Cell surface ceramide controls translocation of transferrin receptor to clathrin-coated pits

Abo Bakr Abdel Shakor a,b,⁎, Mona Mohamed Atia a, Katarzyna Kwiatkowska c, Andrzej Sobota c,⁎⁎

a Laboratory of Molecular Cell Biology, Zoology Department, Assiut University, 71516 Assiut, Egypt
b Department of Biology, Faculty of Science, King Khalid University, Abha, Saudi Arabia
c Laboratory of Molecular Cell Biology, Zoology Department, Assiut University, 71516 Assiut, Egypt

⁎⁎ Corresponding author at: Laboratory of Molecular Cell Biology, Zoology Department, Assiut University, 71516 Assiut, Egypt.
⁎ Corresponding author: Laboratory of Molecular Cell Biology, Zoology Department, Assiut University, 71516 Assiut, Egypt.

1. Introduction

Transferrin receptor (TfR) belongs to a group of receptors which deliver their ligands into the cell via internalization mediated by clathrin-coated vesicles [1]. TfR mediates cellular uptake of ferric ions associated with transferrin (Tf), an 80-kDa serum glycoprotein. The affinity of Tf to its receptor increases upon ferric ions binding and the formed receptor/ligand complex accumulates in clathrin-coated pits as a result of recognition of a signal motif of TfR by AP2, an accessory protein of clathrin. After pinching off, clathrin-coated vesicles deliver their contents to early endosomes from which TfR recycle back to the cell surface, while ferric ions are used up by the cells [2,3].

The molecular mechanisms governing clathrin-mediated receptor endocytosis have been thoroughly studied. Recently, a regulatory function of lipids in this process has attracted increased attention [4–8]. For example, an involvement of phosphatidic acid and phosphatidylinositol-4,5-bisphosphate in receptor endocytosis has been demonstrated by inhibition or mutation of enzymes which modify those lipids [9–11].

Besides clathrin-coated pits other invaginations, such as caveolae, and raft-based membrane platforms coexist in the plane of the plasma membrane. All these structures function as endocytic portals for distinct receptor/ligand complexes. Studies on the endocytosis of TfR, HM1.24 protein and tetanus toxin indicate that there is a cross-talk between clathrin-dependent and non-canonical clathrin-independent endocytic mechanisms [12–14]. It is still unknown how cells control the sorting of different receptors to distinct endocytic pathways. It has been hypothesized that redistribution of membrane receptors can be forced by ceramide, a lipid generated at the onset of activation of distinct receptors, like CD95, CD40 and FcyRIIa [15–18]. In the plasma membrane, ceramide has a tendency to self-aggregate and form ceramide-rich platforms [19,20]. These properties of ceramide promote clustering of receptor molecules within ceramide-rich platforms resulting in a local concentration of the receptors [15,17]. Such clustering of receptors facilitates their association with the signaling molecules and promotes exclusion of others, e.g., phosphatases [21]. Our earlier studies showed that at the first stage of FcyRIa activation, ceramide was generated in the outer leaflet of the plasma membrane. This generation of ceramide was required for association of the activated FcyRIa with plasma membrane rafts and phosphorylation of the receptor, triggering signaling cascades leading to phagocytosis...
Ceramide can be generated by hydrolysis of sphingomyelin catalyzed by three sphingomyelinases: acid, neutral or alkaline [20,22]. Among them, acid sphingomyelinase (ASMase) has been shown to be critically involved in many cellular activities [23]. ASMase exists in two forms, lysosomal and secretory, both derived from the same gene but differing in their linked oligosaccharides and the requirement of zinc ions for activity [24]. The fast generation of ceramide in the outer leaflet of the plasma membrane during activation of distinct receptors is attributed to ASMase extruded onto the cell surface, whereas neutral and alkaline sphingomyelinases seem to act slowly [18,25].

In the present study we demonstrate that at the onset of interaction of TIR with Tf/ferric ion complex, ceramide is generated in close proximity to the receptor. The ceramide precludes trapping of TIR with its cargo by membrane rafts.

