A PKC/RAS/ERK SIGNALING PATHWAY ACTIVATES MYELOID FIBRONECTIN RECEPTORS BY ALTERING β1 INTEGRIN SIALYLATION


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Running title: Regulation of β1 integrin sialylation and function by PKC/ras/ERK

Here we report that myeloid cells differentiating along the monocyte/macrophage lineage downregulate the ST6Gal-I sialyltransferase via a PKC/ras/ERK signaling cascade. In consequence, the β1 integrin subunit becomes hyposialylated, which stimulates the ligand binding activity of α5β1 fibronectin receptors. Pharmacologic inhibitors of PKC, ras, and MEK, but not PI3K, block ST6Gal-I downregulation, integrin hyposialylation and fibronectin binding. In contrast, constitutively active MEK stimulates these same events, indicating that ERK is both a necessary and sufficient activator of hyposialylation-dependent integrin activation. Consistent with the enhanced activity of hyposialylated cell surface integrins, purified α5β1 receptors bind fibronectin more strongly upon enzymatic desialylation, an effect completely reversed by re-sialylation of these integrins with recombinant ST6Gal-I. Finally, we have mapped the N-glycosylation sites on the β1 integrin in order to better understand the potential effects of differential sialylation on integrin structure/function. Notably, there are three N-glycosylated sites within the β1 I-like domain, a region that plays a crucial role in ligand binding. Our collective results suggest that variant sialylation, induced by a specific signaling cascade, mediates the sustained increase in cell adhesiveness associated with monocytic differentiation.

The U937 and THP-1 cell lines represent well-accepted model systems for studying myeloid differentiation along the monocyte/macrophage lineage. Following treatment with phorbol myristate acetate (PMA), these cells exhibit phenotypic changes that are characteristic of cell differentiation, including increased respiratory burst activity, enhanced phagocytotic capability, and markedly elevated cell adhesiveness to extracellular matrix ligands such as fibronectin. In vivo, the increased adhesiveness of monocytes/macrophages contributes to the extravasation of cells from the vasculature, as well as tethering of cells within inflamed tissues.

Differentiating myeloid cells bind to fibronectin through the integrin family of cell adhesion receptors, including the α5β1 integrin species. The molecular mechanisms underlying PMA-dependent cell adhesion have not been well-defined, although it has been reported that PMA increases the synthesis of both α5 and β1 integrin subunits (1-4). However, myeloid cells (U937 and THP-1) express an abundant amount of α5β1 in the absence of PMA treatment, and yet these cells bind very poorly to fibronectin. This suggests that myeloid α5β1 receptors are normally in an inactive state and that increased expression alone cannot account for the dramatically increased fibronectin binding induced by PMA.

In our prior study (5), we observed that PMA stimulated a rapid, but transient, increase in fibronectin binding that was likely due to the activation of integrins already present on the cell surface. However, following this initial transient event, there was a second phase of...
elevated fibronectin binding that was sustained over many hours. The onset of this second phase of integrin activation was temporally correlated with the synthesis of a β1 integrin isoform that lacked α2-6-linked sialic acids, a sugar modification directed by the ST6Gal-I sialyltransferase. Our laboratory has previously determined that β1 integrins serve as a substrate for ST6Gal-I in several different cell types (5-7). In differentiating myeloid cells, expression of hyposialylated β1 integrins results from PMA-induced downregulation of ST6Gal-I (5).

Given that PMA is a known activator of Protein Kinase C (PKC), our goal in this investigation was to identify the signaling molecules that direct ST6Gal-I downregulation and, correspondingly, integrin hyposialylation. Other studies have suggested that ERK (Extracellular signal-Regulated Kinase) signaling is required for monocytic differentiation (8-11), however the mechanism by which ERK regulates integrin function in differentiated cells has not been elucidated. Our current results suggest that a PKC/ras/ERK signaling cascade mediates the sustained phase of fibronectin binding by inhibiting β1 integrin sialylation.

**MATERIALS AND METHODS**

**Cell culture:** A U937 myeloid cell subclone selected for GM-CSF sensitivity was obtained from Dr. Elizabeth Eklund (Northwestern University). The cells were maintained in Dulbecco’s modified Eagle’s medium with 4.5 gm ml⁻¹ glucose, L-glutamine (Cellgro), 10% fetal bovine serum and gentamicin. U937 cells expressing constitutively active MEK were generated by using Lipofectamine Plus (Invitrogen) to transfecnt cells with an HA-tagged activated MEK construct (available from Upstate). A pooled population of clones expressing activated MEK was obtained by selection in G418. Verification of MEK expression was accomplished by immunoblotting for the HA tag.

