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Shed gangliosides provide detergent-independent evidence for Type-3 glycosynapses

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Abstract

Membrane microdomains, or rafts, at the plasma membrane have been invoked to explain many cellular processes. Protein–protein interactions within such microdomains including, for example, the tetraspanin web are reported to provide a scaffold for signal transduction. However, the nature of such protein–protein interactions is not fully elucidated. Hakomori [S.I. Hakomori, The glycosynapse, Proc. Natl. Acad. Sci. USA 99 (2002) 225–232] has advanced the concept that glycosphingolipids, particularly gangliosides, provide the intermediary link between transmembrane receptors and signal transducers and has redefined membrane rafts as Type-1, -2 or -3 glycosynapses. Here, using simple immunofluorescent analysis of the ganglioside complexes naturally released from cellular microprocesses (termed "footprints") we show that the ganglioside can determine the nature of protein–protein associations. Specifically, we demonstrate that CD36 and the tetraspanin CD151, both of which interact with β1 integrins, associate together only in the presence of the gangliosides GD2/GD3. These results substantiate the glycosynapse hypothesis and suggest that the nature of the tetraspanin web may be determined by gangliosides.

Keywords: Ganglioside; Tetraspanin web; Glycosynapse; Footprints

Cell surface gangliosides are recognised as playing a fundamentally important role in numerous cell biological processes including growth, signal transduction, cell migration, and tumor metastasis. Instrumental in these regulatory functions are physical associations between gangliosides and a diverse array of protein families including growth factor receptors, Src-family tyrosine kinases, integrins, and tetraspanins [1,2]. Various members of the tetraspanin family themselves have been shown to form associations with β_1 integrins and, in association with gangliosides, to modify cell migration [2–4]. Different tetraspanin family members have a high degree of homology and can co-associate with each other and with signalling molecules [4,5] and these associations with partner proteins have led to the concept of the 'tetraspanin web', considered as a network of molecular interactions, whereby tetraspanins facilitate the lateral positioning of partner proteins [6,7].

Both gangliosides and the tetraspanin web are reported to locate to microdomains or rafts at the plasma membrane, and these have been shown to be distinct from the structural entities known as caveolae [8–10]. Lipid rafts have received much attention in recent years with numerous papers suggesting their critical role in signal transduction, cell adhesion and migration, synaptic transmission, apoptosis, protein sorting, and cytoskeletal organization. They are considered to be formed as the result of lateral lipid assemblies between glycosphingolipids, sphingolipids, and cholesterol where the attractive forces between the saturated hydrocarbon chains of the sphingolipids and cholesterol can form a liquid ordered (Lo) phase which, in cell membranes, forms a domain discrete from the disordered (Ld) unsaturated glycerophospholipids which surround them

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[11]. Such phases have been well established in biophysical analyses of model membrane systems; however, despite their apparent importance, unequivocal proof of their existence in plasma membranes has proved elusive [12]. All of the methods used to identify plasma membrane rafts are indirect, and the very existence of such microdomains has been challenged [12,13]. In particular, the commonly used method of detergent resistance at low temperatures (DRM) [14] has been strongly criticised, with the suggestion made that these cannot be assumed to resemble biological rafts in size, structure, composition or even existence [15]. Such problems are exacerbated in analyses of the tetraspanin web since the tetraspanin-associated proteins are generally identified after extraction in detergent, and the stringency of the detergent influences both the size and composition of the complex; in addition, the nature of the detergent used determines whether or not the tetraspanins are found within DRM [16]. Despite this, several groups have proposed that tetraspanin-enriched microdomains (TEMs) form specific microdomains that are physically and functionally distinct from other lipid rafts [7,16–19].

Rafts are not homogeneous [9], and because of the potential importance of such heterogeneity it is important that 'rafts' be categorized towards a greater understanding of their influence. Hakomori [20] has recently begun this process in a concept known as the glycosynapse, in which different kinds of microdomains are defined as Type-1, -2 or -3 glycosynapses depending upon their lipid/cholesterol and protein content. In the glycosynapse concept glycosyl epitopes within microdomains determine the nature of the microdomain. Type-3 glycosynapse contains transmembrane adhesion receptors complexed with tetraspanin and gangliosides, where one adhesion receptor typically is an integrin [20]. In support of this, his group has shown that one tetraspanin, CD9, is a proteolipid and that its association with $\alpha 3\beta 1$ integrin is enhanced by the ganglioside GM3 in different cell types [2,21]. The challenge remains to more precisely define the protein and lipid content of each type of glycosynapse, and how this might vary in different cell types.