2. Materials and methods

2.1. Reagents

TF-Alexa Fluor 488, goat anti-mouse IgM-Alexa Fluor 546 and goat anti-mouse IgG-Alexa Fluor 546 were from Invitrogen. Anti-clathrin heavy chain mouse IgG and rabbit IgG were from ABR Affinity Bioreagents and Cell Signaling, respectively. Anti-Lyn rabbit IgG and anti-TIR mouse IgG were from Santa Cruz Biotechnology, anti-ceramide mouse IgM (clone MID1584) was from Alexis Biochemicals, anti-LAT (linker for activation of T cells) mouse IgG was kindly provided by Dr. V. Horejsi (Institute of Molecular Genetics, Prague, Czech Republic). SuperSignal West Pico Chemiluminescent Substrate was from Pierce Biotechnology. C6-NBD-ceramide and C6-NBD-sphingomyelin were from Avanti. ASMase-specific and scrambled siRNA were from Ambion. Other chemicals were purchased from Sigma if not mentioned otherwise.

2.2. Cell culture and Tf treatment

Jurkat human T lymphocyte cell line was cultured in RPMI 1640 supplemented with 10% calf serum in humidified atmosphere with 5% CO₂ at 37 °C. For experiments, cells were kept in serum-free medium for 30 min at 37 °C. In a series of experiments cells were treated with 30 μM imipramine, 0.01% DMSO (imipramine carrier), 10 μg/ml anti-nermall IgM, or 10 μg/ml irrelevant mouse IgM for 1 h at 37 °C. Later, cells were chilled on ice and supplemented with 5 μg/ml of human TF-Alexa Fluor 488 or unlabeled human holo-Tf for 1 min at 4 °C. To start Tf internalization, cells were transferred to 37 °C for up to 30 min and eventually washed with ice-cold PBS. For immunofluorescence studies of internalized TF-Alexa Fluor 488, cells were fixed with 2% formaldehyde for 10 min at 4 °C followed by 20 min at room temperature and processed as described below. For spectrofluorometric measurements of internalized TF-Alexa Fluor 488, cells (2x10⁶/sample) were washed for 15 s at 4 °C, with 100 mM NaCl, 50 mM acetate buffer, pH 4.5, to remove plasma membrane-bound Tf. After additional washing with PBS, cells were lysed in 1% NP-40, 50 mM Tris–HCl, pH 7.2, and centrifuged at 10,000 ×g for 5 min. Supernatant was collected and TF-Alexa Fluor 488 fluorescence was measured at excitation/emission of 495/519 nm.

2.3. K⁺ depletion of cells

Jurkat cells were incubated in serum-free medium for 30 min at 37 °C and cellular potassium ions were reduced as described [26]. Briefly, cells were incubated in hypotonic K⁺–free medium for 2 min at 37 °C, followed by incubation in isotonic K⁺–free buffer for 30 min at 37 °C and used for experiments.

2.4. Confocal microscopy

Cells fixed at different time points of TF-Alexa Fluor 488 uptake were cytopspun onto glass slides, and either examined for Tf distribution or permeabilized with 0.1% Triton X-100 for 10 min at room temperature for clathrin labeling. For that, after blocking with 1% bovine serum albumin (30 min, room temperature), cells were incubated with mouse anti-clathrin heavy chain IgG for 2 h at room temperature, followed by goat anti-mouse IgG conjugated with Alexa Fluor 546 (30 min, room temperature). Cells were examined with a Leica confocal microscope. The colocalization of Tf and clathrin was estimated by counting cells displaying yellow gatherings of endosomes in merged confocal images of at least 200 cells per each treatment and the results were expressed as percentage of cells. In a series of experiments, after addition of TF-Alexa Fluor 488 (1 min, 4 °C) cells were fixed and incubated without permeabilization with mouse anti-TIR IgG followed by goat anti-mouse IgG-Alexa Fluor 546. For visualization of cell surface ceramide, unpermeabilized cells were treated with 1 μg/ml anti-ceramide IgM for 1 h at room temperature followed by goat anti-mouse IgM-Alexa Fluor 546 and then were examined with an Olympus fluorescence microscope (BX60-34FB1).