**Western blotting:** U937 cells were treated with or without 50 ng ml⁻¹ PMA for 15 hours. Cells were then lysed in 50mM Tris-HCl buffer (pH 7.4) containing 1% Triton X-100, 0.5 mM PMSF, 20 µg ml⁻¹ leupeptin, 4 mM sodium fluoride, and 200 µM sodium pervanadate. Protein concentrations in the lysates were determined using a modified Bradford assay (Sigma). Lysates were resolved by reducing SDS-PAGE, and β1 integrins were Western blotted using a monoclonal antibody from BD Transduction Labs. Western blot analysis of ST6Gal-I was accomplished using a polyclonal antibody generously provided by Dr. Karen Colley (University of Illinois, Chicago).

**Enzyme inhibitor studies:** Cells were incubated for 20 minutes at 37°C with one of the following enzyme inhibitors; 10 µM R031-8220 (Calbiochem), 30 µM manumycin A (Sigma), 50 µM PD98059 (Calbiochem), or 30 nM wortmannin (Sigma). PMA was then added to a final concentration of 50 ng ml⁻¹, and cells were incubated in the presence of both PMA and the selected inhibitor for an additional 15 hours at 37°C.

**Lectin affinity analyses:** Cell lysates (600 µg) were incubated for 3 hours at 4°C with 4 µg of the biotinylated lectins, SNA or ECL (Vector Laboratories). Streptavidin-agarose (20 µl, Sigma) was then added, and samples were incubated for an additional 2 hours at 4°C with rotation. Lectin/glycoprotein complexes were collected by brief centrifugation and then washed 3 times with lysis buffer, followed by one wash with phosphate-buffered saline (PBS). Glycoproteins were released from the complexes by boiling in SDS-PAGE sample buffer, resolved by reducing SDS-PAGE, and immunoblotted to detect the β1 integrin.

**Cell attachment assays:** Cells were treated with or without 50 ng ml⁻¹ of PMA and also with enzyme inhibitors in some trials, and were then seeded onto tissue culture dishes that had been precoated with 20 µg ml⁻¹ fibronectin. Adhesion was quantified as previously described using a crystal violet staining method (5).

**Sialidase and ST6Gal-I enzyme treatment of purified α5β1 and modified ELISA integrin**
binding assay: As previously reported (5), purified α5β1 integrins (Chemicon) were suspended in 50 mM Tris buffer containing 150 mM NaCl, 2 mM MgCl2, 0.1 mM CaCl2, and 0.1% Triton X-100 ("ELISA buffer"), adjusted to pH 6.5. Agarose-conjugated Vibrio cholerae sialidase (200 milliunits, CalBiochem) was added to the integrin solution, and samples were incubated for 6 hours at 37°C with rotation. An equal amount of the integrin solution was incubated for 6 hours in buffer without sialidase as a control. Following this incubation, the agarose-conjugated sialidase was removed by centrifugation. Both control and sialidased-treated integrin solutions were subdivided into two tubes, and then treated with or without 5 milliunits/mL of rat recombinant ST6Gal-I (CalBiochem) in the presence of 50 µM CMP-Neu (the activated sugar donor substrate required by ST6Gal-I) for 4 hours at 37°C. Following this incubation, the integrin solution buffer was adjusted to pH 7.4 by addition of pH 8.0 ELISA buffer. Integrin solutions were loaded onto 12-well tissue culture dishes precoated with fibronectin (100 µg mL⁻¹). A final amount of 350 ng purified α5β1 integrin was added to each well. Samples were also loaded onto wells that were precoated with denatured BSA to control for nonspecific binding. Purified integrins were allowed to adhere for 1.5 hours at 37°C; the wells were then washed 3 times with ELISA buffer (pH 7.4) and exposed for one hour to the glycosylation-insensitive anti-β1 integrin monoclonal antibody, MAB2000 (Chemicon). After washing, wells were incubated with an HRP-coupled secondary antibody (Amersham), followed by the colorimetric HRP substrate, Chromogen (BIOSOURCE International). Integrin binding was quantified by absorbance spectroscopy at a 450 nm wavelength. Specific binding values were obtained by subtracting BSA binding values ("nonspecific") from total fibronectin binding.

