Published in final edited form as:

Biochim Biophys Acta. 2008 March ; 1780(3): 486–496. doi:10.1016/j.bbagen.2008.01.010.

The role of membrane microdomains in transmembrane signaling through the epithelial glycoprotein Gp140/CDGP1

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Abstract

Cell adhesion to the extracellular matrix (ECM) via integrin adhesion receptors initiates signaling cascades leading to changes in cell behavior. While integrin clustering is necessary to initiate cell attachment to the matrix, additional membrane components are necessary to mediate the transmembrane signals and the cell adhesion response that alter downstream cell behavior. Many of these signaling components reside in glycosphingolipid-rich and cholesterol-rich membrane domains such as Tetraspanin Enriched Microdomains (TEMs)/Glycosynapse 3 and Detergent-Resistant Microdomains (DRMs), also known as lipid rafts. In the following article, we will review examples of how components in these membrane microdomains modulate integrin adhesion after initial attachment to the ECM. Additionally, we will present data on a novel adhesion-responsive transmembrane glycoprotein Gp140/CUB Domain Containing Protein 1, which clusters in epithelial cell-cell contacts. Gp140 can then be phosphorylated by Src Family Kinases at tyrosine 734 in response to outside-in signals- possibly through interactions involving the extracellular CUB domains. Data presented here suggests that outside-in signals through Gp140 in cell-cell contacts assemble membrane clusters that associate with membrane microdomains to recruit and activate SFKs. Active SFKs then mediate phosphorylation of Gp140, SFK and PKC δ with Gp140 acting as a transmembrane scaffold for these kinases. We propose that the clustering of Gp140 and signaling components in membrane microdomains in cell-cell contacts contributes to changes in cell behavior.

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Dedication: This article is dedicated to S.I. Hakomori in recognition of his sustained original thinking in science and his outstanding example to his students.

INTRODUCTION

The extracellular matrix (ECM) is composed of a meshwork of glycoproteins, proteoglycans and polysaccharides that provide a stable support for cell attachment. Early research contributions from the laboratory of S-I Hakomori increased our understanding of how the ECM promotes cell adhesion, and how it provides instruction to influence specific cell behavior. The Hakomori lab identified “galactoprotein a” as the ECM protein now referred to as fibronectin, and “galactoprotein b3” as the cell adhesion receptor, integrin alpha 3 subunit [1, 2]. Studies of these and other adhesion receptors and ECM ligands established that there is a “cell-dependent adhesion response” to the initial adhesive event leading to changes in downstream cell behaviors [3, 4]. Subsequently, research in the Hakomori lab has focused on glycolipids and proteolipids in membrane microdomains and their contribution to cell-dependent adhesion response (reviewed in [5]).

Upon integrin-mediated cell adhesion, membrane microdomains rich in glycosphingolipids and glycoproteins are recruited to the sites of receptor clustering to initiate signaling cascades that alter the cell's behavior [6]. In immune cells, components of microdomains are recruited to sites of antigen presentation in the immunological synapse-an adhesion and signaling site of immune cells- and affect cell signaling [7]. Further, it has been suggested that sphingolipids such as ceramide may be important in a T cell response.

Glycosphingolipids (GSLs) are not as well characterized and are frequently used as markers for membrane microdomains without regard to their function in these domains [5].

Hakomori's more recent work has focused on the role of GSL in carbohydrate-dependent cell-adhesion sites, termed glycosynapses, that, similar to an immunological synapse, recruit lipid raft components to transmembrane signaling sites [5]. As defined, glycosynapses are distinct from other glycosphingolipid and cholesterol rich domains known as Detergent Resistant Microdomains (DRMs), or lipid rafts. DRMs are biochemically characterized as membrane domains that are resistant to detergent extraction at four degrees Celsius.

A variety of lipid reporters have been used to demonstrate that membrane lipids reorganize in response to clustering of integrin receptors during adhesion [8-10]. For example, experiments with Laurdan D, a lipophilic dye that emits different wavelengths depending on how ordered the lipids are surrounding the dye, showed that sites of adhesion have a more ordered lipid content when compared to the rest of the cell [9]. This ordered membrane was dependent on cell-substrate adhesion; cell detachment disrupts this highly organized membrane structure and treatment with Methyl- β -cyclodextrin (M β CD), which also disrupts DRMs, promoted a more uniform order of lipids around the entire cell membrane [9]. In agreement with this data, GM1, a glycosphingolipid that contributes to higher lipid order in DRMs, reorganizes in response to adhesion and upon cell detachment or treatment with M β CD, GM1 is internalized [8]. Recently, a fluorescent analogue to the sphingolipid C8-lactoceramide was shown to aggregate in membrane patches of fibroblasts when incubated with a polyvalent, but not monovalent, antibody to the integrin β 1 subunit [10]. This suggests that integrin clustering in response to ECM ligands might be a crucial first step towards reorganizing membrane lipids.

