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Galectin-1 signaling in leukocytes requires expression of complex-type *N*-glycans

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Dimeric galectin-1 (dGal-1) is a homodimeric lectin with multiple proposed functions. Although dGal-1 binds to diverse glycans, it is unclear whether dGal-1 preferentially binds to specific subsets of glycans on cell surfaces to transmit signals. To explore this question, we selectively inhibited major glycan biosynthetic pathways in human HL60, Molt-4, and Jurkat cells. Inhibition of *N*-glycan processing blocked surface binding of dGal-1 and prevented dGal-1-induced Ca^{2+} mobilization and phosphatidylserine exposure. By contrast, inhibition of *O*-glycan or glycosphingolipid biosynthesis did not affect dGal-1 binding or dGal-1-induced Ca^{2+} mobilization and phosphatidylserine exposure. These results demonstrate that dGal-1 preferentially binds to and signals through glycoproteins containing complex-type *N*-glycans in at least some leukocyte subsets.

Keywords: galectin/inflammation/leukocytes/*N*-glycans/signaling

Introduction

Galectins constitute a family of soluble β-galactoside-binding lectins that are expressed by all metazoans (Barondes, Castronovo, et al. 1994; Barondes, Cooper, et al. 1994; Cooper and Barondes 1999; Leffler et al. 2004). Dimeric galectin-1 (dGal-1) is a homodimer consisting of two ∼14.5 kDa subunits that are noncovalently associated (Cho and Cummings 1995, 1996; Giudicelli et al. 1997). dGal-1 functions in cell adhesion (Moiseeva et al. 2003; He and Baum 2004; Martinez et al. 2004), development (Colnot et al. 1996), inflammation (Rubinstein et al. 2004), leukocyte apoptosis (Perillo et al. 1998; Rubinstein et al. 2004), neutrophil turnover (Dias-Baruffi et al. 2003; Stowell et al. 2007; Stowell, Qian, et al. 2008), cancer (Rubinstein et al. 2004; van den Brule et al. 2004), and immunity (Rabinovich et al. 2007; Toscano et al. 2007; Salatino et al. 2008). Like other galectins, dGal-1 weakly recognizes lactose (Galβ1-4Glc) and *N*-acetyllactosamine disaccharides such as the type 2 sequence Galβ1-4GlcNAcβ-R (LN) and the type 1 sequence Galβ1-3GlcNAcβ-R (Leffler and Barondes, 1986). However, dGal-1 binds with higher affinity to long-chain type 2 sequences $(\text{-3Gal}\beta1\text{-4GlcNAc}\beta1\text{-})_n$ called poly-*N*-acetyllactosamines (PLs) (Stowell et al. 2004; Stowell, Arthur, et al. 2008; Leppanen et al. 2005).

dGal-1 reportedly binds to both *N*- and *O*-glycans on various cell surface glycoproteins as well as to glycosphingolipids and the ganglioside GM1 (Ohannesian et al. 1995; Perillo et al. 1995; Kopitz et al. 1998; Pace et al. 1999; Nguyen et al. 2001; Gauthier et al. 2002; Carlow et al. 2003; Lanteri et al. 2003; Andre et al. 2005; Elola et al. 2005; Ideo et al. 2005; Suzuki et al. 2005b; Walzel et al. 2006). These apparently conflicting results raise questions as to the nature of the glycans required for dGal-1 signaling in cells.

dGal-1 induces Ca^{2+} mobilization in and phosphatidylserine (PS) exposure on activated human neutrophils and promyelocytic HL60 cells; however, these responses are not accompanied by apoptosis (Dias-Baruffi et al. 2003; Stowell et al. 2007; Stowell, Qian, et al. 2008). The galectin signaling to expose surface PS in the absence of cell death has been termed preaparesis (Stowell, Qian, et al. 2008). These results prompted us to further explore the glycans required for dGal-1-induced preaparesis and Ca^{2+} mobilization in leukocytes. We used human HL60 cells, leukemic MOLT-4 T cells, and leukemic Jurkat T cells, which have been extensively used as models for dGal-1 signaling. Cell lines are particularly useful because they permit the use of selective inhibitors of glycosylation to remodel the cellular glycome. We examined the effects of defined, commonly used inhibitors of *N*- and *O*-glycan and glycosphingolipid biosynthesis and/or elongation on dGal-1 binding and on the ability of dGal-1 to induce Ca^{2+} mobilization and preaparesis.

Results

dGal-1 binds to complex-type N-glycans on HL60 cells

dGal-1 binds to and signals through glycans containing PL sequences on HL60 cells (Leppanen et al. 2005; Stowell, Qian, et al. 2008). Such sequences might be expressed on *N*- or *O*-glycans or on glycosphingolipids. To explore the types of PL-containing glycans required for dGal-1 signaling, we treated HL60 cells with inhibitors of glycosylation and examined the effects of the inhibitors on binding and signaling by dGal-1.

dGal-1 bound well to HL60 cells (Figure 1A). The addition of 20 mM lactose, a weak inhibitor of dGal-1, reduced dGal-1 binding, confirming that binding was to cell surface glycans. We used plant lectins and specific antibodies to explore the effects of biosynthetic inhibitors on glycan structure. Treating cells with kifunensine, an inhibitor of α-mannosidase I, prevents trimming and processing of high mannose-type *N*-glycans, and thus, blocks the formation of complex-type *N*-glycans

