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Biochimica et Biophysica Acta 1467 (2000) 144–152

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Glycosylphosphatidylinositol-anchored proteins are not required for crosslinking-mediated endocytosis or transfection of avidin bioconjugates into biotinylated cells

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Received 22 February 2000; received in revised form 28 March 2000; accepted 28 March 2000

Abstract

Even though glycosylphosphatidylinositol (GPI)-anchored proteins lack direct structural contact with the intracellular space, these ubiquitously expressed surface receptors activate signaling cascades and endocytosis when crosslinked by extracellular ligands. Such properties may be due to their association with membrane microdomains composed of glycosphingolipids, cholesterol and some signaling proteins. In this study, we hypothesize that GPI proteins may be required for crosslinking-mediated endocytosis of extracellular bioconjugates. To test this hypothesis, we first biotinylated the surface membranes of native K562 erythroleukemia cells versus K562 cells incapable of surface GPI protein expression. We then compared the entry of fluorescently labeled avidin or DNA condensed on polyethylenimine–avidin bioconjugates into the two biotinylated cell populations. Using fluorescence microscopy, nearly 100% efficiency of fluorescent avidin endocytosis was demonstrated in both cell types over a 24 h period. Surprisingly, plasmid DNA transfer was slightly more efficient among the biotinylated GPI-negative cells as measured by the expression of green fluorescence protein. Our findings that GPI proteins are not required for the endocytosis of avidin bioconjugates into biotinylated cells suggest that endocytosis associated with general membrane crosslinking may be due to overall reorganization of the membrane domains rather than GPI protein-specific interactions.

Keywords: GPI protein; GPI microdomain; Endocytosis; Biotin; Avidin; Gene transfer vector

1. Introduction

Proteins covalently attached to glycosylphosphatidylinositol (GPI proteins) are ubiquitously expressed on the external cell membranes of human cells. They fulfill many functions including intracellular and intercellular signaling [1]. GPI proteins associate with glycosphingolipids and cholesterol in the membrane forming so called 'GPI microdomains' or 'lipid rafts' [2,3]. GPI microdomains were first identified as detergent-insoluble complexes of membrane floating to a low density during sucrose gradient centrifugation [4]. Further, GPI microdomains ranging in size from 70 to 370 nm were directly visualized in cell membranes by biophysical measurements such as fluorescence resonance energy transfer, immunofluorescence microscopy, single particle tracking and chemical crosslinking [5–8]. Some structural diversity among

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0005-2736/00/$ – see front matter
PII: S0005-2736(00)00213-3

GPI microdomains was also demonstrated in association with functionally different GPI proteins [9]. Although GPI proteins have no direct connection with the cytoplasm, many studies have demonstrated biologic effects after GPI protein crosslinking with antibodies or natural ligands. Crosslinking results in rearrangements of several acylated signaling proteins localized on the inner leaflet of the GPI microdomains such as the Src family tyrosine kinases or G-proteins leading to signaling cascades [10–14]. Crosslinking also triggers endocytosis of the crosslinked GPI proteins [15,16]. These endocytosis events occur in flask-shape endocytotic invaginations called caveolae or in the non-coated membrane invaginations and require cholesterol, sphingolipids and an intact actin cytoskeleton [15,17,18].

Crosslinking-mediated endocytosis of the GPI-anchored proteins is also useful for the delivery of biologically active substances to cells. Examples include the GPI-anchored folate receptor for the delivery of folate and its analogues into neoplastic cells [19,20]. We have further demonstrated gene delivery via the highly abundant CD59 or CD55 GPI proteins on hematopoietic cells [21]. In addition to GPI protein targeting, we demonstrated that generalized endocytosis and targeting can be achieved by avidin crosslinking of biotin covalently attached to the cell surface proteins [22]. As GPI proteins are an integral part of GPI microdomains, we hypothesized that these proteins may be required for the entry of avidin and avidin bioconjugates into biotinylated cells. Using fluorescence microscopy, we compared the crosslinking effect of fluorescent avidin in biotinylated K562 erythroleukemic cells (GPI(+)) with that of K562 cells incapable of surface GPI protein expression (GPI(−)). Also, we compared plasmid DNA entry and expression of polyethyleneimine–avidin (PEI–avidin) bioconjugates in both cell types.