In response to changes in adhesion, the reorganization of lipids can alter the localization of signaling components associated with these lipids. For example, caveolin, a cholesterol-binding protein commonly associated with DRMs and GM1, is internalized along with GM1 after cell detachment [11]. Moreover, phospho-caveolin has been shown to localize to focal adhesion sites [11], in agreement with the finding that adhesion sites have a higher lipid order than the cell membrane as a whole [9]. Cell adhesion can also control Rac, a GTP-binding protein that regulates actin structure, which translocates to the DRMs at the cell surface in response to adhesion. Both Rac and caveolin contribute to changes in cell migration and adhesion, with Rac promoting lamellipodia assembly and phospho-caveolin promoting integrin internalization [11]. A more detailed explanation of how Rac and caveolin modulate the cell adhesion response can be found in a recent review by del Pozo and Schwartz [12]. Additionally, adhesion to the ECM can transiently activate Src Family Kinases (SFKs), a third signaling component in DRMs. SFKs phosphorylate focal adhesion kinase and regulate disassembly of focal adhesions necessary for cell migration [13]. In cases where the constitutively active oncogenic form of Src is present, there is dysregulation of normal cell adhesion [14] resulting in assembly of invadopodia, structures that mediate cell invasion [15]. Other DRM components are required for the dynamic regulation of SFK that may be necessary for cell migration and avoiding invasion. For instance, active SFKs phosphorylate C-terminal src kinase (CSK) binding protein (Cbp), a protein that resides in DRMs. Phosphorylation of Cbp then recruits CSK which phosphorylates SFK at Y527 and results in SFK inactivation, and knockdowns of Cbp or CSK impaired cell spreading after adhesion [16]. These results establish that integrin-mediated adhesion regulates GM1 and DRMs at the cell membrane. Moreover, the reorganization of these components at the cell surface regulates SFK localization and activity, modifying the initial adhesion signal.

Another membrane microdomain that is involved in adhesion and cell behavior changes is the glycosynapse 3, also known as Tetraspanin Enriched Microdomain (TEM). Tetraspanins are small hydrophobic cell membrane proteins with four transmembrane domains and have been implicated in a variety of biological functions in a wide range of organisms-including the immunological synapse, sperm-egg fusion in mammals, early neuromuscular synapse formation in *Drosophila*, and fungal leaf penetration [17, 18]. Reports on tetraspanin involvement in integrin function in keratinocyte adhesion during wound repair prompted the designation of tetraspanins as “molecular facilitators” and “transmembrane linkers” [19]. This is because $\beta 1$ integrins can bind to the large extracellular loop of tetraspanins while cytoplasmic signaling components like PKC [20] and PI-4 Kinase [21] bind to the carboxy tail of various tetraspanin members. Consequently, $\beta 1$ integrins can signal through PKC and PI-4 kinase via a tetraspanin linker. Tetraspanins can also associate with each other to form the glycosynapse 3/TEM within the cell membrane. The glycosynapse 3 has some similarities to DRMs in that palmitoylation of proteins found in this domain is important for tetraspanin-tetraspanin and tetraspanin-integrin associations within the glycosynapse 3 [22]. However, reports suggest that the glycosynapse 3 is physically and functionally distinct from DRMs. One example of this distinction is during antigen presentation, where it was shown that a membrane microdomain enriched in tetraspanins have a distinct set of peptides associated with the MHC receptor, while MHC receptors in lipid rafts displayed a peptide profile that was more representative of the cell’s entire antigen presenting repertoire [23].

Various tetraspanin members contribute to changes in outside-in signaling through the initial adhesion generated by integrin binding to the ECM. CD9 is one tetraspanin that has been shown to associate with the laminin-binding integrins $\alpha 3\beta 1$ and $\alpha 6\beta 4$ [24], and can modulate the adhesion response generated through these subunits. For example, addition of an anti-CD9 antibody during $\alpha 6$ -mediated adhesion of HMEC-1 endothelial cells enhanced cell spreading while the antibody had no effect when adhesion was mediated through other integrin receptors [25]. Anti-CD9 antibody treatment enhanced cell spreading of MDA-MB-435 breast carcinoma cells in $\beta 4$ dependent signaling as well [22]. Further, when CD9 was transfected into a poorly motile human B cell cell line, motility was enhanced on substrates that utilized $\beta 1$ for motility [26]. CD9 siRNA also increased lymphocyte detachment in shear flow assays [27]. In all of these cases, initial adhesion to the substrate was similar to the respective controls and reinforces the idea that CD9 can help initiate signaling cascades to modify the initial attachment response.