2.5. Determination of sphingomyelinase activity

Activity of sphingomyelinases was analyzed by a modification of methods described by Okazaki et al. [27] and Loidl et al. [28]. Briefly, cells (2.5 × 10⁶) were lysed in 1 ml of lysis buffer containing 1% Triton X-100, 1 mM EDTA, 10 mM Tris–HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A and 2.5 μg/ml aprotinin (10 min, 4 °C) and sonicated for 30 s at 4 °C. Cell lysates were centrifuged at 12,000 ×g for 10 min at 4 °C, and the supernatants were used for determination of enzymatic activity. Protein concentration was estimated using Protein Assay Kit (Bio-Rad). For analysis of neutral sphingomyelinase (NSMase) activity, cell lysates containing 20 μg of protein/sample were mixed with the reaction buffer composed of 0.1% Triton X-100, 10 mM MgCl₂, 5 mM diethiothreitol, 100 mM Tris–HCl, pH 7.5 and 54 μM C6-NBD-SM (total volume 100 μl). For assessment of ASMase activity, cell lysates were mixed with reaction buffer containing 0.1% Triton X-100, 100 mM acetate buffer, pH 5.0 and 54 μM C6-NBD-SM (total volume 100 μl). The mixtures were incubated for 30 min at 37 °C; the reaction was stopped by adding 2 ml of chloroform/methanol (2:1, v/v) and then 900 μl of H₂O. The lower phase was collected and the solvent evaporated. After dissolving in chloroform/methanol (2:1, v/v) aliquots were applied to TLC silica gel plates (Whatman) that were then developed in chloroform/methanol/12 mM MgCl₂ (65:25:4, by volume). The fluorescence of C6-NBD-ceramide (excitation/emission of 460/515 nm) was quantified in an image analyzer (LAS-3000, Fuji) in relation to a C6-NBD-ceramide standard and results were expressed as pmoles of C6-NBD-ceramide per μg of protein in the sample.

2.6. Ceramide measurement by diacylglycerol kinase assay

Ceramide was quantified using diacylglycerol kinase (DGK) assay as described elsewhere [29]. Briefly, at different time points of Tf binding and internalization cells (2.5 × 10⁶/sample) were pelleted and cellular lipids were extracted [30] and dried. The lipids were solubilized in 7.5% octyl-β-D-glucoside and 25 mM diethanol phosphate/glycerol solution. Lipid micelles formed were mixed with reaction buffer containing 5 mM LiCl, 12.5 mM MgCl₂, 1 mM EGTA, 19.4 μM DTPA, 50 mM imidazole, pH 6.6, 100 μM ATP, 4 μCi [γ-32P]ATP (Amersham) and 3.8 μg/ml DGK membranes derived from DGK-overexpressing Escherichia coli (kindly provided by Dr. Ali M. Eldib, Alexandria University, Egypt) to give a total volume of 100 μl. The reaction was carried out for 30 min at 37 °C and stopped by adding chloroform/methanol mixture (2:1, v/v). Extracted lipids were
were produced using siRNA construction kit obtained from Ambion.

2.8. ASMase gene silencing

To suppress ASMase mRNA expression, small interfering RNA (siRNA) specific for this enzyme and non-specific scrambled siRNA were produced using siRNA construction kit obtained from Ambion [32]. siRNA (100 pmol) was transfected to Jurkat cells (2 × 10⁶ per sample) using Amaxa Nucleofector according to the manufacturer’s recommendations.

3. Results

3.1. Tf interaction with cells induces transient generation of ceramide

To synchronize Tf uptake, Jurkat cells were exposed to 5 μg/ml Tf-Alexa Fluor 488 for 1 min at 4 °C (binding) and later shifted to 37 °C (internalization). During the binding stage distinct labeling of the plasma membrane by Tf-Alexa Fluor 488 was detected by confocal microscopy. As soon as 1 min after warming of cells, substantial amounts of Tf were accumulated inside the cells, whereas after 15 min the level of Tf in cells was reduced (Fig. 1A). Spectrofluorimetric quantitation of internalized Tf revealed that its accumulation in the cells proceeded for up to 4 min (Fig. 1B). In the course of binding and internalization, significant changes of the ceramide level in the outer leaflet of the plasma membrane were detected. The cell surface ceramide was generated transiently, peaking within 1 min of Tf binding and then the level of ceramide returned within 5 min to that seen in unstimulated cells. The amount of the cell surface ceramide tended to increase slightly again after 10 min of Tf internalization (Fig. 2A). The cell surface ceramide constituted a significant portion of total cellular ceramide since measurements of cellular ceramide by DGK assay revealed a 2.5-fold elevation of the lipid level after 1 min of Tf binding and a following drop during Tf internalization (Fig. 2B). Hence, challenging of the cells with Tf induced a rapid generation of ceramide on the cell surface which preceded Tf uptake. Then, when Tf started to accumulate in the cells, the ceramide disappeared from the cell surface.