2. Materials and methods

2.1. Cells, antibodies and chemicals

K562 erythroleukemia cells capable of cell surface GPI-anchored proteins expression (GPI(+) cells) were obtained from the American Type Culture Collection. Mutated K562 cells incapable of GPI-anchored protein expression (GPI(−)) were provided as a generous gift from Dr. M. Edward Medof from the Department of Pharmacology, Case Western Reserve University, Cleveland, OH, USA [23]. Both cell lines were cultured in DMEM media supplemented with 10% fetal bovine serum (FBS; Biofluids, Rockville, MD, USA) and with 25 μg/ml gentamicin (Life Technologies, Gaithersburg, MD, USA). Biotinylated monoclonal antibodies: anti-CD71 (IgG2a), anti-CD55 (IgG2a) and anti-CD59 (IgG2a) were purchased from Pharmingen (San Diego, CA, USA). Isotypic control antibody (IgG2a–fluorescein isothiocyanate (FITC)) was obtained from Coulter Corporation (Hialeah, FL, USA). FITC-conjugated avidin (avidin–FITC) was purchased from Pierce (Rockford, IL, USA). 10^6 cells were stained with 20 μl of the antibody in 100 μl of phosphate-buffered saline (PBS), incubated at 4°C for 30 min and washed with PBS. Cells were incubated for 30 min with avidin–FITC at the concentration of 0.1 ng/cell, in 1 ml of PBS, at 4°C, and washed twice prior to analysis by flow cytometry. All chemicals were purchased from Sigma (St. Louis, MO, USA), unless otherwise stated in the text.

2.2. Labeling cells with biotin and avidin–FITC

Direct biotinylation of all cultured cells was performed as follows: 10^6 cultured cells were incubated in a final concentration of 0.5 ng sulfo-*N*-hydroxysuccinimide–biotin (sulfo-NHS–biotin) per cell (1 ml PBS final volume; 30 min at 4°C) and washed twice with PBS. The sulfo-NHS group conjugated to biotin permits covalent binding of biotin to free amine groups in proteins. Cells labeled with biotin were then incubated with avidin–FITC (10^6 cells at a final concentration of 0.1 ng avidin–FITC per cell, in 1 ml of PBS, for 30 min at 4°C) and washed twice with PBS. In the time-course studies, cells labeled with avidin–FITC were incubated at 37°C, collected at each time-point, fixed with 2% paraformaldehyde and analyzed by flow cytometry and fluorescence microscopy.

2.3. Flow cytometry and fluorescence microscopy

Flow cytometry was used to analyze the presence or absence of non-GPI-anchored CD71 or GPI-

anchored CD55 and CD59 proteins on the cells and for measurements of green fluorescence protein expression in gene transfer studies. All flow cytometry analyses were performed using an EPICS ELITE ESP flow cytometer (Coulter, Hialeah, FL, USA). In each experiment, 10 000 cells were analyzed using argon laser excitation and 525 nm (FITC and GFP) bandpass emission filters. Fluorescence microscopy was carried out using an Axiophot microscope with standard filter sets (Zeiss, Germany).

2.4. Preparation of PEI–avidin conjugate

Conjugation of PEI to avidin was performed as described previously [22]. Briefly, branched polymer PEI (Fluka, Switzerland) of molecular weight 800 kDa was prepared as a 5% w/v hydrochloride salt solution (800 μl of commercial PEI, 7 ml of water, 200 μl of 36% hydrochloric acid). As avidin is glycosylated, sodium periodate oxidation was used for the introduction of aldehyde residues onto the carbohydrate moieties. The aldehyde residues were then reacted with amine groups of PEI (Schiff base formation and reductive amination in the coupling buffer). 218 μl of 20 mg/ml sodium periodate solution was added to 20 mg of lyophilized avidin dissolved in 2 ml of PBS (pH 7.4), and the sample was incubated for 60 min at 25°C. The reaction was quenched with glycerol followed by gel filtration on a Sephadex G-25 superfine (PD10 Pharmacia) column. PEI was added at the molar ratio of 1:4 (60 mg PEI in 1.2 ml) to the fraction of avidin in PBS, and the sample was mixed vigorously for 1 h at 25°C. One ml of coupling buffer (20 mM Na_3PO_4, pH 7.5, 0.2 M NaCl and 3 mg/ml $NaCNBH_3$) was added to each sample followed by 1 h incubation. The addition of the coupling buffer was repeated twice at 1 h intervals with a total of 3 ml of coupling buffer added to each sample prior to overnight incubation. Glycine in molar excess quenched the avidin for 1 h at 25°C. Finally, the conjugate was purified on the Macro-Prep High S cation-exchanger (Bio-Rad, Hercules, CA, USA) with the 0.5–3 M NaCl gradient in 20 mM HEPES, pH 7.5, using Gilson high performance liquid chromatography system. The main conjugate fraction (eluted between 1.3 and 3.0 M salt) was pooled, concentrated to 6 ml by ultrafiltration and dialyzed overnight against 3×1 l of PBS, pH 7.4. The avidin content of each conjugate preparation was determined at 280 nm and PEI content by ninhydrin assay (NIN-SOL ninhydrin reagent from Pierce) at 570 nm. The reaction yielded 12 mg PEI conjugated to 4.36 mg avidin at the molar ratio of 1:4.36 (PA4). The overall yield of these conjugates based on PEI was 20.0%; yield based on avidin was −21.8%. The conjugates were aliquoted and stored at −80°C.