While CD9 and other tetraspanin members like CD151 can associate with integrin α subunits to affect the adhesion response, association of these tetraspanins within the glycosynapse 3 also appears to have a consequence in modifying cell behavior. In the glycosynapse 3, tetraspanins associate with each other through their transmembrane domains and palmitoylation of tetraspanins is an important mediator of these interactions [22, 28]. When palmitoylation sites of CD151 are mutated, CD151 no longer interacts with other tetraspanin members, including CD9 [29] and CD81 [30]. In addition, expression of this mutation alters cell signaling in Rat-1 cells [30] and generates morphological changes in the B12 kidney epithelial cell line [29]. Thus, similar to detergent resistant membrane microdomains, the glycosynapse 3 has the ability to modulate outside-in signaling through integrins and promote changes in cell behavior and morphology.

We have found that these domains are also important for transmitting a signal through Gp140/CDCP1, a novel adhesion-responsive transmembrane glycoprotein. Gp140, also known as Cub Domain Containing Protein 1 (CDCP1) is phosphorylated at tyrosine 734 by Src Family Kinases in response to adhesion changes [31, 32]. This Src-dependent phosphorylation has been associated with adhesion changes in MDA-468 breast cancer cells [33] as well as anchorage independent growth of lung carcinoma cells [34]. Here we present evidence that further characterizes outside-in signaling through Gp140 that promotes this phosphorylation and show that phosphorylation at tyrosine 734 requires DRM components and possibly CD9. Thus, DRMs appear to be important for transmitting an outside-in signal through Gp140/CDCP1 during the process of adhesion-dependent transmembrane signaling.

MATERIALS AND METHODS

Antibodies and other reagents

Rabbit polyclonal antibodies against SFKpY416 (Cat #2101s), and mouse mAbs against Non-phospho SFKY416 (Cat # 2102, clone 7G9, IgG2a, Cell Signaling, Beverly, MA) were purchased from the designated suppliers and used according to manufacturers suggestions. Mouse mAb anti-human CD9 tetraspanin (P1D7) was prepared by Diane Frank in the Carter lab. Mouse mAb against the L6 tumor antigen [35] was a generous gift from Ingegard Hellstrom (Seattle Genetics, Seattle, WA). For cholesterol staining, filipin III was purchased

from Sigma (Cat # F9765) and used at 50 µg/ml in PBS with 10% FBS. GM1 was detected using a FITC conjugated cholera toxin B subunit from Sigma (Cat #1655) and used at 8µg/ml.

Cell culture, drug treatments and cell extraction

Human neonatal foreskin keratinocytes were isolated and grown in monolayer culture using defined media (KGM, Clonetics, San Diego, CA). All experiments were performed using keratinocytes between passages one and three. For phosphorylation studies, cells were a) treated with or without indicated antibody activators prior to b) extraction with 1% Triton X100 in PBS containing 1mM each PMSF, N-ethyl maleimide (NEM), sodium fluoride (NaF) and sodium orthovanadate (Na₃VO₄) for 30 minutes at 4°C.

Expression of recombinant Gp140 and preparation of mouse mAbs against extracellular Gp140

MAbs against the extracellular domain of Gp140 were prepared by expressing and immunizing mice with a recombinant form of the extracellular domain of human Gp140.

Preparation of recombinant extracellular Gp140—A full-length Gp140/CDCP1 cDNA was constructed as previously described [31] and cleaved with AflIII and HindIII restriction enzymes and the resulting ends were blunted with T4 DNA Polymerase to generate a ExoGp140 cDNA. The ExoGp140 cDNA (nucleotides 1-1989 starting from the initialing ATG, amino acids 1-663) was inserted into a blunt ended fusion expression cassette with murine IgG2a Fc in a pcDNA3.1 vector. The resulting open reading frame encoded a fusion protein consisting of N-terminal ExoGp140 and C-terminal murine IgG2aFc. ExoGp140 was expressed in NS0 mouse myeloma cells or 293T cells and secreted as a 140 kDa fusion protein under reducing conditions and as a dimer of 280 kDa under non-reducing conditions.

Preparation of Mouse anti-Gp140 mAbs—ExoGp140 was used as an immunogen in mice to prepare a mAb library. Candidate hybridoma culture supernatants were screened for reaction with recombinant ExoGp140 in ELISA assay followed by immunoprecipitation of Gp140 from cell extracts (P1C3, P2E2, P2E5, P3D8, P3D9, P5H10).