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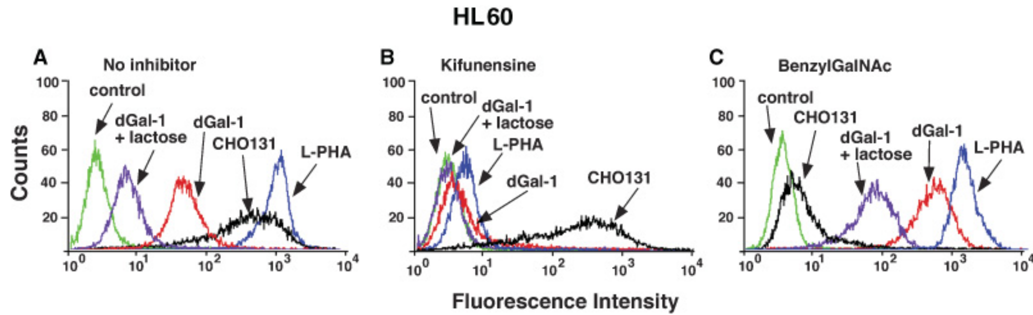

Fig. 1. dGal-1 binding to HL60 cells requires expression of complex-type *N*-glycans. HL60 cells were incubated with dGal-1 in the presence or absence of lactose to measure glycan-specific binding to surface ligands, with L-PHA to measure complex *N*-glycans, or with mAb CHO-131 to measure complex *O*-glycans. Binding assays were performed on control cells that were not incubated with a glycosylation inhibitor (**A**), on cells preincubated with kifunensine for 7 days to inhibit complex-type *N*-glycan synthesis (**B**), or with cells preincubated with benzylGalNAc for 3 days to inhibit complex-type *O*-glycan synthesis (**C**). The data are representative of three separate experiments.

(Elbein et al. 1990). The effects of kifunensine were monitored by reduced binding of the plant lectin, *Phaseolus vulgaris* leukoagglutinin (L-PHA), which recognizes tri- and tetraantennary complex *N*-glycans containing outer galactose residues and an α-linked mannose residue substituted at the C-2 and C-6 positions (Cummings and Kornfeld 1982a, 1982b). Treating cells with benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (benzylGalNAc) affects *O*-glycan biosynthesis by competing with enzymes that elaborate complex-type *O*-glycans (Kuan et al. 1989; Huet et al. 1998; Gouyer et al. 2001). The effects of benzylGalNAc were monitored by reduced binding of mAb CHO-131, which recognizes fucosylated and sialylated complex *O*-glycans on a core 2 backbone (Walcheck et al. 2002).

Both L-PHA and CHO-131 bound to HL60 cells (Figure 1A). Kifunensine treatment of HL60 cells reduced binding of L-PHA but did not affect binding of CHO-131 (Figure 1B). These results demonstrate that kifunensine treatment shifted the distribution of *N*-glycans from complex, fucosylated, and sialylated structures, known to be expressed in HL60 cells, to high mannose-type structures, but did not detectably alter expression of complex *O*-glycans identified by mAb CHO-131. Importantly, much less dGal-1 bound to HL60 cells treated with kifunensine, indicating that dGal-1 receptors contain complex-type *N*-glycans.

BenzylGalNAc treatment of HL60 cells reduced binding of CHO-131 but did not affect binding of L-PHA, indicating that the inhibitor blocked expression of at least some complex *O*-glycans but did not detectably affect expression of complex *N*-glycans (Figure 1C). Somewhat more dGal-1 bound to HL60 cells treated with benzylGalNAc than to untreated cells (Figure 1C), which could be due to enhanced access of dGal-1 to cell surface *N*-glycans in the absence of *O*-glycans. These results demonstrate that the receptors for dGal-1 on HL60 cells require expression of complex *N*-glycans.

dGal-1-induced exposure of surface phosphatidylserine on HL60 cells requires expression of complex-type N-glycans

Binding of dGal-1 to HL60 cells and activated human neutrophils signals exposure of PS on their surfaces, without accompanying apoptosis (Dias-Baruffi et al. 2003; Stowell et al. 2007). Compared to nontreated cells, kifunensine-treated HL60 cells exposed much less PS after incubation with dGal-1 for 4 h (Figure 2A), whereas benzylGalNAc-treated HL60 cells exposed more PS after incubation with dGal-1 (Figure 2B). The inclusion of lactose with dGal-1 decreased PS exposure to control levels, indicating the specificity of dGal-1-induced PS mobilization.

dGal-1-induced Ca^{2+} mobilization in HL60 cells requires expression of complex-type N-glycans

Binding of dGal-1 to neutrophils induces a Ca^{2+} flux (Karmakar et al. 2005). To determine whether dGal-1 similarly mobilized Ca^{2+} in HL60 cells, Fluo-4-loaded HL60 cells were incubated with dGal-1. The addition of dGal-1 elicited an immediate rapid rise in cytoplasmic Ca^{2+}, which triggered an influx of extracellular Ca^{2+} that maintained Ca^{2+} above basal levels for several minutes (Figure 3A). dGal-1 agglutinated the HL60 cells, as manifested by small oscillations in the fluorimeter tracing. Inclusion of 20 mM lactose reversed dGal-1-induced agglutination and prevented the Ca^{2+} flux, indicating that signaling required binding of dGal-1 to cell surface glycoconjugates. Preloading HL60 cells with the intracellular Ca^{2+} chelator BAPTA-AM (*1*,*2*-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid/acetoxymethyl ester) abrogated the sustained Ca^{2+} flux (Figure 3A), indicating that dGal-1 first released Ca^{2+} from intracellular stores, which triggered the extracellular Ca^{2+} influx.