Transfection of N-glycosylation site mutants: CHO-K1 cells were transfected with a V5-tagged ST6Gal-I construct (provided by Dr. Karen Colley, University of Illinois, Chicago), and stable clones were generated by selection in G418. The pECE plasmid containing the human β1 integrin sequence (12) was obtained from Dr. Erkki Ruohola (The Burnham Institute), and site-directed mutagenesis was accomplished using the QuickChange Site-Directed Mutagenesis Kit (Stratagene Inc.) Reactions were performed in a thermal cycler with 40 ng template DNA, complementary primers at 300 nM, and with the following cycling steps repeated 16 times: 30s at 94°C, 60 s at 55°C, 12 min. at 68°C. Mutant clones were verified by DNA sequence analysis. The mutated constructs were transiently transfected into ST6Gal-I-expressing CHO-K1 cells using Lipofectamine Plus according to the vendor protocol (Invitrogen). Forty-eight hours following transfection, cells were lysed and detection of the constructs was accomplished by Western blotting with antibodies that recognized only the transfected form of the β1 integrin. Two antibodies were used; one from BD Transduction Labs (cat.# 610468), and one from Chemicon (cat.# MAB1965). Of note, the N74Q mutant (numbering begins with first amino acid following the signal sequence) was only detectable with the Chemicon antibody, possibly because this mutation alters epitope recognition by the Transduction Labs antibody.

RESULTS

Activation of PKC/ras/ERK induces expression of hyposialylated β1 integrins.

Previously we reported that β1 integrins expressed by PMA-treated U937 and THP-1 cells have a smaller apparent molecular mass when analyzed by SDS-PAGE, and it was subsequently shown this was due to the PMA-induced synthesis of integrins lacking α2-6 sialic acids (5). To evaluate whether the expression of hyposialylated integrins was regulated by PKC, we pretreated U937 cells with the PKC inhibitor, R031-8220, stimulated cells with PMA (plus inhibitor), and then examined the electrophoretic mobility of β1 integrins. As shown (Fig. 1A), mature β1 integrins from PMA-treated cells migrated more rapidly than β1 integrins from control cells, reflecting the expression of the hyposialylated glycoform. Pretreatment with R031-8220
blocked the PMA-induced mobility shift, implicating PKC as a modulator of integrin sialylation. In contrast to mature β1, neither PMA nor R031-8220 had any effect on the mobility of precursor β1 integrins, a species that resides in the endoplasmic reticulum (13-16) and is therefore not a substrate for sialyltransferases.

Other studies from our laboratory have shown that forced expression of oncogenic ras in epithelial cells causes altered α2-6 sialylation of β1 integrins (7); we therefore speculated that ras may act as a regulator of integrin sialylation in myeloid cells. To test this hypothesis, cells were treated with manumycin A, a compound which blocks ras activation by preventing farnesylation (17,18). Similar to results with R031-8820, manumycin A prevented the PMA-induced electrophoretic mobility shift (Fig. 1A).

We next sought to identify downstream effectors of ras that might be involved in regulating integrin sialylation. Ras can activate multiple signaling cascades, however, the phosphoinositide 3-kinase (PI3K) and ras/raf/MEK/ERK signaling pathways are among the best characterized mediators of ras-dependent cellular responses (19). Accordingly, we treated cells with an inhibitor (PD98059) of the ERK activating kinase, MEK, as well as with an inhibitor of PI3K (wortmannin). As shown in Fig. 1A, the MEK inhibitor blocked PMA-induced expression of the hyposialylated glycoform, whereas the PI3K inhibitor was without effect. These data suggest that PKC regulates integrin sialylation by activating a ras/raf/MEK/ERK signaling cascade.

To more directly examine integrin sialylation, we performed a lectin affinity assay. Briefly, cell lysates were incubated with SNA, a lectin that binds specifically to α2-6-linked sialic acids. Sialylated proteins were precipitated, electrophoresed, and β1 integrins were subsequently detected by Western blotting. Consistent with results from mobility shift assays, SNA failed to precipitate β1 integrins from PMA-treated cells, indicating that these integrins are lacking α2-6 sialic acids (Fig. 1B). However, SNA reactivity could be restored when PMA-treated cells were preincubated with R031-8220, manumycin A, and PD98059, but not with wortmannin.