Preparation of rabbit polyclonal Abs against Gp140 and Gp140 phosphorylated at Y734

Affinity purified Abs against Gp140 phosphorylated on Y734 was prepared in rabbits by ProSci (Poway, CA): A Gp140 phosphopeptide (n-R⁷²⁶KDNDSHVpY⁷³⁴AVIEDTMVYGHLLQDS⁷⁵⁰-c) was synthesized and used to immunize rabbits. Rabbit sera positive for reaction with the Gp140pY734 phosphopeptide were pooled and affinity purified by passage over immobilized non-phosphoGp140Y734 peptide to remove antibodies against non-phosphoGp140 (Fraction A) then affinity purified on immobilized Gp140pY734 phosphopeptide (Fraction B). A rabbit polyclonal antibody was against a synthetic peptide representing the carboxy terminal amino acids of human Gp140/CDCP1 (SSKDTDIPLLNTQEPMEPAE) was prepared and provided by Dr. Hubert Kabacker, Intefaculatative Institute for Biochemistry, Universty of Tübingen, Tübingen, Germany.

Preparation and binding of antibody-beads to adherent cells

FluoroSphere sulfate microspheres (4.0 μm , blue fluorescent, Molecular Probes/Invitrogen, Eugene OR) were coated with purified antibodies and proteins following the basic protocol recommended by the manufacturer. The conjugated beads were washed, blocked with heat denatured BSA (5 mg/ml in PBS), and added in suspension to cells adhered on glass cover slips. Beads were centrifuged onto the apical surface of the adherent cells (1000 $\times$ g for 10 min) followed by incubation for 15 min and washing to remove unbound beads. Cells were incubated with the bound beads for times from 0 to 24 h, fixed with formaldehyde (2% in NaCacodylate/Sucrose, 10 min) and then permeabilized with detergent (0.1% Brij 99 in PBS containing 2 mM NEM, 1 mM PMSF and 1 mM NaVanadate; 5 min). Where indicated, cell and beads were extracted with 0.5% Triton X-100 detergent or 0.1% Brij 99 detergent prior to fixation to remove membrane components not bound directly or indirectly to the beads.

Immunoblotting

Western blotting was performed with specific primary antibodies after transfer of proteins to nitrocellulose and the blocking of non-specific binding sites with 0.5% heat-denatured BSA, 0.1% TX-100 in PBS. Primary antibodies were then incubated with species-specific Alexa Fluor 680- or IRDye 800-conjugated secondary Abs (Molecular Probes, Eugene, OR, and Rockland, Gilbertsville, PA, respectively) and visualized with an Odyssey near infrared imager (LiCor, Lincoln, NE).

Tissue sections and immunostaining

Four millimeter punch biopsies of 1 hour wounds in human skin was embedded in O.C.T. (FineTek, Sakura, Inc., Torrance, CA). Tissue sections (6 μm) were fixed in 2% paraformaldehyde for 10 minutes. L6 antibody was used at 1:1000 dilution. Vector Labs (Burlingame, CA) and biotinylated horse anti-mouse was used as secondary antibody. Images were viewed using a Nikon Microphot-SA microscope using differential interference contrast (DIC). Image acquisition was controlled through IP Lab Spectrum® software (Scanalytics, Fairfax, VA). Cells were fixed in 2% formaldehyde in 0.1M Na cacodylate, 0.1 M sucrose, pH 7.2 containing both vanadate and NEM to inhibit phosphatases then permeabilized with 0.1% Triton X-100 detergent and stained with Rhodamine conjugated secondary Ab. Images were collected with a Zeiss fluorescence microscope with a Photometric SenSys cooled CCD digital camera (Roper Industries, Trenton NJ) using MetaMorph software (Universal Imaging, Downtown, PA).

RESULTS AND DISCUSSION

Clustering and redistribution of membrane components is necessary for cell-dependent adhesion responses

Wounding of quiescent epidermis causes keratinocytes at the wound edge to transition from a quiescent state where they are stably anchored to the basement membrane via integrin $\alpha 6\beta 4$ in hemidesmosomes. Wound activation increases cell migration via $\beta 1$ integrins over exposed dermal ligands into the wound bed to promote wound closure and re-epithelialization [36]. Thus, wounding generates a signaling cascade that changes cell

behavior. Upon wounding, there is a dramatic alteration of membrane properties at the wound edge including the up-regulation of the tetraspanins CD9 and the transient expression of ATF3, regulated by the phosphorylation of p38 map kinase [6]. Additionally, we have identified L6, a tetraspanin-like protein as an example of a membrane protein that is up-regulated soon after wounding [6] (Fig. 1). Prior studies originally identified the up-regulation of L6 in a variety of human cancers [35]. The data in Fig. 1 indicate that changes in membrane organization and composition play a critical role in the wound response.

