus [32].

Cholesterol determination

The cholesterol content of the membranes was assayed by gas chromatography [33] using a medium polarity RTX-65 capillary column (0.32 mm internal diameter, length 30 m, and film thickness 0.25 m). Calibration was achieved by a weighted standard of cholesterol.

Incubation of cells with cyclodextrin [34]

β1 and β1 null cells (2×10^6) were rinsed twice with PBS and incubated with 10 mM methyl-β-cyclodextrin for the indicated time at 37 °C under constant agitation. After the incubation the cells were rinsed three times with PBS and used for lipid analysis.

Other procedures

The protein concentration was estimated according to the method of Lowry et al. [35]. Data are expressed as means ± SD for at least two independent experiments. Differences between the means were analyzed by Student's *t* test.

Results

To develop a model system of cell lines with and without β1 integrin receptors we used β1 null mouse GD25 cells [18] and the same cells, stably transfected with human β1 integrin. Mixed populations of positive transfectants were obtained by fluorescence-activated cell sorting (see Materials and methods) in order to avoid potential variations associated with the use of single clones. Since functional β1 integrin is capable of localizing to focal adhesions, we analyzed its cellular localization by immunofluorescence with two antibodies that recognize the activated form of β1 integrin—12G10 [19] and 9EG7. Part of the transfected integrin reacted with 12G10 (Fig. 1, β1) and 9EG7 (not shown) antibodies, and co-localized with the focal adhesion marker vinculin (Fig. 1, anti-vinculin). At the same time GD25 β1 null cells were only vinculin-positive (Fig. 1, β1 null). Proper cellular localization and recognition by activation-specific antibodies indicated that the transfected human β1 integrin was functionally active in mouse GD 25 cells.

β1 integrin-expressing and β1 integrin-deficient cells were used as a source of detergent-resistant membrane (DRM) fractions, which are assumed to represent the lipid raft domains preexisting in the cell membranes [10,24]. Part of the transfected β1 integrin was found to be present in these fractions, supporting previous observations [15,36] for association of activated integrins with lipid rafts (Fig. 2, anti-β1, raft fraction). Interestingly, β1 integrin cells expressed significantly higher amount of caveolin, which was associated exclusively with the DRM fraction (Fig. 2, anti-caveolin, raft fraction). The differences in the protein profiles of the DRM fractions were paralleled with differences in the lipid content. It is noteworthy, that the total lipids in the DRM isolated from integrin-expressing and integrin-deficient cells represented 19 and 11%, respectively, of the total plasma membrane lipid content. This initial characterization suggested that the presence of integrin receptors affected the content of lipid raft domains in the cellular plasma membranes.

Given that rafts represent specific membrane lipid domains, we further studied the lipid composition of plasma membranes isolated from β1-expressing and β1-deficient cells (Table 1). Apparently, the percent of sphingomyelin, cholesterol, and phosphatidylethanolamine was higher in plasma membranes of β1 cells compared to β1 null cells. The percentage participation of the other lipid fractions was reduced in membranes of integrin-expressing cells. Investigations were also carried out to monitor cholesterol efflux from the two cell lines induced by cyclodextrin treatment. The results demonstrated that at all time points of cyclodextrin treatment the integrin-expressing cells contained a higher cholesterol level (expressed as percent of the total membrane lipids) compared to integrin-deficient cells (Fig. 3).

Since cholesterol level was found to be higher in integrin-expressing cells, studies were performed to clarify whether the observed differences affected the transmembrane distribution of cholesterol molecules in plasma membranes of the two tested cell lines. The distribution of cholesterol within the membranes was assessed by the localization of NBD-cholesterol analogs. This approach was chosen due to the adequacy and reliability of fluorescent lipid analogs in experiments monitoring the intramembrane distribution of lipid components [27,37]. For this purpose cells were incubated in the presence the fluorescent analog of cholesterol for different time periods as indicated on the abscissa in Fig. 4. Our results demonstrated that the uptake of NBD-cholesterol was higher in β1 compared to β1 null cells at all investigated time points (Fig. 4). The distribution between the membrane leaflets was assessed by the accessibility of the fluorescent sterol probes to sodium dithionite quenching (Table 2). The calculations showed