In order to determine whether other gangliosides also could influence tetraspanin binding to partner proteins by methods not involving the use of detergent, we took advantage of a finding made by Cheresh and colleagues [22,23] and by our own group [24] during studies establishing a physical and functional association between GD2/GD3 gangliosides and $\alpha v\beta 3$ integrin on mouse and human melanoma cells. Multiple assays were utilized to establish the association, but one very simple assay is pertinent to the present study. Cheresh et al. [22,23] noticed that during cell attachment the gangliosides were preferentially distributed into substrate-associated microprocesses emanating from the plasma membrane. Upon detachment of the cells by calcium chelation, the gangliosides from these processes were left behind as deposits that could readily be visualized by immunofluorescent microscopy. Gangliosides are known to be shed, often as vesicles, and such shed gangliosides are extremely important to tumor cell migration and invasion [25,26]. However, the microprocess-derived deposits—which we term "footprints"—were not indiscriminate membrane fragment since, although they contained gangliosides-associated integrins, they did not contain the abundant membrane proteins, melanoma-associated proteoglycan nor class I histocompatibility antigens [22,23]. In the present report, we present further characterization of these ganglioside-containing footprints and present evidence to suggest that the GD2/GD3 gangliosides can influence the binding partners of a tetraspanin, CD151, thereby supporting the concept of the glycosynapse.

Materials and methods

Antibodies. The preparation and specificity of the Mel 3 antibody has been described elsewhere [27,28]. MAb VM58 and rabbit polyclonal antibody (pAb) to CD36 were gifts from Dr. M.C. Berndt, (Monash University, Australia); mAb 2A7 directed against focal adhesion kinase (FAK) was a gift from Dr. J.T. Parsons (University of Virginia, VA), and the anti-FAK pAb330 and 331 from Dr. S.K. Hanks (Vanderbilt University, TN). Anti-CD151, mAb (11B1.G4) [29] was a kind gift from Dr. L.K. Ashman (The University of Newcastle, Australia). The anti-caveolin pAb was purchased from Santa Cruz (CA).

Cell transfection and flow cytometric analysis. The conditions used for the transfection and flow cytometric analysis of COS-7 and MV3 melanoma cells were those described previously [30].

Immunoprecipitation and immunoblotting. For the co-precipitation analysis cells were first lysed in 1% Brij 97 (Sigma) buffer (150 mM NaCl, 5 mM $MgCl_2$, 25 mM Hepes, pH 7.5). All other conditions of precipitation, washing, transfer and blotting were as described previously [30].

Indirect immunofluorescence of cells and 'footprints'. Cells were seeded onto glass coverslips and allowed to adhere overnight before fixation (5% w/v formaldehyde, 2% w/v sucrose) and permeabilization (0.3% v/v Triton X-100 in PBS). Alternatively to prepare 'footprints', cells were removed from the coverslips by incubation with 0.2% EDTA in PBS (90 min at RT) and washed three times with PBS prior to fixation. Indirect immunofluorescence staining was performed as previously described [30] using Alexa-Fluor coupled secondary antibodies (Invitrogen, Australia). Images were collected with an AxioCam HRm digital camera fitted to an Axioplan 2 epifluorescence microscope (Zeiss, Australia).

Glucosylceramide synthase inhibition. Two glucosylceramide synthase inhibitors were used to block ganglioside synthesis: D-*threo*-PDMP (D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol-HCl; Matreya Inc., Pleasant Gap, PA), and PPPP (D-*threo*-1(3′4′-ethylenedioxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol; kindly provided by Dr. J.A. Shayman, University of Michigan Medical School, MI). These were used to inhibit ganglioside synthesis by C32 cells following the protocol described by Lee et al. [31], and each reagent was tested over a range of concentrations (0.1–100 μM for D-*threo* PDMP, 0.1–20 μM for PPPP).

GD2/GD3 ganglioside incorporation. CD36-transfected COS cells were harvested with trypsin, washed in serum-free medium and 2.5×10^5 cells were resuspended in 1.2 ml of the same medium containing the GD2 and GD3 gangliosides, both at 50 μg/ml, (Calbiochem, La Jolla, CA). The cells were kept in suspension with gentle agitation and the gangliosides were allowed to incorporate for 3 h at 37 °C. After washing with serum-free medium, cells were resuspended in DMEM containing 10% FBS and seeded onto glass coverslips overnight before analysis.