Signaling through Gp140/CDCP1 requires components of DRMs and TEMs to transduce outside-in signals

Gp140 is a novel transmembrane receptor that localizes in cell-cell contacts of quiescent epithelial cells (Fig. 2) [31], and can regulate SFKs and PKC δ (Zaitsevkaia et al, manuscript in preparation) that are necessary for cell adhesion responses [13, 37]. Fig. 2 (left panel) identifies Gp140 in cell-cell contacts in undifferentiated basal cells at the basement membrane zone (arrow) and in cell-cell contacts of more differentiated suprabasal and granular cell layers. At higher magnification (Fig. 2 right panel), Gp140 is absent from the basement membrane zone (arrow) but is present in lateral and apical cell-cell contacts. Gp140 was first identified as a membrane receptor in keratinocytes that is phosphorylated by Src Family Kinases and cleaved by proteases in response to changes in cell adhesion in wounds [31, 32]. It was also identified as CDCP1, an mRNA transcript that is up-regulated in colon and lung cancer [38], as well as skeletal muscle, colon, and various human tumor lines [39, 40]. Current observations suggest that CDCP1 expression negatively correlates with the amount of methylation found at the promoter region [41], but only weakly correlates with the metastatic potential of cell lines [39]. Further, Gp140 is regulated at the protein level through SFK-dependent phosphorylation (Zaitsevkaia et al, manuscript in preparation).

Gp140 is a single pass transmembrane glycoprotein that contains two or three CUB domains in the extracellular domain (Fig. 3). CUB domains are found in a variety of proteins in many organisms and function in protein-protein interactions related to adhesion, receptor/protein multimerization in developmental processes, and the complement cascade in innate immunity [42]. The CUB domains of Gp140 exhibit high similarity to Mannose-Binding Lectin (MBL) Associated Serine Protease 1 (MASP 1), where the N terminal CUB domain has been implicated in stabilizing the interaction of MASP1 dimers [43]. On the cytoplasmic tail of Gp140 there are 5 potential tyrosine phosphorylation sites (Fig. 3) and Y734 is phosphorylated by SFKs in response to adhesion changes [31, 33, 44].

In quiescent epidermis, Gp140 localizes in cell-cell contacts but is absent from the basement membrane zone that is rich in integrin-dependent adhesion sites (Fig. 2). The localization of Gp140 in cell-cell contacts of epidermis and the known role of CUB domains in protein-protein interaction suggests that homotypic interactions of the extracellular Gp140 CUB domains in cis and/or trans may activate SFKs in cell-cell contacts. To better understand the possible role of Gp140 in activating SFK in cell-cell contacts, we prepared mAbs against the extracellular CUB domains that could mimic possible interactions in cell-cell contacts of keratinocytes to regulate phosphorylation. For simplicity, these anti-Gp140 mAbs with

ability to bind Gp140 and activate outside-in phosphorylation of Gp140 at Y734 are referred to ActGp140 mAbs. A second class of anti-Gp140 mAbs that bind Gp140 but do not activate phosphorylation are referred to as Non-Activating or NActGp140 mAb. NActGp140 mAbs will be discussed later. Ligation of extracellular Gp140 in cell-cell contacts with ActGp140 mAbs assembles a detergent-resistant “Gp140 membrane cluster” (Fig. 4) and co-aligns with activated SFKs that autophosphorylate at Y416 in the Triton-resistant Gp140 membrane clusters (Fig. 4). Significantly, the ActGp140 mAbs also increased SFK-dependent phosphorylation of Gp140Y734 and this phosphorylation required a parallel signal from substrate adhesion (Fig. 5). Ligation of Gp140 with anti-Gp140 mAb increases phosphorylation of Gp140 in keratinocytes adhered to endogenous laminin 5 and collagen (Fig. 5). However, ligation of suspended cells fails to induce phosphorylation unless the cells are re-adhered to the substratum. This suggests that cell attachment is necessary to phosphorylate Gp140 at Y734 in response to binding of ActGp140 mAbs. Integrin adhesion was sufficient but not necessary since adhesion to poly Lysine was also able to phosphorylate Gp140 when combined with ligation of the ActGp140 mAbs (Fig. 5). In results presented elsewhere, we find evidence that upon induction of Gp140 phosphorylation, Gp140 can be found in higher molecular weight clusters in the presence of a cross-linking agent not seen in un-induced controls (Zaitsevskaja et al, manuscript in preparation). Clustering of receptors may be a driving force for the assembly of DRMs because receptor clustering can increase the affinity or partitioning coefficient for raft domains [45]. Put together, this data indicates that clustering of Gp140 through the extracellular CUB domains may be an important step required to recruit SFK and other DRM components resulting in phosphorylation of cytoplasmic Gp140Y734. Further, phosphorylation of Gp140 by SFKs requires a dual signal from cell adhesion and ligation of extracellular Gp140.