The addition of dGal-1 to kifunensine-treated HL60 cells elicited much smaller Ca^{2+} fluxes than those in untreated cells (Figure 3B), indicating that the major Ca^{2+} signaling receptors for dGal-1 required expression of complex-type *N*-glycans. Thus, the loss of Ca^{2+} mobilization correlated strongly with the loss of surface binding of dGal-1 and abrogation of dGal-1-induced PS exposure on kifunensine-treated HL60 cells. In contrast, the addition of dGal-1 to benzylGalNAc-treated HL60 cells triggered Ca^{2+} fluxes similar to those in untreated cells (Figure 3C).

dGal-1 binding and signaling in Molt-4 and Jurkat cells also require expression of complex-type N-glycans

The above results show that binding and signaling through dGal-1 require expression of complex-type *N*-glycans on HL60 cells. To explore the generality of cellular responses to dGal-1, we examined two lymphocytic cell lines, Molt-4 and Jurkat, both of which are known to respond to dGal-1 (Walzel et al. 1996, 2006; Dias-Baruffi et al. 2003; Hahn et al. 2004; van der Leij et al. 2007). Notably, Jurkat cells lack even

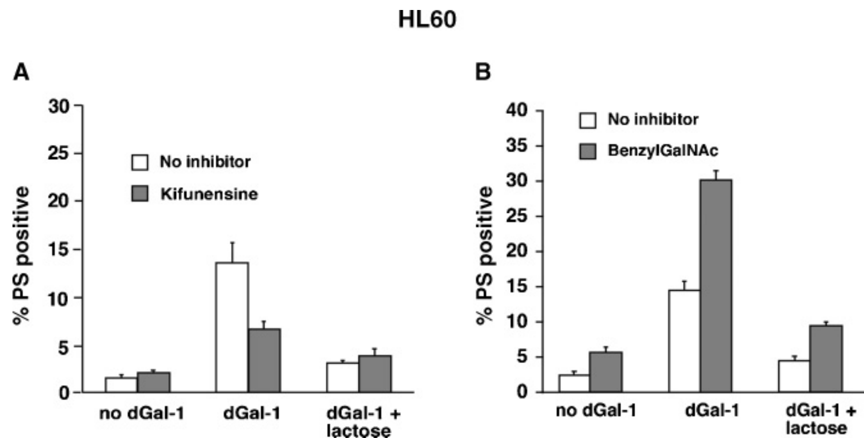

Fig. 2. dGal-1-induced exposure of PS on HL60 cells requires expression of complex-type *N*-glycans. HL60 cells were treated with 10 μM dGal-1 for 4 h, disengaged with lactose, stained with FITC-annexin V and PI, and then analyzed by flow cytometry. The indicated cells were preincubated with kifunensine for 7 days (**A**) or with benzylGalNAc for 3 days (**B**). The results are depicted as the percentage of cells that stained with annexin V above a threshold level, but that remained viable as assessed by staining with PI below a threshold level. The data represents the mean ± SD of three experiments.

simple core 1 *O*-glycans (Piller et al. 1990) because of a mutation in the chaperone Cosmc (Ju and Cummings 2002), which is required for correct folding and expression of core 1 β1-3-galactosyltransferase (T-synthase). As observed for HL60 cells, kifunensine treatment of Molt-4 and Jurkat cells markedly decreased binding of L-PHA and dGal-1 (data not shown). CHO-131 did not bind to Molt-4 or Jurkat cells, indicating that these cells lacked complex sialylated and fucosylated *O*-glycans recognized by this antibody (data not shown). The plant lectin peanut agglutinin (PNA), which recognizes the core 1 *O*-glycan disaccharide (Galβ1-3GalNAcα-Ser/Thr) (Lotan et al. 1975; Novogrodsky et al. 1975), bound well to both Molt-4 and Jurkat cells and also bound to benzylGalNAc-1-treated cells (data not shown). This illustrates the lack of specificity of PNA for core 1 Galβ1-3GalNAcα-Ser/Thr, which is not present on Jurkat cells. The lack of specificity of PNA for the core 1 *O*-glycan is further documented by binding of PNA to a variety of galactose-containing glycans on microarrays, as summarized on the website of the Consortium for Functional Glycomics (http://www.functionalglycomics.org/glycomics/).

Kifunensine treatment of Molt-4 cells decreased PS exposure after incubation with dGal-1 (Figure 4A), whereas benzylGalNAc did not inhibit PS exposure (Figure 4B). Similarly, kifunensine treatment of Jurkat cells decreased PS exposure after incubation with dGal-1 (Figure 4C), whereas benzylGalNAc did not inhibit PS exposure (Figure 4D).