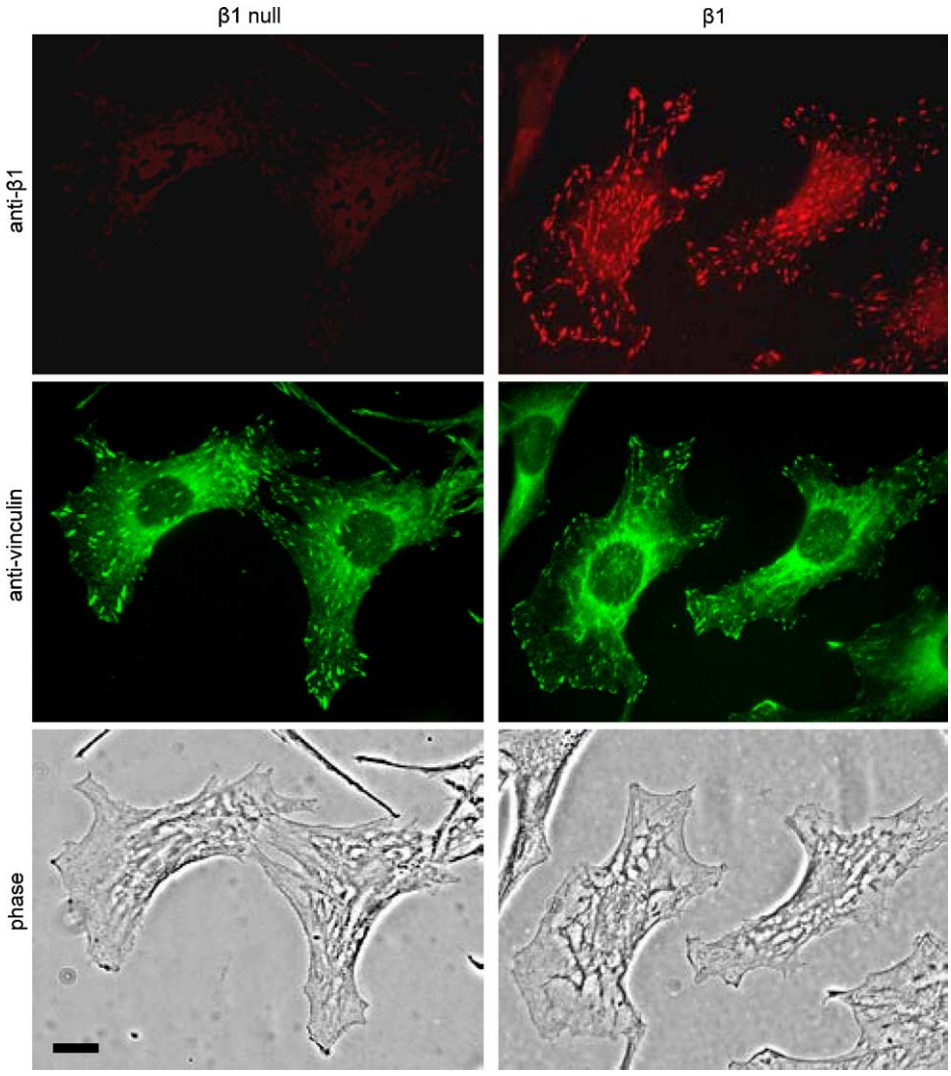

Fig. 1. Immunofluorescent and phase-contrast (phase) images of β1 integrin-null (β1 null) and β1 integrin-expressing (β1) cells stained with antibody 12G10 against activated β1 integrins (anti-β1) and antibody against vinculin (anti-vinculin). Bar—10 μm.