To identify the enzyme participating in ceramide generation, ASMase and NSMase activities were investigated during Tf binding and internalization. ASMase was activated rapidly upon Tf addition to the cells, reaching maximal activity 1 min after the Tf addition, following which it returned to its basal level (Fig. 2C). Hence, activation of ASMase correlated with the transient generation of ceramide on the surface of the cells exposed to Tf (Fig. 2A, B, C). In contrast to ASMase, no changes of NSMase activity were detected during Tf binding and 10 min of Tf internalization (Fig. 2C).

3.2. Ceramide is important for clathrin-mediated Tf uptake

To investigate the role of ceramide generation in Tf uptake, the activity of ASMase was diminished by pretreatment of cells with imipramine. Surprisingly, in those conditions Tf uptake (1 min) was upregulated by 40% (Fig. 3A and B, white bars). Similarly, abrogation of ceramide generation by silencing of ASMase gene expression facilitated the uptake of Tf by 60% after 1 min of exposure to Tf (Fig. 3B, white bars). Measurements of ASMase activity in lysates of cells treated with imipramine or ASMase siRNA confirmed reduction of the enzyme activity by about 85% (Fig. 3C).

Treatment of cells with imipramine and ASMase-specific siRNA affected also later stages of Tf accumulation in the cells. In control cells the amount of Tf accumulated after 15 min of exposure to Tf was only 30% of the Tf content after 1 min. ASMase siRNA treated the uptake of Tf by 60% after 1 min of exposure to Tf (Fig. 3B, white bars). Conversely, in cells treated with imipramine or ASMase siRNA the amount of Tf accumulated in the cells following 15 min of Tf treatment was even slightly higher than after 1 min. The amounts of intracellular Tf were, respectively, ca. 3.5-fold and 5-fold higher than in control cells at the same time point (Fig. 3 A and B, gray bars). Similar results were obtained when Tf binding and internalization were allowed to proceed in the presence of anti-ceramide IgM expected to bind and neutralize the cell surface ceramide (Fig. 3B). Taken together, the results indicate that abrogation of ceramide generation or its neutralizing facilitates internalization of Tf and, especially, its accumulation in the cells, suggesting that in such conditions Tf does not recycle.
The facilitation of Tf uptake and the abrogation of its recycling in the absence of ceramide suggest that in those conditions Tf is internalized through a pathway different than the regular clathrin-mediated one. To examine this possibility we studied Tf/clathrin colocalization at 1 min of Tf internalization, when its uptake was maximal in the control conditions. As expected, in the controls the colocalization of Tf and clathrin was seen in 90–95% of cells (Fig. 4A–D). In contrast, abrogation of ceramide generation by treatment of cells with imipramine or ASMase siRNA led to a loss of Tf/clathrin colocalization (Fig. 4A, B) by 71% or 75%, respectively (Fig. 4D). Similar results were obtained when the cell surface ceramide generated during Tf binding was neutralized by exposure of cells to anti-ceramide antibody (Fig. 4C). In those conditions the reduction was about 50% (Fig. 4D).

Fig. 2. Ceramide generation and ASMase activation are triggered by Tf binding. (A) Visualization of ceramide on the surface of cells with the use of anti-ceramide IgM. (B) Time-course of ceramide generation in cells in response to Tf binding and internalization determined by DGK assay. (C) Activity of ASMase (closed circles) and NSMase (open circles) in whole cell lysates during Tf binding and internalization. Data in B and C are mean ± S.E.M. from 3 or 4 experiments.