2.5. Plasmid DNA and transfection of cells

The plasmid pGT encoding green fluorescence protein was prepared and purified as described elsewhere [24]. Briefly, pGT was subcloned from the 780 bp DNA *Eco*RI/*Not*I fragment encoding eGFP from pEGFP-N1 (Clontech, Palo Alto, CA, USA) inserted into the pGreenLantern™-1 vector (Life Technologies, Gaithersburg, MD, USA) digested with *Eco*RI and *Not*I. Transfection complexes of DNA with PEI or PEI–avidin were prepared based on the optimization studies described earlier [22]. Ten μg of plasmid DNA was added and mixed gently with PEI or PEI–avidin (PA4) conjugates in PBS (total volume 0.5 ml). PEI–DNA or PEI–avidin–DNA complexes were formed at molar ratios of PEI nitrogen to DNA phosphate of 6.4:1. After a 30 min incubation at 25°C, 0.5 ml transfection mixture was added to the cells in 1.5 ml culture medium containing 10% FBS and gently mixed. All transfections were performed in 24 well plates (Costar, Cambridge, MA, USA) with 5×10^5 cells per well. After 4 h incubation with the transfection complexes, 1 ml fresh culture media containing 15% FBS was added to each well. Transfection was assessed based on GFP expression by fluorescence microscopy and flow cytometry 48 h later.

3. Results

3.1. Fluorescent avidin is efficiently endocytosed in GPI(+) and GPI(−) K562 cells

We used flow cytometry analyses to confirm the lack of GPI proteins on the GPI(−) cells versus their expression on GPI(+) cells. Both cell populations demonstrated very little autofluorescence, non-specif-

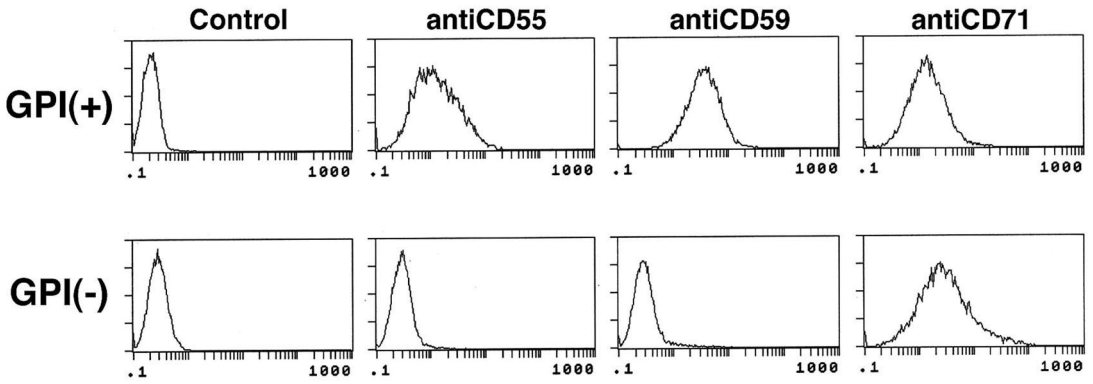

Fig. 1. Flow cytometry analysis of monoclonal antibody labeling in wild-type K562 cells (GPI(+)) and K562 cells deficient in production of GPI anchor (GPI(−)). Cells were labeled using fluorescent isotypic control antibody (first panel) or one of the biotinylated monoclonal antibodies followed by avidin–FITC: anti-CD55 (second panel), anti-CD59 (third panel) or anti-CD71 (fourth panel). *y*-axis: cell counts, *x*-axis: fluorescence. The experiment was repeated twice with the same results.

ic binding with fluorescently labeled antibodies (Fig. 1, first panel), or avidin–FITC (not shown). In contrast, monoclonal antibodies with affinity to GPI-anchored CD55 or CD59 proteins efficiently bound to native K562 cells at a significantly higher level than observed on the controls confirming a high level of GPI-anchored proteins on the surfaces of these cells (Fig. 1, upper panel). The mean fluorescence was highest with anti-CD59 (3.57 ± 2.46 FU) compared with 1.33 ± 1.20 FU with anti-CD55 and 0.229 ± 0.08 FU among the unstained cells. On K562 cells unable to synthesize GPI anchors, staining with both anti-CD59 and anti-CD55 antibodies resulted in no significant increase in fluorescence above the unstained control cells (Fig. 1, lower panel). We also analyzed the surface level of the transferrin receptor (CD71), a transmembranous protein [25]. Anti-CD71 staining demonstrated the expression of CD71 among both GPI(−) and GPI(+) cells (Fig. 1, far right). Interestingly, the level of CD71