The addition of dGal-1 to Fluo-4-loaded Molt-4 cells (Figure 5A) and to Jurkat cells (Figure 6A) caused an immediate rapid rise in cytoplasmic Ca^{2+}, which triggered an influx of extracellular Ca^{2+} that maintained Ca^{2+} above basal levels for several minutes. Inclusion of 20 mM lactose reversed dGal-1-induced agglutination and prevented the Ca^{2+} flux, indicating that signaling required binding of dGal-1 to cell surface glycoconjugates. Preloading cells with the intracellular Ca^{2+} chelator BAPTA-AM abrogated the sustained Ca^{2+} flux (Figure 5A and Figure 5B), indicating that dGal-1 first released Ca^{2+} from intracellular stores, which triggered the extracellular Ca^{2+} influx. The addition of dGal-1 to kifunensine-treated cells elicited much smaller weak Ca^{2+} fluxes than those in untreated cells (Figure 5B and Figure 6B), indicating that the

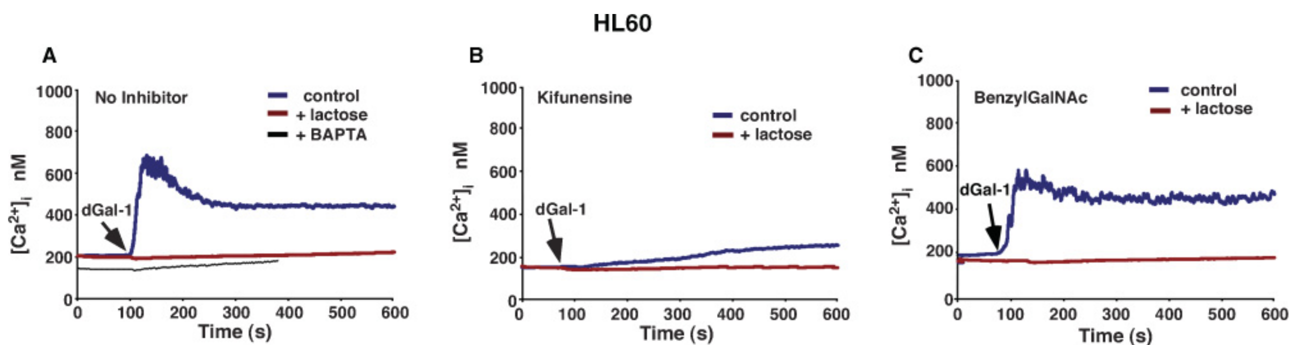

Fig. 3. dGal-1-induced elevation of cytosolic Ca^{2+} in HL60 cells requires expression of complex-type *N*-glycans. Cytosolic Ca^{2+} levels in stirred Fluo-4-labeled HL60 cells were continuously measured in a fluorimeter. (**A**) Control or BAPTA-loaded HL60 cells in a Ca^{2+}-containing buffer were incubated with 10 μM dGal-1 in the presence or absence of 20 mM lactose. (**B**) Kifunensine-treated HL60 cells were incubated with 10 μM dGal-1 in the presence or absence of 20 mM lactose. (**C**) BenzylGalNAc-treated HL60 cells were incubated with 10 μM dGal-1 in the presence or absence of 20 mM lactose. The data are representative of three independent experiments.

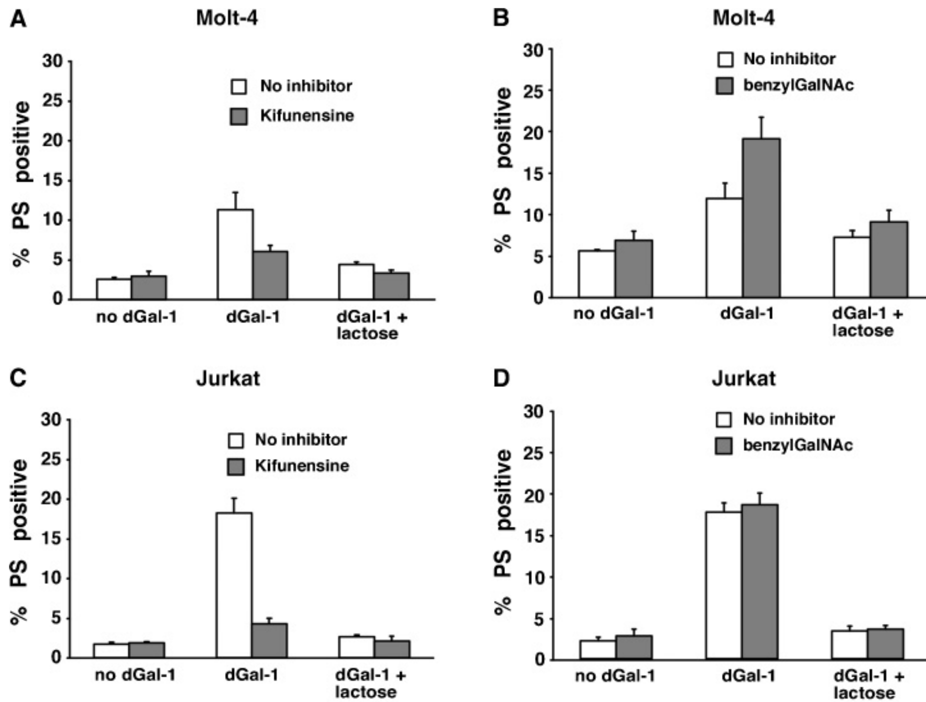

Fig. 4. dGal-1-induced exposure of PS on Molt-4 and Jurkat cells requires expression of complex-type *N*-glycans. Molt-4 cells (**A**, **B**) and Jurkat cells (**C**, **D**) were treated with 10 μM dGal-1 for 4 h, disengaged with lactose, incubated with a mixture of FITC-conjugated annexin V and PI, and analyzed by flow cytometry. The indicated cells were preincubated with kifunensine for 3 days or with benzylGalNAc for 3 days. The data are depicted as the percentage of cells that stained with annexin V above a threshold level, but that remained viable as assessed by staining with PI below a threshold level. The data represent the mean ± SD of three experiments.