Evidence that DRM and TEM components are required for phosphorylation of Gp140Y734

In theory, cell-cell or cell-substrate adhesion may be necessary to assemble DRMs or prevent their internalization- allowing for ActGp140 mAb-induced assembly of Gp140 membrane clusters that leads to recruitment and activation of SFKs. Since SFKs reside in- or are recruited to- membrane microdomains that are rich in cholesterol and glycosphingolipids, and because cell attachment is necessary for the phosphorylation of Gp140, we examined whether other components of DRMs can also localize with Gp140. Consistent with the idea that Gp140 requires DRM components for tyrosine 734 phosphorylation, removal of cholesterol with M β CD functions like cell detachment and prevents ActGp140 mAb-induced phosphorylation of Gp140 similar to detachment (Fig. 6). For these experiments, ActGp140 mAb was coated on microspheres that bound to Gp140 on the cell surface and induced phosphorylation of Gp140Y734 only at the point of bead contact with the cell surface (Fig. 6a, arrow). However, treatment of cells with M β CD to remove membrane cholesterol prevented phosphorylation of Gp140 (Fig. 6b), suggesting that membrane cholesterol was required for the Gp140-dependent transmembrane phosphorylation. We also saw an increase in cholesterol resistance to detergents upon antibody ligation as seen with filipin, a compound from *Streptomyces filipinensis* that binds to membrane cholesterol [46] (Fig. 7). Lastly, we found that cells treated with an anti-Gp140 mAb prior to M β CD treatment prevented the internalization of GM1 ganglioside from the

cell surface (Fig. 8). Binding of cholera toxin to cell surface GM1 increases internalization of GM1. However, if cells are first treated with Non-Activating anti-Gp140 mAb (NActGp140) ligation of Gp140 prevented internalization of GM1 induced by Cholera toxin. This suggests that ligation of extracellular Gp140 with NActGp140 mAb can alter the normal regulation or organization of GM1 via an uncharacterized mechanism. Taken together, these observations suggest that signaling through Gp140 in cell-cell contacts assembles Gp140 membrane clusters that recruit components of DRMs, including GM1 and cholesterol, and leads to the phosphorylation of Gp140 at Y734.

Studies are underway to identify the domains within Gp140 that transmit the outside-in signal that results in the phosphorylation of tyrosine 734. The CUB domain of ADAMTS13 was shown to be important for the constitutive localization of ADAMTS13 to lipid rafts [47]. However, biochemical properties of Gp140 suggest that Gp140 does not always reside in DRMs and may, instead, only transiently associate with these domains upon outside-in signaling or cross-linking (Fig. 4). Protein sequence analysis shows that Gp140 contains a putative palmitoylation site [39] (Fig. 3) with C689 being evolutionary conserved. Palmitate is added by a thioester bond to cysteine residues that are proximal to the transmembrane region of proteins and is the only fatty acid protein modification that can be regulated for proteins found in lipid rafts [48]. Thus the palmitoylation state of Gp140 may contribute to its phosphorylation by regulating when Gp140 associates with DRMs that contain SFKs.

Preliminary data indicates that Gp140 may also associate with component of the glycosynapse 3/TEMs. In a proteomic characterization of the tetraspanin web using two different colon cancer cell lines, the glycosynapse 3/TEMs were isolated by immunoaffinity purification with anti-CD9 Abs. Mass spectrometry analysis of the resulting peptide mixture identified CDCP1 as a possible protein found in the glycosynapse 3 [49]. Observations from our lab strengthen this finding as CD9 staining overlaps with Gp140 in the Gp140 membrane clusters. Further, during prolonged and localized Y734 phosphorylation of Gp140 at the cell surface using ActGp140 mAb coated beads, CD9 can be found around the periphery of this phosphorylation (Fig. 9) and overlaps with Gp140 that has not been phosphorylated (data not shown). This finding suggests that the glycosynapse 3 may be important to either prevent phosphorylation of Gp140 or promote dephosphorylation of Gp140. Interestingly, the tetraspanin member CD82 can suppress cell migration of a metastatic prostate cancer cell line as well as integrin-mediated activation of Src [50]. Moreover, in rat immune cells a phosphatase activity was associated with the tetraspanins CD53 and CD63 immune complexes that was not intrinsic to the tetraspanins themselves [51]. These observations may help explain why CD9 is associated with a non-phosphorylated form of Gp140. Alternatively, CD9 may associate with Gp140 and prevent assembly of the Gp140 membrane clusters in cell-cell contacts. In oocytes that lacked CD9, ligation of integrin $\alpha 6 \beta 1$ yielded bigger clusters than observed in wild-type oocytes. Moreover, ligation of CD9 in wild-type oocytes prevented clustering of integrin $\alpha 6 \beta 1$ and led to alterations in gamete fusion [52]. Similar to these observations, it is possible that CD9 could also be preventing clustering of Gp140 and phosphorylation at tyrosine 734.