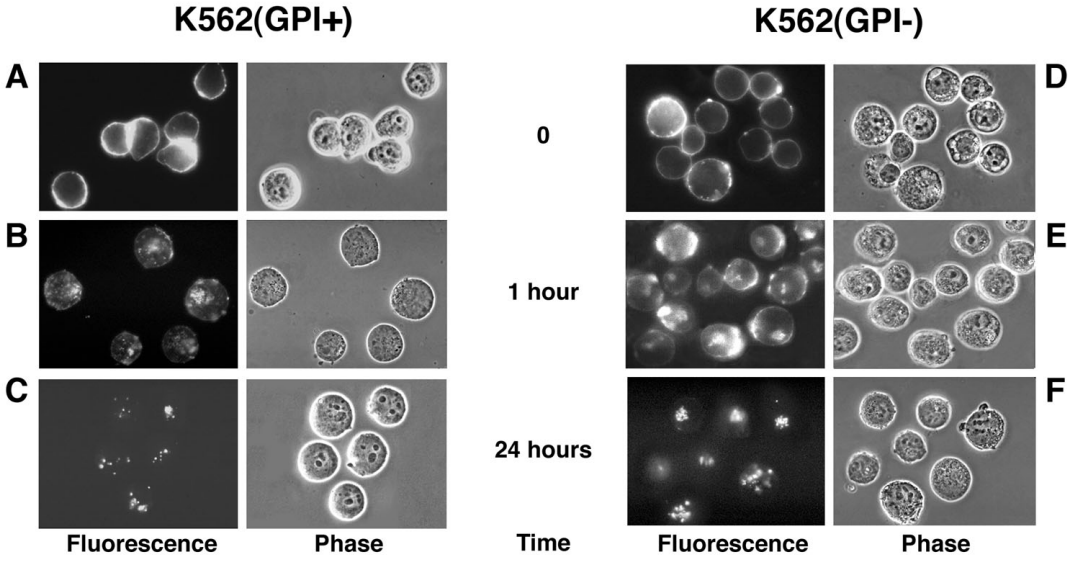

Fig. 2. Addition of avidin–FITC to biotinylated K562 GPI(+) cells (A–C) versus K562 GPI(−) cells (D–F). Matching fluorescence (left) and phase (right) images of biotinylated cells incubated at 37°C after addition of fluorescent avidin are shown (100× objective). Cells treated with avidin–FITC and observed immediately (A, D), after 1 h (B, E) and after 24 h (C, F) were compared. Each experiment was repeated several times with the same results.

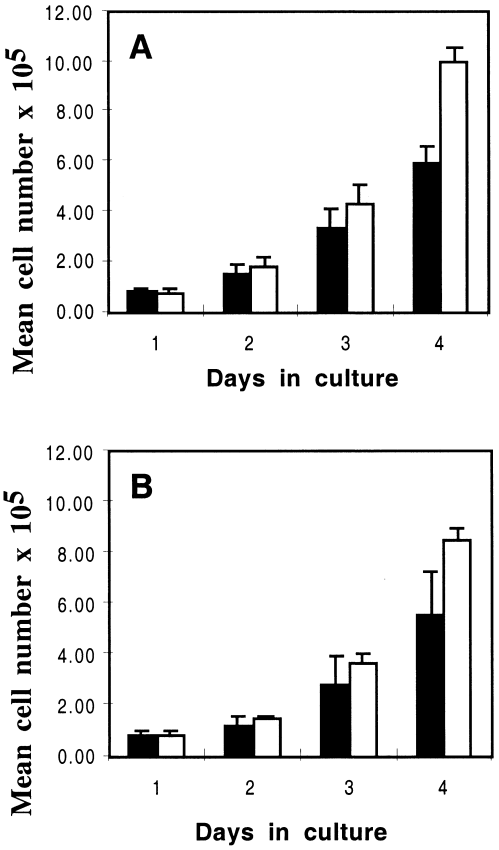

Fig. 3. Cell growth of K562 GPI(+) and GPI(−) cells before (A) and after biotinylation and avidin addition (B). Cell growth was measured on four successive days. Numbers of cells are means of triplicate experiments with S.D. bars. solid bars: GPI(−) cells, open bars: GPI(+) cells.

expression appeared slightly higher among the GPI(−) cells.

Next, we biotinylated the surface membranes of both cell types and incubated those cells with fluorescent avidin. As demonstrated previously [22], avidin–FITC crosslinked to biotin on the cell surface results in the rapid and efficient entry of those molecules. Hence, avidin–FITC endocytosis into biotinylated GPI(+) K562 cells was used here as a positive control (Fig. 2A–C). When avidin–FITC was added to biotinylated GPI(+) K562 cells, 100% of cells became fluorescently labeled. The fluorescent label was initially well dispersed on the cell surfaces with several areas of increased fluorescence (Fig. 2A). Within 1 h, fluorescein was detected as clusters inside endosomal compartments in all the cells (Fig. 2B). Over the next 24 h, the fluorescence gradually shifted from the cell surface to the cell interior in nearly 100% of the cells as demonstrated by comparing the fluorescence and phase images of the same microscopic field (Fig. 2C).