major Ca^{2+} signaling receptors for dGal-1 required expression of complex-type *N*-glycans. Thus, the loss of Ca^{2+} mobilization correlated strongly with the loss of surface binding of dGal-1 and abrogation of dGal-1-induced PS exposure on kifunensine-treated Molt-4 and Jurkat cells. In contrast, the addition of dGal-1 to benzylGalNAc-treated cells elicited Ca^{2+} fluxes similar to those in untreated cells (Figure 5C and Figure 6C).

Taken together, the data with Molt-4 and Jurkat cells closely parallel those obtained with HL60 cells. For all three cell lines, the kifunensine-mediated loss of complex *N*-glycans abrogated binding of dGal-1 and prevented dGal-1-mediated Ca^{2+} mobilization and exposure of PS.

dGal-1 binding to and signaling in HL60 cells do not require expression of glycosphingolipids

Since it has been reported that dGal-1 binds to the ganglioside GM1 (Kopitz et al. 1998; Siebert et al. 2005), we explored whether glycosphingolipids on HL60 cells contribute to dGal-1 binding and signaling. To reduce expression of

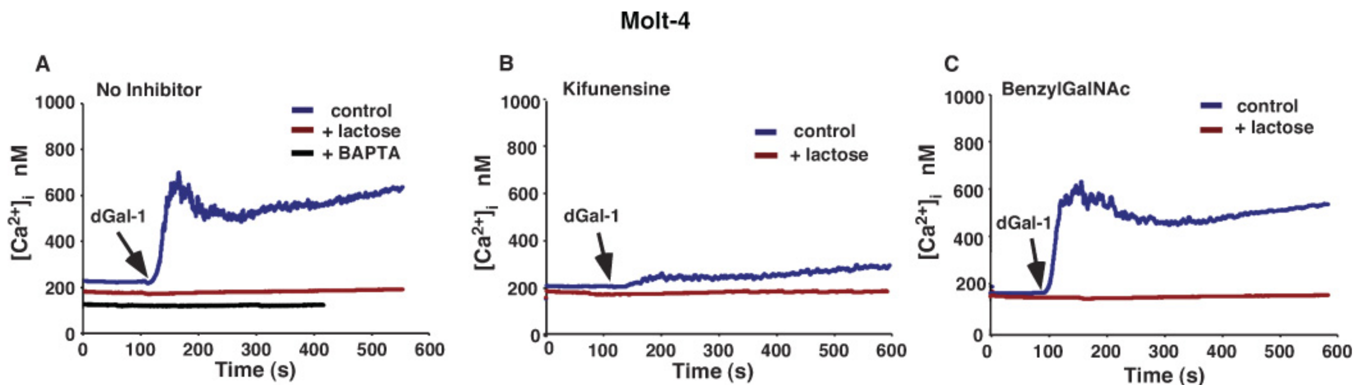

Fig. 5. dGal-1-induced elevation of cytosolic Ca^{2+} in Molt-4 cells requires expression of complex-type *N*-glycans. Cytosolic Ca^{2+} levels in stirred Fluo-4-labeled Molt-4 cells were continuously measured in a fluorimeter. (**A**) Control or BAPTA-loaded Molt-4 cells in a Ca^{2+}-containing buffer were incubated with 10 μM dGal-1 in the presence or absence of 20 mM lactose. (**B**) Kifunensine-treated Molt-4 cells were incubated with 10 μM dGal-1 in the presence or absence of 20 mM lactose. (**C**) BenzylGalNAc-treated Molt-4 cells were incubated with 10 μM dGal-1 in the presence or absence of 20 mM lactose. The data are representative of three independent experiments.