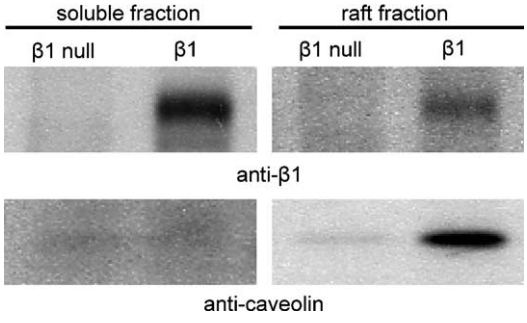

Fig. 2. Western blotting analysis of Triton X-100 soluble (soluble fraction) and insoluble (raft fraction) fractions isolated from β1 integrin-null (β1 null) and β1 integrin-expressing (β1) cells, performed with antibody 4080 against cytoplasmic domain of β1 integrin (anti-β1), antibody against caveolin (anti-caveolin).

Table 1
Lipid composition of plasma membranes from β1 integrin-expressing and β1 integrin-deficient (β1 null) cells expressed as percent of the total membrane lipids

Phospholipids	β1 integrin-expressing cells	β1 integrin-null cells
Sphingomyelin	20.75 ± 2.16	13.44 ± 1.78*
Phosphatidylcholine	33.41 ± 2.95	40.07 ± 3.12**
Phosphatidylserine	5.03 ± 0.68	9.57 ± 0.97*
Phosphatidylinositol	3.04 ± 0.93	7.15 ± 1.07*
Phosphatidylethanolamine	18.64 ± 1.97	15.79 ± 1.67***
Cholesterol	19.13 ± 1.76	14.16 ± 1.25*

Each value represents the mean ± SD of five separate experiments.
* $P < 0.001$.
** $P < 0.01$.
*** $P < 0.05$.

that the outer membrane leaflet of β1 cells contained a higher level of quenchable fluorescent probe (68%) compared to β1 null cells (51%).

Further studies were performed to analyze the possible mechanisms responsible for the differences in cholesterol transmembrane distribution between the

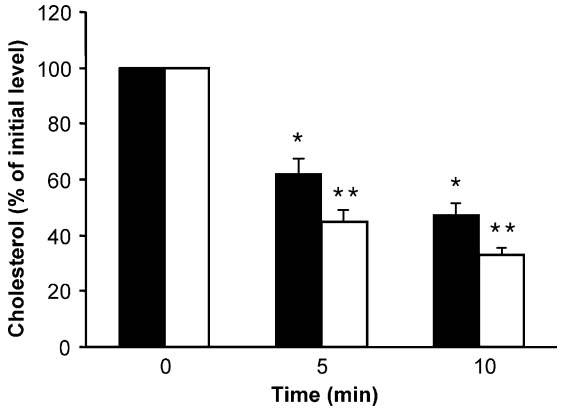

Fig. 3. Cholesterol depletion of β1 integrin-expressing (■) and β1 integrin-deficient (□) cells, mediated by methyl-β-cyclodextrin. Cells were incubated for 5 or 10 min with methyl-β-cyclodextrin as described under Materials and methods. The data represent means ± SD of four separate experiments. *$P < 0.001$ compared to β1 integrin-expressing cells before incubation with methyl-β-cyclodextrin; **$P < 0.001$ compared to β1 integrin-deficient cells before incubation with methyl-β-cyclodextrin.

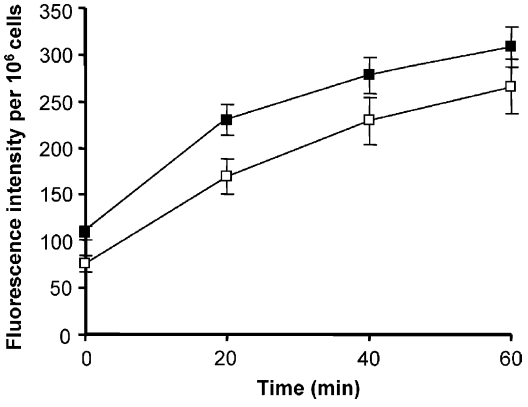

Fig. 4. Uptake of NBD-cholesterol into β1 integrin-expressing (■) and β1 integrin-deficient (□) cells. Cells were labeled with-NBD-cholesterol as described under Materials and methods. Each value was corrected for the intrinsic fluorescence measured before addition of NBD-cholesterol. The data represent means ± SD of three separate experiments.