**Downregulation of ST6Gal-I is mediated by PKC/ras/ERK.**

Given that PMA induces downregulation of ST6Gal-I (5,20), we examined the effects of pharmacologic inhibitors on ST6Gal-I protein levels. Western blots of ST6Gal-I revealed that the PMA-dependent downregulation in ST6Gal-I expression could by blocked by preincubating cells with R031-8220, manumycin A, and PD98059, but not with wortmannin (Fig. 1C).

**Hyposialylated β1 integrins have increased levels of galactose-terminated N-glycans.**

ST6Gal-I directs the addition of sialic acid in an α2-6-linkage to the terminal galactose of N-linked polylactosamine chains. However, this terminal galactose is a potential substrate for other trans-Golgi glycosyltransferases, including several α2-3-sialyltransferases which are known to be active in U937 cells (21). It follows that in cells with downregulated ST6Gal-I, the terminal galactoses of β1 could either remain unmodified, or alternately become capped with other types of sugars or sugar linkages. To establish whether β1 integrins become targeted by competing glycosyltransferases as a result of PMA-induced downregulation of ST6Gal-I, we performed a lectin affinity analysis with ECL, a lectin specific for the unsubstituted terminal galactose of N-linked polylactosamine chains. As shown in Fig. 1D, β1 integrins from PMA-treated cells were much more reactive with ECL, suggesting that a substantial proportion of integrin polylactosamine chains remain uncapped in the absence of ST6Gal-I activity.

**PMA-dependent cell binding to fibronectin is mediated by PKC/ras/ERK.**

Having determined that a PKC/ras/ERK signaling cascade directs ST6Gal-I downregulation and hyposialylated integrin expression, we anticipated that inhibitors of this pathway would block integrin-dependent cell adhesion to β1 substrates. Thus, cells were pretreated with inhibitors as before, stimulated with PMA, and then subjected to standard cell adhesion assays using fibronectin as a substrate. These assays showed that PMA-dependent fibronectin binding was blocked by R031-8220,
manumycin A, and PD98059, but not by wortmannin (Fig. 1E).

**Constitutively active MEK mimics the effect of PMA on integrin sialylation and function.**

To verify that integrin hyposialylation and function are regulated by an ERK-dependent signaling cascade, we generated cells that stably express constitutively active MEK. SNA analyses of integrins harvested from these cells revealed that activated MEK induced the expression of hyposialylated \( \beta_1 \) integrins, in tandem with downregulation of ST6Gal-I (Figs. 2A and B). We also found that MEK-dependent integrin hyposialylation was associated with enhanced cell adhesion to fibronectin (Fig. 2C). These data, combined with results from the pharmacologic inhibitor studies (Fig. 1), indicate that ERK is both a necessary and sufficient regulator of sialylation-dependent integrin activation.

**\( \alpha_2-6 \) sialylation directly regulates \( \alpha_5\beta_1 \) binding to fibronectin.**

To further establish that \( \alpha_2-6 \)-linked sialic acids play a causal role in regulating integrin function, we manipulated the sialylation of purified \( \alpha_5\beta_1 \) integrins and then monitored integrin binding to fibronectin using a modified ELISA. Consistent with our prior results (5), the enzymatic de-sialylation of purified \( \alpha_5\beta_1 \) integrins stimulated fibronectin binding (Fig. 3A). However, we now show that this increased fibronectin binding can be reversed by using recombinant ST6Gal-I to add \( \alpha_2-6 \) sialic acid residues back onto de-sialylated \( \alpha_5\beta_1 \) integrins. These data provide strong evidence that \( \alpha_2-6 \)-linked sialic acids directly regulate \( \alpha_5\beta_1 \) ligand binding activity. Also important, the behavior of purified \( \alpha_5\beta_1 \) integrins recapitulates the behavior of \( \alpha_5\beta_1 \) integrins expressed on the myeloid cell surface; desialylated purified integrins, like cell surface hyposialylated integrins, bind better to fibronectin.