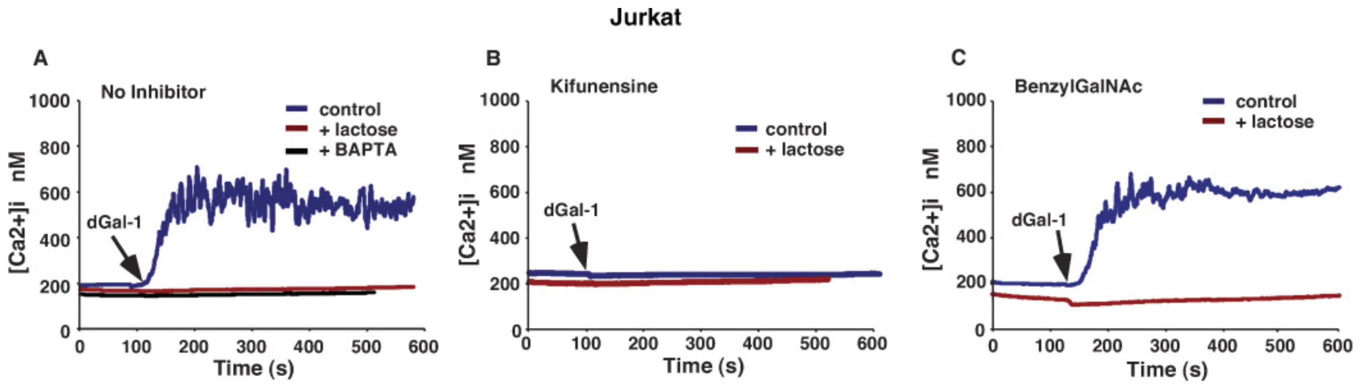

Fig. 6. dGal-1-induced elevation of cytosolic Ca^{2+} in Jurkat cells requires expression of complex-type *N*-glycans. Cytosolic Ca^{2+} levels in stirred Fluo-4-labeled Jurkat cells were continuously measured in a fluorimeter. (**A**) Control or BAPTA-loaded Jurkat cells in a Ca^{2+}-containing buffer were incubated with 10 μM dGal-1 in the presence or absence of 20 mM lactose. (**B**) Kifunensine-treated Jurkat cells were incubated with 10 μM dGal-1 in the presence or absence of 20 mM lactose. (**C**) BenzylGalNAc-treated Jurkat cells were incubated with 10 μM dGal-1 in the presence or absence of 20 mM lactose. The data are representative of three independent experiments.

glycosphingolipids, we used the inhibitor 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), which blocks the addition of glucose to ceramide in the first step in glycosphingolipid synthesis (Rosenwald et al. 1992; Radin et al. 1993). PDMP treatment inhibited glycosphingolipid synthesis in HL60 cells, as documented by reducing the staining of cells with cholera toxin B subunit, which binds specifically to the ganglioside GM1 (Van Heyningen et al. 1976; Spangler 1992) (Figure 7A). However, PDMP treatment did not reduce binding of dGal-1 (Figure 7B), L-PHA (Figure 7C), or PNA (Figure 7D). These results demonstrate that PDMP treatment reduced GM1 on the cell surface but did not affect expression of *N*- or *O*-glycans and dGal-1 receptors. Furthermore, PDMP treatment did not affect exposure of PS after incubation with

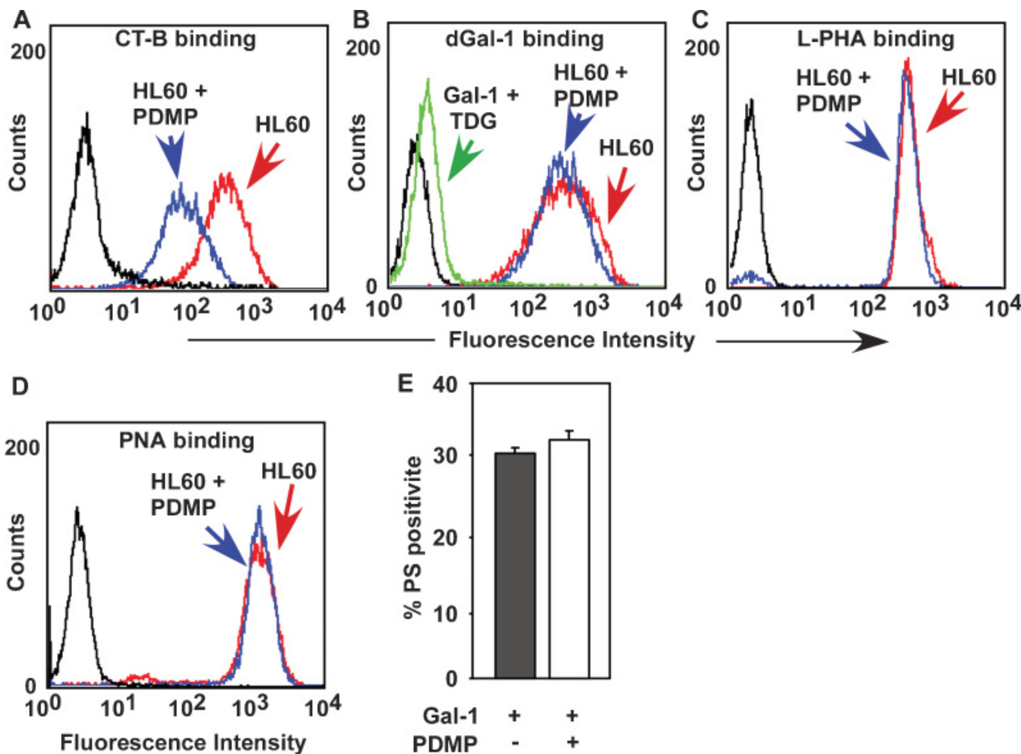

Fig. 7. dGal-1 binding to HL60 cells and dGal-1-induced exposure of PS on HL60 cells do not require expression of gangliosides. Control HL60 cells or HL60 cells incubated with PDMP for 3 days were analyzed by flow cytometry for binding of cholera toxin subunit B (CT-B) (**A**), dGal-1 (**B**), L-PHA (**C**), or PNA (**D**). (**E**) Control or PDMP-incubated HL60 cells were treated with 10 μM dGal-1 for 4 h, disengaged with lactose, incubated with a mixture of FITC-conjugated annexin V and PI, and analyzed by flow cytometry. The data are depicted as the percentage of cells that stained with annexin V above a threshold level, but that remained viable as assessed by staining with PI below a threshold level. The data represent the mean ± SD of three experiments.

dGal-1 (Figure 7E). These results demonstrate that inhibition of glycosphingolipid expression on HL60 cells does not significantly affect either dGal-1 binding or signaling.