investigated cell lines. Taking into consideration the fact that interaction between phosphatidylethanolamine and cholesterol molecules is thermodynamically unfavorable [4,38], we presumed that the observed elevation of phosphatidylethanolamine, which is located predominantly

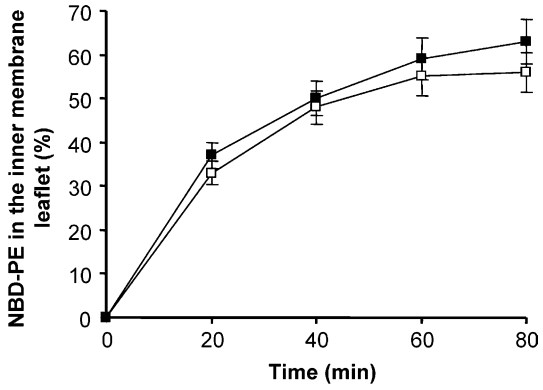

Fig. 5. Inward translocation of NBD-phosphatidylethanolamine (NBD-PE) in membranes of β1 integrin-expressing (■) and β1 integrin-deficient (□) cells. Cells were labeled with NBD-PE as described under Materials and methods. The fraction of fluorescent phospholipids in the inner membrane leaflet was determined by back exchange to serum albumin. The data represent means ± SD of two separate experiments.

in the inner leaflet [1], might induce partial extrusion of cholesterol molecules towards the outer leaflet. Therefore, the transmembrane distribution of phosphatidylethanolamine was monitored to determine whether there was a correlation between the localization of phosphatidylethanolamine and cholesterol within membranes of β1 and β1 null cells. For this purpose, we studied the internalization of NBD-analogs of phosphatidylethanolamine in plasma membranes of the two cell lines. As evident from Fig. 5, there were no significant differences in the intramembrane localization of phosphatidylethanolamine, implying that there was no apparent correlation between the distribution of cholesterol and phosphatidylethanolamine within plasma membranes of β1 and β1 null cells.

Since sphingomyelin and cholesterol exhibit specific molecular interactions [39], we analyzed the effect of sphingomyelin partial depletion on the pattern of cholesterol intramembrane distribution in β1 and β1 null cells. The level of sphingomyelin in the cell membranes was manipulated using exogenous sphingomyelinase. The content of sphingomyelin was reduced by about 64% in integrin-expressing and 75% in integrin-deficient cells as a result of sphingomyelinase treatment (data not shown). The effect of sphingomyelin partial depletion on cholesterol distribution between the membrane leaflets was investigated by measuring the accessibility of cho-

Table 2
Quenching of NBD-cholesterol fluorescence in the outer membrane leaflet of intact cells with sodium dithionite

Cells	F_o	F_r	F_{ap}	% quenchable probe
β1 integrin-expressing	272 ± 10.7	162 ± 9.7	110 ± 8.9	68 ± 4.3
β1 integrin null	261 ± 11.3	180 ± 10.4	102 ± 9.1	51 ± 4.1*

F_o—fluorescence intensity before addition of sodium dithionite; F_r—fluorescence intensity after sodium dithionite treatment; F_{ap}—apparent fluorescence of cells with no NBD-cholesterol. Each value represents the mean ± SD of five separate experiments.
* $P < 0.001$.