Results and discussion

For our initial analyses, we examined a possible association between the adhesion receptor, CD36, and the tetraspanin CD151. This was for a number of reasons. Both

molecules are known to be components of membrane rafts, and we have demonstrated previously that CD36 physically and functionally associates with $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins [30]. Because these particular integrins are known to also associate with several members of the tetraspanin family, we suggested the possibility that CD36 may form a ternary complex with integrin and tetraspanin [30]. Further, since the tetraspanin, CD151, is reported invariably to associate with α3β1 integrin in every tissue examined, and it has been proposed that other tetraspanin–integrin associations may be due to co-precipitation with CD151–integrin complexes [4], we specifically tested the possibility of an association between CD36 and CD151.

We first used immunoprecipitation and found that CD36 indeed associates with CD151 in C32 melanoma cells as shown in Fig. 1A, illustrating co-precipitation of CD36 and CD151 in both directions. With a view to determining the nature of the association with CD36 chimeric constructs, the same immunoprecipitation experiments were carried out with COS-7 cells transfected with CD36. But, precipitation of CD36 and CD151 did not show any associated CD151 (Fig. 1B), and the solubility of CD151 was poor in this detergent with much material remaining in the insoluble pellet (data not shown). This failure to co-precipitate was not attributable to low levels of endogenous CD151 in the COS cells since flow cytometry demonstrated equivalent levels of this antigen on the surface of COS and C32 melanoma cells (Fig. 1C). Therefore, it appears likely that CD36 and CD151 are differently distributed in C32 melanoma cells and COS cells, or possibly an intermediary required for their co-association is absent in the COS cells.

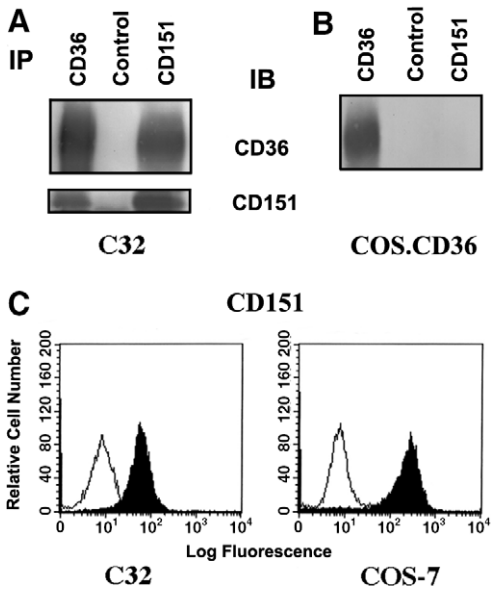

Fig. 1. CD36 and CD151 co-precipitate from C32 melanoma cells (A) but not from CD36-transfected COS cells (B). After immunoprecipitation (IP) from Brij 97 lysates, samples were immunoblotted (IB) under reducing conditions for CD36 (top) or under non-reducing conditions for CD151 (bottom). (C) Both C32 melanoma cells and COS cells display equivalent endogenous cell surface CD151 levels as shown by flow cytometry (open profile, control; black profile, mAb 11B1. G4 staining CD151).

Next, we turned to analysis of footprints using fluorescent staining. It has been recorded previously that GD2/GD3 gangliosides, together with integrins, are deposited as residual 'footprints' by melanoma cells, and these can be detected by staining after gently removing the cells by calcium chelation [22–24]. Since the Type-3 glycosynapse is defined by ganglioside, adhesion receptor—typically an integrin—and a tetraspanin [20], we were interested to determine whether these deposits contained the tetraspanin, CD151, and also its associated CD36 molecule, thereby identifying a novel Type-3 glycosynapse by simple, non-detergent-based analysis. Therefore, preparations of such 'footprints' from cells cultured on coverslips were co-stained for CD36 and gangliosides GD2/GD3, or CD36 and CD151 with a combination of rabbit polyclonal and monoclonal mouse antibodies. The merged profiles obtained from C32 melanoma cells showed striking identity between GD2/GD3 and CD36 (Fig. 2A), and also between CD36 and CD151 (Fig. 2B).

It should be noted that these ganglioside-containing deposits that we call footprints are quite different from the classical focal adhesion plaques that tightly bind spread cells to the subcellular matrix through a series of well-defined proteins connecting integrins to the actin cytoskeleton. Components of these adhesion plaques do remain attached to the matrix after removal of the cells; but, to achieve this, the cells must be physically sheared from the substrate [32]. The residue that remains associated with the integrin after cell shearing includes vinculin, α-actinin, and talin, together with actin itself [32]. We and others have shown previously that footprints contain integrins [24,33]; however, in the present study when we stained the residual footprint material from these cells for focal-adhesion kinase, vinculin, and actin, and all were found to be negative (data not shown). Also, whereas the constituents of focal adhesion plaques remain associated after lysis of the cells with octylglucoside [34], lipid-mediated associations generally are dissociated by this detergent [35]. Upon testing, we found that treatment of the footprints with octylglucoside before fixation and staining caused loss of the constituent components (data not shown). Therefore, the footprints are fundamentally different from classical focal adhesion plaques. In addition, we stained the footprints for caveolin, and although the C32 melanoma cells were rich in this structural component of caveolae, none was contained within the residual deposit (Fig. 2C). Thus, these footprints are not the result of general membrane shedding [22], nor caused by the release of caveolae, but they do appear to be consistent with the specific shedding of a Type-3 glycosynapse.