Discussion

We have demonstrated that dGal-1 preferentially bound to kifunensine-sensitive complex *N*-glycans on HL60, Molt-4, and Jurkat cells. Furthermore, the receptors for dGal-1 on these cells that signal Ca^{2+} mobilization and exposure of PS required complex-type *N*-glycans. By contrast, dGal-1 did not require benzylGalNAc-sensitive *O*-glycans or PDMP-sensitive glycosphingolipids to bind to and signal in these cells.

Our results are consistent with studies of glycans recognized by dGal-1 in Jurkat cells (Walzel et al. 2006) although these authors did not note that Jurkat cells lack core 1 *O*-glycans. Our data also support previous findings that binding of rat dGal-1 and several other mammalian galectins to glycosylation mutants of Chinese hamster ovary (CHO) cells correlates with expression of complex-type *N*-glycans (Patnaik et al. 2006). Galectin-3 receptors on T cells require proper *N*-glycosylation and *N*-glycan branching for effective crosslinking and formation of the T-cell synapse (Demetriou et al. 2001; Dennis et al. 2002; Partridge et al. 2004; Lau et al. 2007). Deficiency of β1,6 *N*-acetylglucosaminyltransferase V (Mgat5), a key enzyme involved in *N*-glycan biosynthesis, lowers the threshold for T-cell activation by enhancing the clustering of the T-cell receptor (Demetriou et al. 2001). Mgat5 initiates β1,6 GlcNAc branching on *N*-glycans, thereby increasing expression of PL ligands that are important for dGal-1 binding and signaling (Leppanen et al. 2005; Suzuki et al. 2005a, 2006; Bianco et al. 2006; Lagana et al. 2006; Chen et al. 2007; Stowell, Arthur, et al. 2008). The modification of complex-type *N*-glycans on CD45 by ST6Gal I sialyltransferase negatively regulates dGal-1-induced signaling (Amano et al. 2003). Human dGal-1 does not bind to sialylated *N*-glycans with terminal α2,6-linked sialic acids of the type elaborated by ST6Gal I (Stowell, Arthur, et al. 2008). A recent study tested the ability of dGal-1 to bind to a very large panel of *N*- and *O*-glycans on a microarray. Of the many glycans examined, dGal-1 bound to a subset of *N*-glycans but failed to bind to core 1 *O*-glycans, including the disaccharide Galβ1-3GalNAcα (Stowell, Arthur, et al. 2008). Although these studies and our current data demonstrate the importance of complex-type *N*-glycans for interactions with dGal-1, some studies have questioned the roles of *N*- or *O*-glycans in dGal-1 binding and signaling (Carlow et al. 2003; Siebert et al. 2003, 2005; Elola et al. 2005). Therefore, it remains possible that some cell types express dGal-1 receptors that do not require complex-type *N*-glycans.

The specific glycoprotein signaling receptors for dGal-1 on HL60, Molt-4, and Jurkat cells are not yet defined. It is possible that each cell displays different receptors that share common complex-type *N*-glycans recognized by dGal-1. dGal-1 mobilizes cytosolic Ca^{2+} and exposes PS on the surfaces of human neutrophils through a pathway that requires action of Src family kinases and phospholipase C-γ (Karmakar et al. 2005).

The major dGal-1 receptors on HL60 cells are PL-containing glycans that are sensitive to endo-β-galactosidase, which degrades linear, unmodified PL (Leppanen et al. 2005; Stowell, Arthur, et al. 2008). The expression of PL-containing glycans is regulated by Mgat5 (Pierce and Arango 1986; Dennis et al. 2002; Guo et al. 2003; Partridge et al. 2004) and by β1,3-*N*-acetylglucosaminyltransferases and β1-4-galactosyltransferases (Ishida et al. 2005; Togayachi et al. 2007). How these enzymes cooperatively synthesize the structures that galectins recognize is an important topic for future studies.

Materials and methods

Materials

The chemicals used and their sources were as follows: kifunensine (Roche Diagnostics, Mannheim, Germany); benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (Calbiochem, San Diego, CA); PNA-biotin and PHA-L-biotin (Vector Laboratories, Burlingame, CA); fluorescein isothiocyanate (FITC)-conjugated annexin V and streptavidin, RPMI 1640 medium (Roche Diagnostics); Fluo-4 AM and propidium iodide (PI); biotinylated cholera toxin subunit B, streptavidin ALEXA 488 (Molecular Probes Invitrogen, Carlsbad, CA); DL-PDMP (Biomol, Plymouth Meeting, PA); Hank's balanced salt solution (Mediatech, Manassas, VA); human serum albumin (ZLB Bioplasma, Glendale, CA); and BAPTA-AM (Amersham, Piscataway, NJ).