While the above evidence indicates that Gp140 may be associating with TEMs and DRMs, the mechanism for how Gp140 interacts with these domains is unclear. Outlined in Fig. 10 is

a working model for assembly of Gp140 membrane clusters leading to the recruitment of DRM components such as SFKs which phosphorylates Gp140 and allows for the recruitment of PKC δ to the complex. Benes et al [44] have shown that phosphorylation at Y734 generates a binding site for SFK. SFK binding is followed by phosphorylation of Y762 to generate a novel docking site on Gp140 for the C2 domain of PKC δ . As a result, a complex is formed between SFK, Gp140 and PKC δ .

Conceivably, Gp140 may act as a transmembrane scaffold to regulate cross-talk between PKC δ and SFK during the adhesion process. Both PKC δ and SFK activity have been implicated in signaling cascades that modify cell-substrate adhesion via integrins [13, 16, 37]. In keratinocytes, activated PKC δ is important to phosphorylate the integrin α 6 and β 4 subunits, and PKC δ - but not PKC α - over-expression in primary keratinocytes reduced the ability of those keratinocytes to adhere to laminin 5 in an adhesion assay [37]. PKC δ is also required for deposition of laminin 5 [53] by leading keratinocytes and deposition is required for both migration and wound repair. As discussed in an earlier section, SFKs are required for turnover of integrin-dependent focal adhesions and null defects in SFKs impair cell migration but not adhesion or assembly of β 1 integrins in focal adhesions [13]. Consistently, Cbp can serve as a sensor of SFK activity, and inhibition of SFK by Cbp and CSK is essential for dynamic regulation of adhesion to mediate spreading and migration [16]. These results suggest that signaling through Gp140 may contribute to SFK and PKC δ adhesion-dependent processes. Interestingly, when CDCP1 is over-expressed in MDA-468 breast cancer cells, they become rounded, but continue to grow in a loosely adherent suspension [33], indicating that Gp140 can promote changes in cell adhesion.

Acknowledgements

SMA would like to thank C.L. Walcher for input and critical review of manuscript. SMA was funded by Public Health Service, National Research Service Award, T32 GM07270, from the National Institute of General Medical Sciences, NIH This work was supported by: National Institutes of Health Grants R01 CA49259 (WGC), R01 AR047963 (WGC/Paul D. Lampe), U54 CA126540 (Stephen R. Plymate/WGC) and DK59221 (John E. Olerud/WGC).

Abbreviations

CDCP1	CUB Domain Containing Protein 1
ECM	extracellular matrix
MβCD	methyl- β -cyclodextrin
TEM	Tetraspanin Enriched Microdomain
DRM	detergent resistant microdomain

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Fig. 1. L6 is up-regulated in leading keratinocytes at the edge of human epidermal wounds

Cryostat sections were prepared from wounds 0 (a), 4 (b), 8 (c) and 24 hours (d) post wounding. The wounds were then stained with an anti-L6 antibody. Black arrows point to the leading keratinocytes at the wound edge of the epidermis. As previously reported, L6 constitutively stains small vasculature in the dermis. Perinuclear staining in epidermal basal cells distal from the wound edge is background melanin staining. Wound margins are in the lower right of all panels and identified by the grey arrow. Bar, 20 μ m.

Fig. 2. Gp140 localizes in cell-cell contacts of quiescent epidermis and is absent from cell-substrate contacts with the basement membrane
(Left panel) Immunoperoxidase staining of normal epidermis with anti-Gp140 mAb shown at low magnification. Arrows indicate basement membrane zone. (Right panel) High magnification immunofluorescence staining of the basement membrane zone showing Gp140 (red) and nuclei (blue with DAPI). Gp140 is absent from the basement membrane zone but present in lateral and apical cell-cell contacts.

Fig. 3. CUB domains, tyrosine (Y) phosphorylation sites and palmitoylation sites of Gp140/CDCP1

The amino acid sequence of Gp140/CDCP1 was plotted using the TOPO2 display page (<http://www.sacs.ucsf.edu/TOPO-run/wtopo.pl>) to generate a schematic of the protein with relevant protein motifs. Gp140/CDCP1 contains 2 or 3 CUB domains on the extracellular side (sequence depicted as orange or yellow). On the cytoplasmic side, Gp140 contains 5 potential tyrosine phosphorylation sites (highlighted as red or purple)- including Y734 and Y762 (shown in purple) that have been shown to be phosphorylated by SFK. In addition a putative palmitoylation site for Gp140, C689/690, is shown in green.