The pattern and time-course of avidin–FITC endocytosis observed among biotinylated GPI(−) K562 cells were comparable to those detected in GPI(+) K562 cells, but some important differences were observed (Fig. 2D–F). Immediately after the addition of avidin–FITC (Fig. 2D), 100% of GPI(−) cells displayed well dispersed surface fluorescence with small areas of increased intensity. Within 1 h, avidin–FITC was also detected within endosomal compartments (Fig. 2E). However, increased capping (gathering of avidin–FITC in clusters on the cell surface preceding endocytosis) and larger fluorescent clusters were present on the surfaces of the GPI(−) cells compared with GPI(+) cells (Fig. 2E,B). This may reflect differences in the surface organization of lipid rafts in the absence of GPI proteins. In both cell types, the surface fluorescence was no longer detected after 24 h. All avidin–FITC remained inside the cells without evidence of recycling to the surface, and no exocytosis of fluorescent complexes was detected. It was also noted that both GPI(+) and GPI(−) cells formed clustered groups directly after avidin labeling, perhaps as the result of avidin bridging among neighbor cells (Fig. 2A,D). After endocytosis of the surface

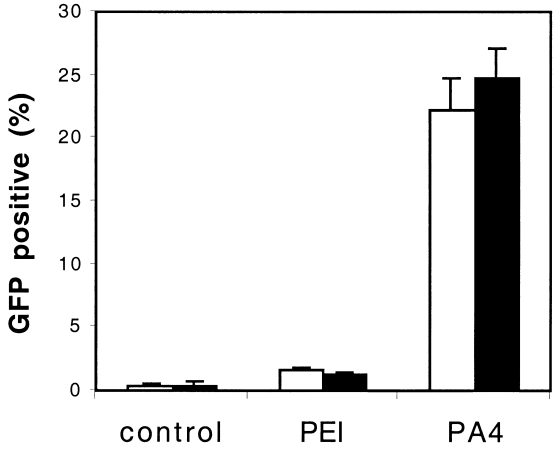

Fig. 4. Comparison of PEI–avidin (PA4) transfection efficiencies in GPI(+) K562 cells (open bars) or GPI(−) cells (solid bars). For each transfection, PEI or PEI–avidin (PA4) was complexed with 10 μg of pGT plasmid at the N:P ratio of 6.4:1. Transfection efficiencies represent the percentage of GFP expressing cells from two independent experiments each performed in duplicate.

avidin, the cells no longer showed the tendency to cluster (Fig. 2C,F).

We also tested the effect of avidin–FITC endocytosis on cell viability. Cell counts revealed that while GPI(−) cells grow slower than GPI(+) cells, no significant change in cell growth was detected after avidin–FITC crosslinking in either biotinylated GPI(+) and in GPI(−) cell populations (Fig. 3). No changes in cell viability were detected by dye exclusion among either population. Unbiotinylated GPI(+) and GPI(−) K562 cells incubated with avidin–FITC alone showed no increases in fluorescence as assessed by flow cytometry or fluorescence. Avidin–FITC endocytosis was not detected in directly biotinylated GPI(+) or GPI(−) cells incubated at 4°C over the 24 h period (not shown).

3.2. *Cells lacking GPI proteins can be efficiently transfected using PEI–avidin–DNA targeted to biotinylated receptors*

We have previously demonstrated transfer of plasmid DNA mediated by avidin conjugate (PEI–avidin–DNA) crosslinking of biotinylated cell surface proteins [22]. As the expression of transferred gene requires its endocytosis and trafficking to the nucleus, we tested the requirement of GPI proteins for the successful transfection using PEI–avidin–DNA in biotinylated cells.

PEI–avidin conjugates were prepared at the molar ratio of PEI to avidin of 1:4 (PA4). For the transfection experiments, complexes of DNA with untargeted PEI or PEI–avidin were formed at an N:P ratio of 6.4:1. Green fluorescent protein (GFP)-encoded plasmid DNA was chosen as a specific marker of successful transfection since endosomal escape, trafficking of plasmid DNA to the nuclear compartment and high-level transgene expression are required for the cells to fluoresce at detectable levels. GFP expressing cells were defined as those cells having a fluorescence at levels at least two S.D.s above the negative control. Gene transfer efficiencies were measured in GPI(+) and GPI(−) K562 cells by flow cytometry after 48 h and are shown on Fig. 4. Untargeted PEI in GPI(+) and GPI(−) cells resulted in only $1.6 \pm 0.2\%$ and $1.2 \pm 0.3\%$ of cells expressing GFP, respectively. Transfection using PEI–avidin (PA4) conjugate in cells directly labeled with biotin resulted in the $22.2 \pm 2.5\%$ GPI(+) and $24.8 \pm 2.3\%$ GPI(−) GFP expressing cells (about 11–30-fold increase over untargeted PEI). Fig. 5 demonstrates the transfection efficiencies and GFP expression levels among GPI(+) and GPI(−) cells incubated with untargeted PEI or PEI–avidin (PA4). Each dot repre-