Fig. 3. Interference with ASMase activity or ceramide sequestration affect Tf uptake and recycling. (A) Distribution of Tf-Alexa Fluor 488 in cells pretreated with 30 μM imipramine, ASMase-directed siRNA or appropriate controls (0.01% DMSO, scrambled siRNA). (B) Spectrofluorimetric quantitation of Tf-Alexa Fluor 488 in lysates of cells obtained after 1 or 15 min of Tf uptake at 37 °C. (C) Influence of imipramine or ASMase siRNA on ASMase activity in cell lysates. Data in B and C are mean ± S.E.M. from 3 experiments.
The reduction of the Tf/clathrin colocalization was in line with the hypothesis that depletion of the cells with ceramide allowed the Tf internalization via a clathrin-independent pathway. In another approach to demonstrate the involvement of a clathrin-independent route in Tf internalization we analyzed the amount of Tf accumulated in the cells after reducing the cellular concentration of K+. As expected, the inhibition of clathrin coat assembly by the K+ depletion resulted in ca. 10-fold reduction of Tf internalization in cells not-exposed to imipramine and those transfected with scrambled siRNA (Fig. 4E). In contrast, in imipramine-treated cells and in the cells transfected with ASMase siRNA, the Tf internalization was reduced following K+ depletion by ca. 2.4- and 2.9-fold, respectively (Fig. 4E).

We verified that the treatment of cells with the ASMase siRNA did not have an unintended effect on the level of TfR or clathrin (Fig. 4F, upper panel) or on the Tf/TfR interaction (Fig. 4F, lower panel). The lack of the latter effect indicated that Tf was not internalized by non-receptor-mediated endocytosis.

As the uptake of ferric ions bound to Tf mediated by the clathrin pathway is required for cell growth [33,34], cell proliferation was measured 24 and 48 h after ceramide generation had been blocked. Each of the three anti-ceramide treatments completely abolished the cell proliferation, showing that the alternative route of Tf uptake activated in the cells depleted of ceramide failed to supply them with usable ferric ions (Fig. 5).

### 3.3. Ceramide prevents TfR trapping in plasma membrane rafts

To investigate the relations between ceramide and Tf during its binding and uptake, colocalization of the two molecules was analyzed. At the stage of Tf binding (1 min, 4 °C) Tf and ceramide colocalized on the cell surface (Fig. 6A). When the Tf internalization started...
(1 min, 37 °C) ceramide disappeared from the cell surface and no longer colocalized with Tf (Fig. 6A).

The transient generation of cell surface ceramide restricted to the stage of Tf binding suggested its role in TfR dynamics in the plane of the plasma membrane. It is commonly accepted that TfR resides in a glycerophospholipid-rich, non-raft areas of the plasma membrane, therefore we performed fractionation studies to analyze the distribution of TfR in Triton X-100-soluble and detergent-resistant membrane fractions (DRM) related to rafts. Fractionation of cell lysates from control cells exposed to Tf for 1 min (binding stage) revealed that TfR was mainly concentrated in Triton X-100-soluble non-raft fractions 4–7. In contrast, in cells depleted of ASMase due to gene silencing the binding of Tf led to a shift of a part of TfR towards Triton X-100-insoluble low density fractions 1–3 (Fig. 6B, upper panel). Similar results were obtained after fractionation of Triton X-100 lysates derived from cells preincubated with imipramine (Fig. 6B, lower panel). Fractions 1–3 were likely to contain raft-derived membrane fragments judging from the presence of two raft markers, Lyn kinase and LAT.
protein (Fig. 6B). The association of Lyn kinase and LAT protein with the raft fractions 1–3 was not affected by the ceramide-interfering agents used (Fig. 6B). On the other hand, the clathrin heavy chain was detected in high density fractions 5–7 derived from both control cells and cells pretreated with imipramine or ASMase siRNA (Fig. 6B).

This indicates that disturbances in ceramide generation during Tf binding induce translocation of TfR to plasma membrane rafts without concomitant changes in distribution of clathrin.

4. Discussion

The uptake of Tf/ferric ions complex by TfR is a well-established example of clathrin-dependent endocytosis. The mechanisms governing accumulation of distinct receptors in coated pits are well known and include recognition of the receptor signaling motifs by AP2 complex [35,36]. Less is known about the forces that facilitate the recruitment of the Tf/TfR complex to the coated pits.