Fig. 4. Treatment of keratinocytes with an ActGp140 mAb increases autophosphorylation of SFK at Y416 that co-localizes with Triton-resistant Gp140 membrane clusters

Incubation of human keratinocytes with ActGp140 mAb for 30 minutes increases the fraction of both Gp140 and SFKpY416 that is resistant to extraction with 0.5% Triton-X100. Cells were incubated without (a,b) and with (c,d) ActGp140 mAb (P1C3) for 30 minutes, after which, they were extracted on ice with cold Triton -X100 detergent, fixed and immunostained for Gp140 (a,c) and SFKpY416 (b,d).

Fig. 5. Ligation of Gp140 in cell-cell contacts with ActGp140 mAb increases phosphorylation of Gp140Y734 in adherent but not suspended keratinocytes

Keratinocytes adhered to deposits of laminin 5 (ECM) were suspended with EDTA then either kept in suspension or re-adhered to poly-lysine or collagen. The adherent, suspended and re-adherent cells were treated with (+) and without (-) ActGp140 mAb (P1C3) as indicated. Extracts of the cells were immunoblotted with anti-Gp140 Ab (total) or anti-Gp140pY734 mAb as indicated. The status (Adherent and Spread) is indicated.

Fig. 6. MβCD inhibits phosphorylation of Gp140 tyrosine 734

Beads (4 μ M) coated with ActGp140 mAb (P3D9) were adhered to the apical surface of keratinocytes that had either been [a] untreated or [b and c, same field] pretreated with 10mM M β CD for thirty minutes. After a 24 hour incubation with the beads, cells were extracted with Brij99 detergent (0.1%, 5 min) to remove cell components not bound by ActGp140-beads. Cells were fixed (2.0% formaldehyde for 10 min), stained with rabbit antibody against Gp140pY734, and visualized by rhodamine anti Rabbit secondary. Arrow in [a] indicates Gp140pY734 at sites of bead (circle) contact. [b] and [c] M β CD prevents

phosphorylation of Gp140 at sites of bead contact. [c] High exposure of [b] identifies beads in the field. White bar, 10µm.

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Fig. 7. ActGp140 mAb ligation of Gp140 alters cholesterol organization detected by staining with filipin

Keratinocytes were treated without [a] or with [b] activating Gp140 mAb for forty minutes. Cells were then fixed (2.0% formaldehyde for 10 min) and permeabilized (0.5% Triton-X100, 5 min). Filipin (50 μ g/ml in PBS-10% FBS) was added to cells for two hours at room temperature to detect changes in cholesterol distribution generated by binding of ActGp140 mAB. Filipin bound to cholesterol was viewed with a 340-380 nm excitation filter and a 400/430 nm reflector/barrier filters. White bar, 50 μ m

Fig. 8. Pretreatment with a Non-activating anti-Gp140 mAb antibody prevents M β CD-induced GM1 internalization

Adherent keratinocytes labeled with FITC-conjugated Cholera toxin B (Sigma) were treated [a] without or [b] with NActGp140 mAb (P5H10). After 20 minutes, cells were treated with 10mM M β CD for thirty minutes and then fixed (2.0% formaldehyde for 10 min). GM1 was detected by FITC conjugated Cholera toxin B. White bar, 20 μ m

Fig. 9. Ligation of extracellular Gp140 assembles Gp140 membrane clusters, phosphorylates Gp140Y734 and recruits CD9

Beads (4 μ M) coated with ActGp140 mAb (P3D9) were adhered to the apical surface of keratinocytes, then extracted with Brij99 detergent (0.1%, 5 min) to remove cell components not bound by ActGp140-beads. Cells were fixed (2.0% formaldehyde for 10 min) and stained with [a] anti-CD9 mAb (green, P1D7) and [b] anti-Gp140 phosphorylated at Y734 (red). Panel [c] is an overlay of the two images. Antibodies were visualized by rhodamine conjugated anti-Rb (pY734 Gp140) and 588-conjugated anti-mouse IgG2a Mouse (CD9). White Bar, 5 μ m.

Fig. 10. A Working Model for Gp140 signaling: Ligation of extracellular Gp140 assembles detergent-resistant Gp140 membrane clusters, recruits components of lipid rafts, and increases phosphorylation of SFKs, PKCδ and Gp140 in the cytoplasm

Outside-in signaling (pink zigzag) through Gp140 CUB domains in adherent cells (shown as orange and yellow circles), assembles Gp140 clusters that recruits or translocates to DRMs (yellow line). In DRMs, Gp140 associates with SFK where Gp140 is phosphorylated at tyrosine 734 and 762. As a result, PKC delta can associate with Gp140. Gp140 both contributes to and responds to adhesion changes through an uncharacterized signaling cascade.