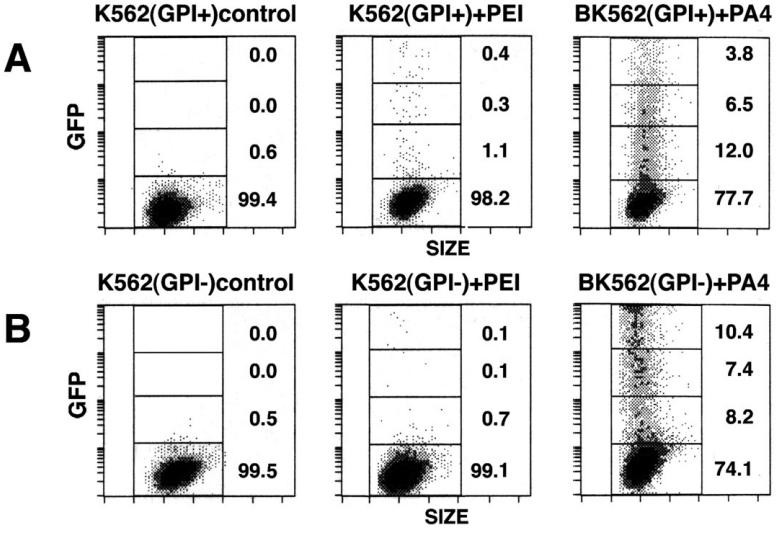

Fig. 5. Transfection of GPI(+) cells (A) and GPI(−) cells (B) with PEI or PEI–avidin (PA4) complexed with pGT. Untransfected control cells, cells transfected with untargeted PEI and biotinylated cells transfected with PEI–avidin (PA4) are compared. Flow cytometry analyses show relative GFP expression on the *y*-axis within the four log scale. The percentage of cells within each fluorescence decade is provided (negative cells in the lowest decade, and cells expressing GFP at low, medium and high levels in the upper three decades).

sents a cell with the corresponding GFP expression intensity shown (*y*-axis; log scale). As shown, the PEI–avidin (PA4) transfection efficiency and the overall distribution of GFP expressing cells was similar in GPI(−) and GPI(+) cells. However, more transfectants demonstrated the highest level of GFP expression among the GPI(−) cells (10.4% GPI(−) versus 3.8% GPI(+) cells).

4. Discussion

GPI-anchored proteins are expressed on the surfaces of human cells where they fulfill various functions. Depending on the cell type, some GPI proteins function as receptors (CD14, u-PAR, folate receptor, prion protein) or cell adhesion molecules (CD48, CD58 or CD90) [1,14,26]. Other GPI proteins (CD55, CD59) protect hematopoietic host cells against complement-mediated cell lysis [27]. In contrast to the considerable functional diversity of GPI proteins, their lipid anchors result in shared properties including association with sphingolipids, cholesterol and some signaling proteins in the membrane [3]. Recent data suggest that GPI microdomains cover large areas of the cell surface and, depending on the specific structure, can cluster functionally different GPI proteins [9]. It was proposed that GPI microdomains serve as membrane-specialized structures that associate GPI proteins and signaling molecules for cell-specific signal transduction events upon GPI proteins crosslinking [2]. Another typical feature of GPI proteins is their crosslinking-mediated endocytosis. In cells able to form caveolae (membrane domains structurally different from GPI microdomains [3]), antibody binding results in redistribution of GPI proteins into caveolae prior to endocytosis [18,28–30]. In cells unable to form caveolae, such as T-cell leukemic Jurkat cells or erythroleukemic K562 cells, antibody binding also results in the endocytosis of GPI proteins [15,16,31]. The rearrangements in membrane structure following GPI protein crosslinking are presumably required for crosslinking-mediated endocytosis to occur [2]. Based on this assumption, we investigated whether GPI-anchored proteins themselves are required for endocytosis triggered by membrane crosslinking.

Based upon investigations into the pathogenesis of paroxysomal nocturnal hemoglobinuria, K562 cell lines have become available that are defective in the synthesis of GPI [23]. This defect results in the lack of all GPI proteins on the surface of those cells. The specific absence of GPI-anchored proteins on the GPI(−) K562 cells was confirmed in this study by flow cytometry examination after anti-CD59, anti-CD55 and anti-CD71 staining. The use of sulfo-NHS–biotin permitted covalent attachment of biotin primarily to epsilon amine groups of a lysine residue in all proteins available on the cell surface [32]. Extremely high affinity ($K_a = 10^{15}$ M^{-1}) and tetravalency of avidin for biotin provided very efficient crosslinking of proteins on the whole cell surface leading to random bridging of most surface proteins. The clustering of biotinylated proteins on the cell surface and the entry of avidin–FITC in both GPI(+) and GPI(−) cells began within 1 h and resulted in complete clearance of the avidin within 24 h. Hence, efficient endocytosis of fluorescent avidin into biotinylated cells occurred even in the absence of GPI proteins on the cell membrane.

In addition to the endocytosis of avidin–FITC bioconjugates, we studied transfection of PEI–avidin–DNA bioconjugates into cells lacking GPI proteins as a means of determining the role of GPI proteins for endocytosis-mediated transfection. After internalization of surface PEI–avidin–DNA complexes into endosomes, further transfection requirements include DNA escape into the cytoplasm promoted by PEI-driven osmotic swelling and disruption of endosomes, entry of DNA into the nucleus, transcription, polyadenylation and proper translation of the encoded gene [33–35]. We wanted to experimentally determine whether GPI proteins additionally affect endosomes trafficking, disruption and release of DNA into the cytoplasm. The avidin–PEI bioconjugates used in this study transfected K562 cells with efficiencies comparable to PEI targeted to transferrin receptor or adenoviral transduction of these cells [36,37]. Surprisingly, we found slightly higher transfection efficiencies among the GPI(−) cell populations. In addition, a relatively high percentage of GPI(−) cells demonstrated extremely high transgene expression. These results suggest that GPI proteins are not required for the entry, trafficking or endosomal escape of transfected bioconjugates. However, these data have not ruled out the possibilities that

slower growth of GPI(−) cells or differences in the expression levels of other surface molecules like CD71 may influence transfection efficiencies in those cells.