Table 3
Quenching of NBD-cholesterol fluorescence in the outer membrane leaflet of sphingomyelinase-treated cells

Cells	F_o	F_r	F_{ap}	% quenchable probe
β1 integrin-expressing	260 ± 10.4	168 ± 8.7	105 ± 5.5	59 ± 4.1
β1 integrin null	210 ± 9.1	161 ± 7.3	98 ± 6.2	44 ± 3.7*

F_o, F_r, and F_{ap} are described in Table 2. Each value represents the mean ± SD of five separate experiments.
* $P < 0.001$.

lesterol fluorescent analogs to quenching with dithionite (Table 3). The results showed that the percent of quenchable fluorescent analogs of cholesterol present in the outer membrane monolayer was reduced in both β1 and β1 null cells due to sphingomyelinase treatment (compare Tables 2 and 3). Interestingly, the content of cholesterol in membranes of sphingomyelinase-treated cells occurred to be lower by about 14% compared to sphingomyelinase-untreated ones, which was rather unexpected, because cholesterol level was not manipulated (values not shown). Thus, sphingomyelin depletion seemed to affect the content and distribution of cholesterol in membranes of both integrin-expressing and integrin-deficient cells, a phenomenon which needs further clarification. Nevertheless, the differences in cholesterol asymmetry observed between β1 and β1 null cells were preserved after sphingomyelinase treatment, suggesting that the presence of β1 integrins was a factor, stimulating the preferential localization of cholesterol in the extracellular leaflet.

Discussion

In this study, we tested the hypothesis that certain protein components of lipid rafts may play an active role in the formation and/or stabilization of these cholesterol-rich membrane domains. One very well documented property of lipid rafts is that they can take in or exclude a variety of signaling proteins [40], including integrins [12], thus influencing their activation state. However, the effect of proteins like integrin receptors on membrane raft lipids still remains unclear.

Here, we investigated the intramembrane distribution of the major lipid raft component cholesterol in plasma membranes of β1 integrin-expressing and β1 integrin-deficient cells. The main reason for the choice of these cells as an experimental model was the fact that they differed by the presence of β1 integrin receptors, which were reported to be associated with the membrane cholesterol-rich raft domains [41]. Our results demonstrated that integrin presence was accompanied by changes in the plasma membrane lipid profile, expressed as increased content of the major raft components—cholesterol and sphingomyelin as well as a non-raft constituent—phosphatidylethanolamine (Table 1). The observed higher level of cholesterol and sphingomyelin may serve as a prerequisite for formation of more lipid raft domains in β1 cells. This possibility was experimentally confirmed by the fact, that we obtained a higher percent of DRM fractions from β1 integrin-expressing cells.

In view of the fact that cholesterol transmembrane distribution is dynamic and depends on the cell's physiological state [7,42], it seemed of interest to analyze whether the presence of β1 integrin receptors, which was accompanied by elevation of the membrane level of cholesterol, also affected its intramembrane localization. The results obtained by NBD-cholesterol fluorescence quenching demonstrated that integrin-expressing cells contained a higher percent of cholesterol molecules in the outer membrane leaflet compared to integrin-lacking cells (Table 2).

To analyze the mechanism underlying the differences in cholesterol distribution within the membranes, we presumed that one possible reason could be the observed alterations in the level of other membrane lipids [4,43]. Boesze-Battaglia et al. [37] reported intramembrane migration of cholesterol and phosphatidylethanolamine in opposite directions within platelet membranes upon stimulation with collagen. Thus, it is not unlikely that eventual alterations in the transmembrane distribution of phosphatidylethanolamine, which was elevated in membranes of β1 fibroblasts, could induce redistribution of cholesterol molecules. Since the interactions between phosphatidylethanolamine and cholesterol are thermodynamically unfavorable [4,38,43] it is also possible that elevation of phosphatidylethanolamine, which is concentrated mainly in the inner membrane leaflet, could induce partial extrusion of cholesterol from the inner to the outer monolayer. Such migration would make a larger part of the cholesterol molecules accessible to quenching by dithionite. Studies on the localization of NBD-phosphatidylethanolamine within the membranes showed that despite of the different level of this phospholipid, its transmembrane distribution was quite similar in the two cell lines, making it unlikely to affect cholesterol intramembrane migration. This observation made us consider another possible mechanism, which could be responsible for the differences in cholesterol distribution within the membranes of β1 and β1 null cells. As already mentioned, cholesterol molecules have a high affinity for sphingomyelin [40,44], which makes this lipid capable of affecting cholesterol distribution. Indeed, partial depletion of membrane sphingomyelin by exogenous sphingomyelinase altered the accessibility of NBD-cholesterol fluorescence to quenching. In both