To confirm the activity of both the sialidase and ST6Gal-I enzymes in our assays, treated integrins were precipitated with SNA, and then Western blotted for \( \beta_1 \) integrin. As shown in Fig. 3B, sialidase treatment of \( \alpha_5\beta_1 \) led to significantly reduced SNA reactivity, suggesting that the sialidase was very effective in removing \( \alpha_2-6 \)-linked sialic acids. The subsequent incubation of de-sialylated \( \alpha_5\beta_1 \) integrins with recombinant ST6Gal-I restored SNA reactivity to baseline levels, indicating re-addition of \( \alpha_2-6 \)-linked sialic acids. Treatment of control \( \alpha_5\beta_1 \) integrins, which are already heavily sialylated, with ST6Gal-I slightly increased \( \alpha_2-6 \) sialylation, although this did not appear to affect ligand binding activity (Fig. 3A).

**\( \beta_1 \) integrins are glycosylated on 10 out of 12 of the asparagine residues that have the appropriate consensus sequence for \( N \)-glycosylation, including 3 sites within the functionally important I-like domain.**

Our understanding of the role of glycosylation in regulating integrin structure/function has been limited by the lack of information concerning specific sites of \( N \)-glycosylation. To address this deficiency, we used a mutagenesis approach to identify the sites carrying \( N \)-linked glycans. Specifically, the asparagine residues within the \( NxS/T \) glycosylation consensus sequence were mutated to glutamine; these mutated cDNAs were then transfected into CHO-K1 cells, and expression of the constructs was detected by Western blotting using antibodies that recognize only the transfected \( \beta_1 \) isoform. We anticipated that the loss of an \( N \)-glycan at a given site would result in a reduced apparent molecular mass when compared with wild type \( \beta_1 \) integrins. As shown in Figs. 4A and B, 10 out of the 12 mutant constructs demonstrated increased electrophoretic mobility by SDS-PAGE, indicating decreased molecular mass (N461Q is shown in 4A as a representative example). These data suggest that the \( \beta_1 \) integrin is typically \( N \)-glycosylated on 10 sites, including 3 sites within the \( \beta_1 \) I-like domain, a region crucial for ligand binding. In contrast, the mobilities of the N564Q and N74Q constructs were identical to that of the wild type \( \beta_1 \) isoform, suggesting that these sites do not normally carry \( N \)-linked glycans.

Interestingly, only one band was typically observed for the transfected \( \beta_1 \) construct (Fig 4A), and this band migrated to the expected...
position of the partially-glycosylated precursor isoform. In a few blots, a small amount of mature β1 was detected upon extended exposure of the films (not shown). The mature band, when observed, also showed an electrophoretic mobility shift, as expected. It is currently unclear why the majority of the transfected β1 construct remains in the endoplasmic reticulum.

DISCUSSION

The regulation of Golgi glycosyltransferases by signaling mechanisms has been little studied, and even less is known about how such regulation affects the function of specific substrates targeted by these enzymes. We and others have shown that forced expression of oncogenic ras alters ST6Gal-I expression in epithelial cells (7) and fibroblasts (22-25), however, the current study describes regulation of ST6Gal-I by an endogenous, multistep, signaling cascade. These results are noteworthy because they indicate that Golgi enzymes such as ST6Gal-I can be dynamically regulated, and further imply that variantly-glycosylated substrates may be expressed in response to extracellular stimuli that activate appropriate signaling cascades.

Signaling through ERK has been suggested as an essential step in the differentiation of myeloid cells along the monocyte/macrophage lineage (8-11), and the heterologous expression of constitutively active ras or MEK stimulates monocytic cell behaviors including phagocytosis and adhesion to fibronectin (8,26,27). However, the molecular mechanisms linking ERK to specific changes in integrin structure/function have not been well-defined. Our results indicating that expression of hyposialylated integrins is induced by PKC/ras/ERK signaling, combined with our prior observation that the synthesis of hyposialylated integrins is temporally correlated with enhanced adhesion to fibronectin, strongly support variant β1 sialylation as a mechanism for activation of myeloid fibronectin receptors during monocytic differentiation.