Our original hypothesis was that GPI proteins may be required for microdomain crosslinking and subsequent endocytosis of biotin covalently attached to cell surface proteins. This hypothesis was based on the suggestion that GPI protein clustering introduces rearrangements in GPI microdomains that trigger crosslinking-mediated endocytosis [2]. Therefore, one might expect an incomplete clearance of crosslinked surface molecules in the absence of GPI proteins. Such incomplete clearance occurs in the absence of crosslinking [22]. While slight differences between the pattern of avidin–FITC capping and entry into GPI(−) versus GPI(+) cells were observed, complete endocytosis and clearance of avidin from the cell surface occurred within 24 h in both cell types. Also, further steps of endocytosis including endosomes trafficking and release of plasmid DNA were not diminished by a lack of GPI proteins. Thus, endocytosis and intracellular trafficking may be generically associated with crosslinking and reorganization of membrane domains rather than dependent on the presence of specific molecules, like GPI proteins, located within those domains.

Acknowledgements

We thank J. Muthoni Njoroge for technical assistance with the flow cytometry aspects of this work.

References

[1] J.L. Miller, Release and extracellular transit of glycosylphosphatidylinositol proteins, J. Lab. Clin. Med. 131 (1998) 115–123.
[2] V. Horejsi, K. Drbal, M. Cebecauer, J. Cerny, T. Brdicka, P. Angelisova, H. Stockinger, GPI microdomains: a role in signalling via immunoreceptors, Immunol. Today 20 (1999) 356–361.
[3] N.M. Hooper, Detergent-insoluble glycosphingolipid/cholesterol-rich membrane domains, lipid rafts and caveolae (review), Mol. Membr. Biol. 16 (1999) 145–156.
[4] T. Cinek, V. Horejsi, The nature of large noncovalent complexes containing glycosyl-phosphatidylinositol-anchored membrane glycoproteins and protein tyrosine kinases, J. Immunol. 149 (1992) 2262–2270.
[5] J. Matko, M. Eddin, Energy transfer methods for detecting molecular clusters on cell surfaces, Methods Enzymol. 278 (1997) 444–462.
[6] T. Harder, P. Scheiffele, P. Verkade, K. Simons, Lipid domain structure of the plasma membrane revealed by patching of membrane components, J. Cell Biol. 141 (1998) 929–942.
[7] E.D. Sheets, G.M. Lee, R. Simson, K. Jacobson, Transient confinement of a glycosylphosphatidylinositol-anchored protein in the plasma membrane, Biochemistry 36 (1997) 12449–12458.
[8] T. Friedrichson, T.V. Kurzchalia, Microdomains of GPI-anchored proteins in living cells revealed by crosslinking, Nature 394 (1998) 802–805.
[9] N. Madore, K.L. Smith, C.H. Graham, A. Jen, K. Brady, S. Hall, R. Morris, Functionally different GPI proteins are organized in different domains on the neuronal surface, EMBO J. 18 (1999) 6917–6926.
[10] I. Stefanova, V. Horejsi, I.J. Ansotegui, W. Knapp, H. Stockinger, GPI-anchored cell-surface molecules complexed to protein tyrosine kinases, Science 254 (1991) 1016–1019.
[11] K.R. Solomon, C.E. Rudd, R.W. Finberg, The association between glycosylphosphatidylinositol-anchored proteins and heterotrimeric G protein α subunits in lymphocytes, Proc. Natl. Acad. Sci. USA 93 (1996) 6053–6058.
[12] P.E. Korty, C. Brando, E.M. Shevach, CD59 functions as a signal-transducing molecule for human T cell activation, J. Immunol. 146 (1991) 4092–4098.
[13] B.P. Morgan, C.W. vandenBerg, E.V. Davies, M.B. Hallett, V. Horejsi, Cross-linking of CD59 and of other glycosyl phosphatidylinositol-anchored molecules on neutrophils triggers cell activation via tyrosine kinase, Eur. J. Immunol. 23 (1993) 2841–2850.
[14] V. Horejsi, M. Cebecauer, J. Cerny, T. Brdicka, P. Angelisova, K. Drbal, Signal transduction in leucocytes via GPI-anchored proteins: an experimental artefact or an aspect of immunoreceptor function?, Immunol. Lett. 63 (1998) 63–73.
[15] M. Deckert, M. Ticchioni, A. Bernard, Endocytosis of GPI-anchored proteins in human lymphocytes: role of glycolipid-based domains, actin cytoskeleton, and protein kinases, J. Cell Biol. 133 (1996) 791–799.
[16] U. Wojda, J. Njoroge, J. Miller, Protection of cells from complement mediated attack: CD59 receptor clustering for the entry of macromolecules into hematopoietic cells, Immunol. Invest. 28 (1999) 127–135.
[17] K. Kasahara, Y. Sanai, Possible roles of glycosphingolipids in lipid rafts, Biophys. Chem. 82 (1999) 121–127.
[18] R.G. Parton, B. Joggerst, K. Simons, Regulated internalization of caveolae, J. Cell Biol. 127 (1994) 1199–1215.
[19] C.P. Leamon, P.S. Low, Delivery of macromolecules into living cells: a method that exploits folate receptor endocytosis, Proc. Natl. Acad. Sci. USA 88 (1991) 5572–5576.
[20] R.J. Lee, P.S. Low, Folate-mediated tumor cell targeting of