Hakomori and colleagues have suggested that the nature of the ganglioside determines the constituents of the proteins associated with specific glycosynapses [2,20]. We wished to test whether the nature of the protein constituents of the footprints from the C32 melanoma cells would be altered by suppressing GD2/GD3 ganglioside synthesis; however, attempts to analyse this were unsuccessful in

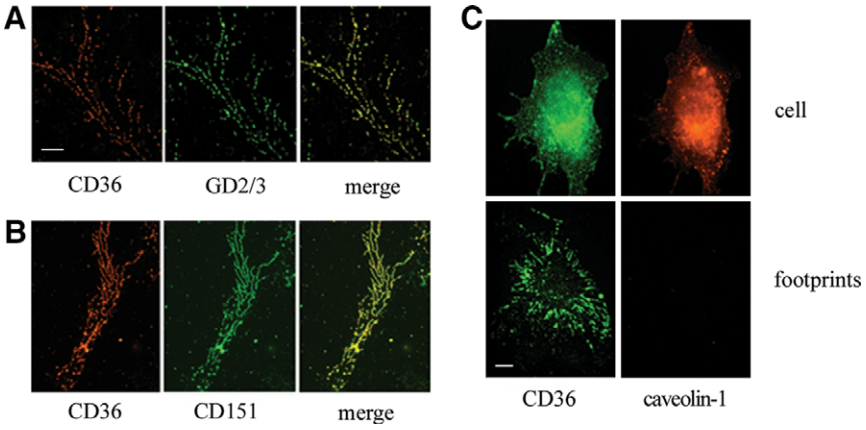

Fig. 2. Visualization of the constituents of the complex deposited in 'footprints' from C32 cells by immunofluorescent staining after detachment with EDTA (see Materials and methods). Bar = 10 μM.

these cells since the cells rounded up, detached, and began to die upon treatment with both of the inhibitors tested. This result is consistent with findings that in melanoma cells in particular, GD2/GD3 ganglioside appear to play a vital but undetermined role in cell attachment, growth and survival [36,37]. Therefore, we turned to COS-7 cells transfected with CD36. These cells are known to express different gangliosides from melanoma cells [38] and, accordingly, flow cytometric analysis demonstrated their relative lack of GD2/GD3 ganglioside expression (Fig. 3A). In keeping with this, no GD2/GD3 was detected in the footprints deposited by these cells (data not shown). Also, and consistent with the co-immunoprecipitation data (Fig. 1), footprints from these cells contained CD36 but not CD151 (Fig. 3B). This result was not the consequence of CD36 transfection since MV3 melanoma cells that express GD2/GD3 but not CD36 yielded footprints that were indistinguishable from C32 cells (GD2/GD3+ve, CD151+ve, CD36+ve) upon transfection with CD36 (data not shown). Therefore, we incorporated GD2/GD3 gangliosides into the CD36-expressing COS cells, confirmed incorporation by cell surface staining, and repeated the analysis. Now it was found that the footprints deposited by the transfected COS cells were almost indistinguishable from those of the melanoma cells, with co-staining for GD2/GD3 and CD36, and CD36 and CD151 (Fig. 3C).