Preparation of dGal-1

Recombinant human dGal-1 expressed in *Escherichia coli* was purified on lactosyl-Sepharose and dGal-1 was biotinylated as described (Dias-Baruffi et al. 2003).

Cell lines

HL60, Molt-4, and Jurkat cells from the American Type Culture Collection were maintained at 37°C and 5% CO_2 in a complete RPMI 1640 medium containing 10% fetal calf serum, glutamine (2 mM), penicillin (100 milliunits/mL), and streptomycin (100 μg/mL).

Treatment of cells with kifunensine or benzylGalNAc to inhibit complex N-glycan and complex O-glycan biosynthesis

HL60 cells were incubated in a medium containing 10 μg/mL kifunensine for 7 days before use in experiments. Molt-4 and Jurkat cells were incubated in a medium containing kifunensine for 3 days before use. In other experiments, cells were incubated in a medium containing 2 mM benzylGalNAc for 3 days before use. Control, treated cells were incubated with dGal-1 in the presence or absence of 20 mM lactose. HL60, Molt-4, and Jurkat cells were suspended in either the complete RPMI medium or Ca^{2+}–Mg^{2+}-containing Hank's balanced salt solution (HBSS) with 0.5% human serum albumin (HSA). To monitor changes in PS exposure, control- and inhibitor-treated cells were incubated with 10 μM dGal-1 at 37°C in the presence or absence of 20 mM lactose for the times indicated. Prior to flow cytometry analysis, agglutination of dGal-1-treated cells was reversed by the addition of 20 mM lactose.

Treatment of cells with PDMP to inhibit glycosphingolipid synthesis

HL60 cells were treated with 1.2 μM PDMP for 72 h. Following treatment, the cells were washed twice in a HBSS buffer before flow cytometry analysis. To determine the extent of ganglioside

inhibition following PDMP treatment, cells were resuspended in HBSS containing 1 μg/mL biotinylated cholera toxin subunit B, a specific marker for GM1 (Reed et al. 1987), and incubated for 1 h at 4°C. After incubation, cells were washed twice with HBSS, incubated with 2 μg/mL streptavidin ALEXA 488 for 1 h at 4°C, washed twice again, and resuspended in HBSS. To analyze changes in PS exposure following PDMP treatment, control and treated HL60 cells were incubated with 10 μM dGal-1 for 4 h in complete RPMI and then analyzed for PS exposure by flow cytometry as detailed below.

Flow cytometry

Cells were incubated with biotinylated dGal-1 (2 μg/mL), PHA-L, PNA, or MAL (*Maackia amurensis* agglutinin) lectins (10 μg/mL), or CHO-131 mAb (5 μg/mL) for 45 min on ice in HBSS/HSA, washed once with HBSS, and then incubated with streptavidin-FITC or anti-mouse $(Fab)_2$-FITC for 30 min on ice in HBSS/HSA. After washing twice with HBSS, cells were resuspended in HBSS and analyzed by flow cytometry. To measure PS exposure, a mixture of FITC-conjugated annexin V and PI was incubated with cells for 15 min on ice as described previously (Dias-Baruffi et al. 2003). The cells were diluted into HBSS and analyzed immediately on a FACS Calibur instrument (Becton–Dickinson) using Cell Quest software. The fluorescence intensity for binding of both annexin V and PI was measured for the entire cell population. For most experiments, the data are represented as the percentage of cells that stained with annexin V above a threshold level, but that remained viable as assessed by staining with PI below a threshold level.

Ca^{2+} flux measurements

Ca^{2+} flux experiments were carried out as described (Karmakar et al. 2005). Briefly, HL60, Molt-4, and Jurkat cells were loaded with 3 μM Fluo-4 AM at 37°C for 30 min in the presence of 4 mM probenecid to minimize dye leakage. The cells were washed with HBSS, incubated for 30 min at room temperature to allow the Fluo-4 dye to completely de-esterify, washed twice more, and resuspended at 10^7 cells/mL in HBSS/HSA. In some cases, cells were incubated with BAPTA in HBSS for 30 min along with Fluo-4-AM. Fluo-4-labeled cells (3×10^6/mL) were treated with 10 μM dGal-1 at 37°C in the presence or absence of 20 mM lactose. Fluorescence readings were obtained in a stirring cell fluorimeter (PerkinElmer Life Sciences LS-50) equipped with a water-jacketed cuvette holder. After obtaining the basal signal, fluorescence intensities were acquired at 0.1 s intervals for 10-15 min with continuous stirring of the cell suspension.

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Conflict of interest statement

None declared.

Abbreviations

AM, acetoxymethyl ester; BAPTA, *1*,*2*-bis(*o*-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid); benzylGalNAc, benzyl-*2*-acetamido-2-deoxy-α-D-galactopyranoside; CHO, Chinese hamster ovary; dGal-1, dimeric galectin-1; FITC, fluorescein isothiocyanate; HBSS, Hank's balanced salt solution; L-PHA, *Phaseolus vulgaris* leukoagglutinin; MAL, *Maackia amurensis* agglutinin, PI, propidium iodide; PDMP, DL-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol•HCl; PL, poly-*N*-acetyllactosamine; PNA, peanut agglutinin; PS, phosphatidylserine.

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