β1 and β1 null cells with degraded sphingomyelin the differences in the accessibility of outer monolayer NBD-cholesterol to quenching were reduced compared to cells with intact sphingomyelin (see Tables 2 and 3). Thus it seems likely that the level of sphingomyelin in the tested membranes played a significant role for cholesterol intramembrane distribution. Interestingly, the reduction of outer leaflet-associated fluorescence, expressed as percent of the fluorescence before sphingomyelinase treatment, was almost similar (about 25%) for the two cell lines (see Tables 2 and 3). It is possible that the partial membrane depletion of sphingomyelin destroyed some of the sphingomyelin–cholesterol interactions, thus rendering part of the cholesterol molecules free to redistribute between the membrane leaflets. These specific interactions might as well underlie the observation, that despite of the higher level of sphingomyelin in integrin-containing cells, a higher percent of this sphingolipid was hydrolyzed in β1 null cells. This could be due to the fact that β1 cells contained also an elevated level of cholesterol, which, together with sphingomyelin formed a higher content of raft domains as confirmed by the isolated DRM fractions. The more compact structure of raft domains could make sphingomyelin less accessible to sphingomyelinase-induced degradation, which would explain why sphingomyelin hydrolysis was more effective in β1 null cells. Another possibility is that the presence of integrins and caveolin in β1 cells may also play a certain sphingomyelin-protective role.

The fact that sphingomyelin partial depletion was accompanied by redistribution and reduction of cholesterol in both β1-expressing and β1-deficient fibroblasts demonstrated that the interactions between sphingomyelin and cholesterol were essential for maintenance of cholesterol intramembrane distribution [45]. However, the presence of membrane-bound proteins like integrin receptors was also an important factor, since the differences in cholesterol transmembrane localization, observed between the two cell lines before sphingomyelinase treatment, were preserved after sphingomyelin depletion.

The observation that the level of membrane cholesterol was higher in integrin-expressing cells at all time points of cyclodextrin treatment could as well be attributed to the specific sphingomyelin–cholesterol interactions within the raft domains. It is possible that the presence of more raft domains in β1 cells made cholesterol in their membranes less accessible to cyclodextrin.

Finally, it seems likely, that the presence of functionally active membrane-associated β1 integrin receptors induced formation and/or stabilization of already existing raft domains. This is confirmed not only by the higher level of the raft-forming lipids in these membranes, but also by the higher percent of DRM fractions isolated from integrin-expressing fibroblasts, compared to integrin-deficient ones. It is still unclear how integrin receptors affect the components and stability of raft domains. One possible explanation involves formation of specific lipid–protein interactions in the plane of the membrane [46], leading to organization and/or stabilization of raft domains on the surface of β1 integrin-expressing cells. Another option takes into account the ability of integrins to regulate different cellular processes including gene expression [47–49]. A pathway from β1 integrin to stimulated caveolin expression may lead to increased cholesterol transfer to plasma membrane and induction of raft formation. This possibility is supported by the elevated caveolin content in β1 integrin-expressing cells (Fig. 2). Furthermore, it is well established that caveolin is a cholesterol-binding transport protein [50,51], whose mRNA levels are linked to cholesterol levels [52,53]. Caveolin may have a role in regulating intracellular-free cholesterol distribution since its dominant-negative mutant decreases cholesterol levels in the plasma membrane [54]. Thus, caveolin can stabilize the cell surface expression of raft domains by regulating their cholesterol content or by affecting the dynamic of their endocytosis as suggested by Nabi and Le [55]. Although the exact mechanism could not be specified at present, it is clear that the optimal functioning of β1 integrin receptors requires the presence of raft domains [56,57], which possibly triggers a feedback elevation of the raft lipid components.

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