Unequivocal evidence that α2-6 sialic acids play a direct role in regulating the activity of α5β1 fibronectin receptors is provided in this report by purified integrin receptor/ligand binding assays. The enzymatic removal of sialic acids from purified α5β1 integrins increases fibronectin binding, whereas the re-addition of these sugars by recombinant ST6Gal-I attenuates binding to basal levels. Importantly, the behavior of purified α5β1 integrins in a cell-free assay system mimics the behavior of integrins on the cell surface; α5β1 integrins lacking α2-6 sialic acids bind better to fibronectin. We postulate that the enhanced fibronectin-binding activity of hyposialylated integrins contributes to the recruitment of leukocytes to sites of inflammation. This hypothesis is consistent with results from a recent animal study in which ST6Gal-I expression was reduced in selected tissues by mutating one of the multiple ST6Gal-I promoter sequences. In these ST6Gal-I deficient mice, introduction of a bacterial pathogen stimulated enhanced recruitment of leukocytes into the peritoneum (28).

Elegant work from Luo et al. suggests that glycosylation can affect integrin conformation (29). These investigators engineered an artificial N-glycosylation site into the β1 integrin by substituting an asparagine residue for proline at amino acid 333, which created the Nxs consensus sequence for N-glycosylation. Upon transfection of this construct in CHO-K1 cells, the β1 subunit was N-glycosylated, and this variant glycoform was shown to have increased ligand binding activity. Site 333 is in the C-terminal end of the β1 I-like domain, and it was hypothesized that the addition of a glycan at this site caused an increase in the distance between the β1 head and stalk domains, thus inducing the integrin heterodimer to assume a more extended (activated) integrin conformation. While P333N is not a naturally occurring mutation, these data are important because they provide proof of concept that changes in the glycan structure within key regions of the integrin molecule can alter integrin conformation and activity. Notably, the specific sites on β1 that normally carry N-linked glycans have not previously been identified, which is surprising given the fact that there is currently intense interest in delineating integrin structure. Our studies now suggest that ten of the twelve consensus asparagine residues are elaborated with N-linked glycans. Three of
these sites lie within the β1 I-like domain, a region critical for ligand binding, and seven other sites are distributed among the PSI, hybrid, I-EGF, and β tail domains. We hypothesize that sialic acid, a negatively charged sugar, either alters the overall conformation of the integrin receptor, or more directly regulates ligand binding. Negative charges within the β1 I-like domain are very important for integrin function, as negatively-charged amino acids within this region are known to complex with divalent cations. In turn, the binding of divalent cations is a requisite event in integrin activation. We speculate that the addition or subtraction of sialic acids within this domain could either influence coordination of divalent cations, or alter positioning of the ligand within the ligand binding surface.

Results generated from integrins with artificial N-glycosylation sites, combined with our ligand-binding studies using enzymatically-manipulated purified integrins, provide much-needed causal evidence that integrin function can be regulated by changes in glycosylation. However, it is important to note that there is an extensive literature showing that naturally-occurring changes in integrin glycan structure are associated with dramatic alterations in cell behaviors such as adhesion, migration and invasion. Variant β1 glycoforms have been observed in numerous cell types including fibroblasts, myeloid cells, keratinocytes, cytrophoblasts, T lymphocytes, and several kinds of epithelial cells (reviewed in 30,31). Importantly, the altered β1 glycosylation described in most of these studies occurred in response to physiologic events or stimuli, not merely as a consequence of in vitro molecular manipulations of cell lines. Thus, differential glycosylation likely represents an important feature of the natural biology of integrins containing the β1 subunit. The current study adds to the prior body of literature by defining a specific endogenous signaling mechanism that regulates variant β1 glycosylation, and by demonstrating that hyposialylation is the likely mechanism underlying the known involvement of ERK in regulating the increased cell adhesiveness associated with monocytic/macrophage differentiation.

REFERENCES


**FOOTNOTES**

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FIGURE LEGEND

**Fig 1:** A PKC/ras/ERK signaling cascade regulates integrin sialylation and function.

A. Cells were pretreated with R031-8220, manumycin A, PD98059, or wortmannin, and then further incubated with the respective inhibitor plus PMA for 15 hours. Cells were lysed and the electrophoretic mobility of the β1 integrin was evaluated by Western blotting. PMA treatment induced increased mobility of the mature β1 integrin species, indicating reduced sialylation, whereas no alteration was noted in the endoplasmic reticulum-resident, precursor β1 integrin isoform.