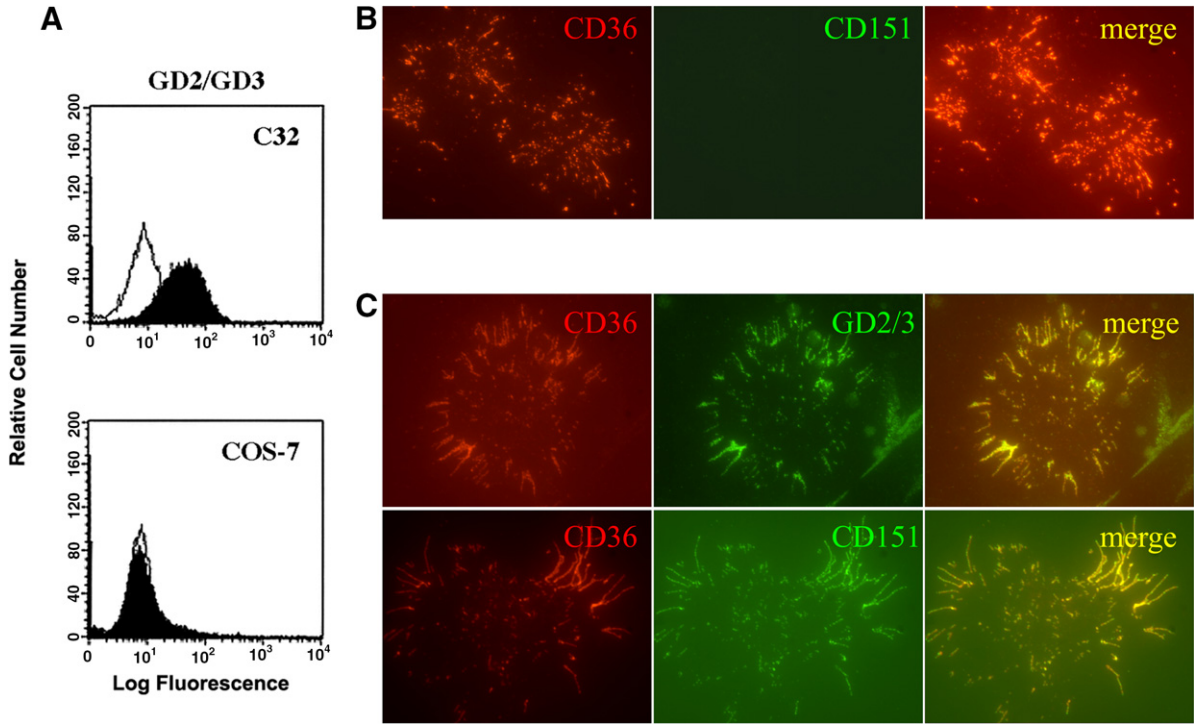

Fig. 3. (A) C32 melanoma cells but not COS-7 cells display endogenous cell surface GD2/GD3 gangliosides by flow cytometry (open profile, control; black profile, mAb Mel 3 staining GD2/GD3). Footprint analysis in COS-7 cells expressing CD36 before (B) and after (C) addition of exogenous gangliosides.

Together, these data support the notion suggested by Hakomori and colleagues that the nature of the ganglioside dictates the composition of the glycosynapse.

Cheresh and Klier [23] demonstrated that GD2 preferential redistributed into microprocesses that make contact with the substrate during melanoma cell attachment. Therefore the simplest interpretation for the footprints is that the tips of the microprocesses bud off after attachment has been initiated and the appropriate signals generated. This event could also occur in order to facilitate cell migration on the substrate, a suggestion in keeping with the influence of the Type-3 glycosynapse on cell motility [2]. Identification of the physical association shown here between CD36 and CD151 was not unexpected, indeed anticipated because of their mutual association with $\alpha3\beta1$ and $\alpha6\beta1$ integrins [4,30]. But it was unexpected to find that these two molecules no longer co-associated in the context of CD36-transfected COS cells, particularly since we have shown previously that CD36 associates with β_1 integrins and also localizes to GEM in these cells [30]. CD36 from the transfected COS cells was deposited onto the matrix, therefore it is likely that in these cells CD36 is associating with a different ganglioside not detected with the Mel 3 antibody which, in addition to GD2/GD3, binds GT_3 and GQ_{1b} but not GM1, GM2, GM3, GD_{1a}, GD_{1b} or GT_{1b} [28]. Notably, CD151 was not deposited onto the matrix, indicating that it is not contained within the same glycosynapse as CD36 in the COS cells. Recently, by the use of inhibition of glycosphingolipid synthesis, Berditchevski and colleagues [39] pointed to the importance of gangliosides in the maintenance of the TEM by mammary epithelial cells. We suggest that a different tetraspanin will be found in the CD36 positive glycosynapses from COS cells, thereby implicating the ganglioside, rather than the tetraspanin as directing the constituents of particular glycosynapses. In support of this, the introduction of GD2/GD3 gangliosides into the transfected COS cells caused a redistribution of the CD151 which was now deposited in the same 'footprints' as CD36 and GD2/GD3 ganglioside. Also, in platelets, in which the main ganglioside is GM3 [40], CD36 is found to associate with CD9 as well as integrins [41,42]. This interpretation also expands the range of glycosynapses that remain to be identified, and introduces a subtlety that is not evident from the biochemical study of 'rafts'.

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