liposome-entrapped doxorubicin in vitro, Biochim. Biophys. Acta 1233 (1995) 134–144.
[21] U. Wojda, J.L. Miller, Targeted transfer of polyethylenimine-avidin-DNA bioconjugates to hematopoietic cells using biotinylated monoclonal antibodies, J. Pharmacol. Sci. 89 (2000) 674–681.
[22] U. Wojda, P. Goldsmith, J.L. Miller, Surface membrane biotinylation efficiently mediates the endocytosis of avidin bioconjugates into nucleated cells, Bioconjug. Chem. 10 (1999) 1044–1050.
[23] R.P. Mohney, J.J. Knez, L. Ravi, D. Sevlever, T.L. Rosenberry, S. Hirose, M.E. Medof, Glycoinositol phospholipid anchor-defective K562 mutants with biochemical lesions distinct from those in Thy-1-murine lymphoma mutants, J. Biol. Chem. 269 (1994) 6536–6542.
[24] A.N. Gubin, S. Koduru, J.M. Njoroge, R. Bhatnagar, J.L. Miller, Stable expression of green fluorescent protein after liposomal transfection of K562 cells without selective growth conditions, Biotechniques 27 (1999) 1162–1170.
[25] A.L. Schwartz, Receptor cell biology: receptor-mediated endocytosis, Pediatr. Res. 38 (1995) 835–843.
[26] P.C. Pauly, D.A. Harris, Copper stimulates endocytosis of the prion protein, J. Biol. Chem. 273 (1998) 33107–33110.
[27] B.P. Morgan, Physiology and pathophysiology of complement: progress and trends, Crit. Rev. Clin. Lab. Sci. 32 (1995) 265–298.
[28] S. Mayor, K.G. Rothberg, F.R. Maxfield, Sequestration of GPI-anchored proteins in caveolae triggered by cross-linking, Science 264 (1994) 1948–1951.
[29] T. Fujimoto, GPI-anchored proteins, glycosphingolipids, and sphingomyelin are sequestered to caveolae only after crosslinking, J. Histochem. Cytochem. 44 (1996) 929–941.
[30] F.R. Maxfield, S. Mayor, Cell surface dynamics of GPI-anchored proteins, Adv. Exp. Med. Biol. 419 (1997) 355–364.
[31] I. Parolini, M. Sargiacomo, F. Galbiati, G. Rizzo, F. Grignani, J.A. Engelman, T. Okamoto, T. Ikezu, P.E. Scherer, R. Mora, E. Rodriguez-Boulan, C. Peschle, M.P. Lisanti, Expression of caveolin-1 is required for the transport of caveolin-2 to the plasma membrane. Retention of caveolin-2 at the level of the Golgi complex, J. Biol. Chem. 274 (1999) 25718–25725.
[32] E.P. Diamandis, T.K. Christopoulus, The biotin-(strept)avidin system: principles and applications in biotechnology, Clin. Chem. 37 (1991) 625–636.
[33] Y. Kato, Y. Sugiyama, Targeted delivery of peptides, proteins, and genes by receptor-mediated endocytosis, Crit. Rev. Ther. Drug Carrier Syst. 14 (1997) 287–331.
[34] O. Boussif, F. Lezoualc'h, M.A. Zanta, M.D. Mergny, D. Scherman, B. Demeneix, J.P. Behr, A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine, Proc. Natl. Acad. Sci. USA 92 (1995) 7297–7301.
[35] W.T. Godbey, K.K. Wu, A.G. Mikos, Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery, Proc. Natl. Acad. Sci. USA 96 (1999) 5177–5181.
[36] R. Kircheis, A. Kichler, G. Wallner, M. Kursa, M. Ogris, T. Felzmann, M. Buchberger, E. Wagner, Coupling of cell-binding ligands to polyethylenimine for targeted gene delivery, Gene Ther. 4 (1997) 409–418.
[37] J.S. Smith, J.R. Keller, N.C. Lohrey, C.S. McCauslin, M. Ortiz, K. Cowan, S.E. Spence, Redirected infection of directly biotinylated recombinant adenovirus vectors through cell surface receptors and antigens, Proc. Natl. Acad. Sci. USA 96 (1999) 8855–8860.