B. Cell lysates harvested from cells treated with PMA and inhibitors as described above were incubated with biotinylated SNA lectin, followed by precipitation with streptavidin-coupled agarose beads. Lectin-glycoprotein complexes were resolved by SDS-PAGE, then Western blotted for β1 integrins. Note that only the mature integrin species is precipitated by SNA, since precursor β1 isoforms are never sialylated. Loss of SNA reactivity in samples treated with PMA only or PMA plus wortmannin reflects the expression of mature integrins lacking α2-6 sialic acid.

C. Cells treated as above were subjected to Western blot analysis to determine levels of ST6Gal-I.

D. Cell lysates were incubated with biotinylated ECL lectin, and glycoproteins with terminal galactoses were precipitated using streptavidin-agarose. β1 integrins precipitated by ECL were detected by Western blot.

E. Cells were treated with inhibitors and PMA as previously described, and were then seeded onto fibronectin-coated tissue culture dishes. Cell adhesion was quantified using a standard crystal violet staining method. Values represent the means and SEMs for three independent experiments performed in duplicate.

**Fig 2:** Constitutively active MEK induces loss of integrin sialylation and enhanced cell adhesion to fibronectin.

A. Cells stably expressing constitutively active MEK (ca MEK) were generated using standard protocols. Lysates harvested from parental cells or cells expressing activated MEK were Western-blotted to detect ST6Gal I.

B. SNA was used to precipitate α2-6 sialylated proteins; the precipitated proteins were resolved by reducing SDS-PAGE, and β1 integrins were detected by Western blot.

C. Control or ca MEK-expressing cells were seeded onto fibronectin-coated tissue culture dishes, and adhesion was quantified as previously described. Values represent the means and SEMs for 3 independent experiments performed in triplicate (* represents $p < 0.05$)

**Fig 3:** α2-6 sialylation of purified α5β1 integrins modulates binding to fibronectin.

A. Purified α5β1 integrins were treated with or without sialidase to remove sialic acids. The integrins were then treated with or without recombinant ST6Gal-I to restore α2-6-linked sialic acids. The purified integrins were added to fibronectin-coated dishes, and binding was quantified using a modified ELISA assay. Values represent the means and SEMs for three independent experiments performed in duplicate (* indicates $p < 0.05$).

B. The treated integrins from above were subjected to lectin precipitation using SNA. The precipitates were resolved by SDS-PAGE, and the β1 subunit was detected by Western blotting.

**Fig 4:** Expression of N-glycosylation-site mutants.
A. Site-directed mutagenesis was used to generate β1 integrin constructs containing glutamine substitutions for asparagine residues lying within N-glycosylation consensus sequences. The constructs were transfected into CHO-K1 cells engineered to express ST6Gal-I, and expression of the constructs was verified by Western blotting. Ten of the twelve mutant constructs exhibited reduced molecular mass, as compared with the wild-type β1 isoform (WT), reflecting the loss of an N-linked glycan. The construct containing the N461Q substitution is shown in the left panel as a representative example. Mutants N564Q (right panel) and N74Q (not shown) did not show mobility shifts, suggesting that these sites are not normally glycosylated.

B. Summary of results from transfections of N-glycosylation site mutants. Ten out of twelve sites appear to carry N-linked glycans, including three sites within the I-like domain.
Figure 1

A

Cont  PMA  PMA + R031  man A  PD98  wort

β1 integrins with uncapped galactoses

B

Cont  PMA  R031  man A  PD98  wort

Sialylated β1

C

Cont  PMA  R031  man A  PD98  wort

β1 integrins with uncapped galactoses

D

Cont  PMA

β1 integrins with uncapped galactoses

E

Fibronectin Binding (Abs 540)

Cont  PMA  R031  man A  PD98  wort

PMA +

β1

precursor β1

Mature β1

ST6Gal-I
Figure 2

A
Cont  ca MEK

B
Cont  ca MEK

C

Fibronectin binding (abs 540)

Cont  ca MEK

*
Figure 3

A

![Bar graph showing Fibronectin binding with Sialidase and ST6Gal I](image)

B

![Image of sialylated β1](image)
Figure 4

A

WT         N461Q

WT N564Q

B

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A PKC/ras/ERK signaling pathway activates myeloid fibronectin receptors by altering β1 integrin sialylation
Eric C. Seales, Faheem M. Shaikh, Alencia V. Woodard-Grice, Pooja Aggarwal, Alexis C. McBrayer, Kristin M. Hennessy and Susan L. Bellis

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