We addressed this issue by studying whether translocation of TfR to coated pits upon Tf binding can be affected by plasma membrane ceramide. Several recent studies indicated that activation of some plasma membrane receptors induced hydrolysis of sphingomyelin, concentrated in the outer leaflet of the plasma membrane, leading to ceramide generation. This reaction is likely to be catalyzed by secretory form of ASMase. This assumption is supported by two lines of evidence. First, a correlation of increased activity of ASMase and production of cell surface ceramide has been reported following activation of CD95, CD40, FcγRlla and TNFα receptor [15,17,37,38]. Second, a transient appearance of ASMase on the cell surface has been demonstrated during activation of the receptors by confocal and electron microscopy, and by FACS analysis [17,39,40]. Recently, a C-terminal region of ASMase including serine 508 was identified as required for ASMase secretion and generation of certain forms of ceramide in cells [41,42]. These data have established the participation of secretory ASMase in the generation of cellular ceramide [17,37].

The plasma membrane ceramide formed is quickly metabolized by hydrolysis to sphingosine or by phosphorylation to ceramide-1-phosphate, or converted to sphingomyelin by sphingomyelin synthase 2. Ceramide itself and all its derivatives can function as signaling molecules, but ceramide also affects the biophysical properties of the plasma membrane as indicated by its ability to induce domain formation in model membranes [19,43–46].

In this study we showed that ceramide is generated in the outer leaflet of the plasma membrane upon binding of Tf/ferric ions to TfR. The ceramide is most likely generated by ASMase since its activity, but not the activity of NSMase, was increased substantially at the time when ceramide appeared. It was generated in close vicinity of activated TfR. It is generally accepted that this receptor is located in the plasma membrane in glycerophospholipid-rich regions, outside so-called sphingomyelin/cholesterol-based rafts. This suggests that during binding of Tf/ferric ions to TfR, non-raft sphingomyelin is converted to ceramide. The plasma membrane location of the sphingomyelin pool utilized by ASMase during receptor activation is a matter of an ongoing debate. Our earlier data on FcγRlla activation indicated that the ceramide was derived from sphingomyelin located outside rafts. In line with those data, we also found earlier that clustering of non-raft TfR with specific antibodies induced even more pronounced ceramide production than that following activation of the raft-directed FcγRlla [17,18]. Once formed ceramide self-assembles into domains that are often attributed to rafts [18,21,37]. Such redistribution of plasma membrane lipids is likely to affect the distribution of activated plasma membrane receptors. The intrinsic properties of the receptors, like the hydrophobicity of their transmembrane regions could facilitate fusion of the receptors with the ceramide enriched rafts or, conversely, exclusion of the receptors from the rafts and their direction to, e.g., clathrin-coated pits. TfR follows the second scenario, unless the generation of ceramide is disturbed. Depletion of ceramide after silencing of ASMase expression or treatment of cells with imipramine led to the association of activated TfR with raft-related regions (Fig. 6) and its internalization by a non-canonical clathrin-independent route (Fig. 3, 4).

The mechanisms of clathrin-dependent endocytosis are becoming increasingly understood. Recent years have brought ample data on new clathrin-independent endocytotic pathways [1,47,48]. Now reports suggesting a possibility of dual clathrin-mediated and clathrin-independent internalization routes of receptors are scarce. It has been found that knock down of clathrin heavy chain or expression of a dominant negative mutant of dynamin 2 favors internalization of EGF and TGFβ receptors via a clathrin-independent pathway instead of the normal clathrin-dependent one. Notably, both receptors were directed towards caveolae and rafts, caveolae-related structures. The receptors internalized via clathrin-independent pathway lost their signaling function and underwent degradation [49,50].

In line with those data we found that during Tf binding in ceramide-depleted cells, a part of TfR was associated with the raft fraction of the plasma membrane. Thus, rafts can be involved in the internalization of TfR when cell surface ceramide generation is disturbed. The uptake of the Tf/TfR complex through the clathrin-independent pathway disturbed the turnover of Tf. The protein remained accumulated in the cells while under physiological conditions is quickly returned to the cell surface. This intracellular arresting of Tf was likely responsible for the cessation of cell growth upon inhibition of ceramide production—the cells could no longer be supplied with the iron required for proliferation. It was shown earlier that iron delivered to lymphocytes in an abnormal manner can be toxic to these cells. To sum up, our data indicate that the cell surface ceramide generated by ASMase during Tf binding is required for proper lateral sorting of TfR governing its concentration in coated pits.

References
