A Protein Kinase C/Ras/ERK Signaling Pathway Activates Myeloid Fibronectin Receptors by Altering β1 Integrin Sialylation*

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Here we report that myeloid cells differentiating along the monocyte/macrophage lineage down-regulate the ST6Gal-I sialyltransferase via a protein kinase C/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 protein kinase C, Ras, and MEK, but not phosphoinositide 3-kinase, block ST6Gal-I down-regulation, 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 resialylation of these integrins with purified fibronectin more strongly upon enzymatic desialylation, an effect completely reversed by resialylation of these integrins with purified ST6Gal-I sialyltransferase. Finally, we have mapped the N-glycosylation sites on the β1 integrin to better understand the potential effects of differential sialylation on integrin structure/function. Notably, there are three N-glycosylated sites within the β1 1-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),4 these cells exhibit phenotypic changes that are characteristic of myeloid cells the expression of hyposialylated 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 the expression of hyposialylated β1 integrins results from PMA-induced down-regulation 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 down-regulation and, correspondingly, integrin hyposialylation. Other studies have suggested that extracellular signal-regulated kinase (ERK) 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.

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 NORMAL 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 the expression of hyposialylated β1 integrins results from PMA-induced down-regulation of ST6Gal-I (5).

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MATERIALS AND METHODS

Cell Culture—A U937 myeloid cell subclone selected for granulocyte/macrophage colony-stimulating factor sensitivity was obtained from Dr. Elizabeth Eklund (Northwestern University). The cells were maintained in Dulbecco’s modified Eagle’s medium with 4.5 mg ml−1 glucose, 1-glutamine (Cellgro), 10% fetal bovine serum, and gentamicin. U937 cells expressing constitutively active MEK were generated by using Lipofectamine Plus (Invitrogen) to transfect cells with a hemagglutinin-tagged, activated MEK construct (available from Upstate Biotechnology). A pooled population of clones expressing activated MEK was obtained by screening in G418. Verification of MEK expression was accomplished by immunoblotting for the hemagglutinin tag.
Western Blotting—U937 cells were treated with or without 50 ng ml\(^{-1}\) PMA for 15 h. Cells were then lysed in 50 mM Tris-HCl buffer (pH 7.4) containing 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 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 provided by Dr. Karen Colley (University of Illinois, Chicago, IL). Following this incubation, the 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 Laboratories (catalog number 610468) and one from Chemicon (catalog number 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 BD Transduction Laboratories 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 that blocks Ras activation by preventing farnesylation (17, 18). Similar to results with R031-8220, 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 and 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 phosphoinositide 3-kinase (wortmannin). As shown in Fig. 1A, the MEK inhibitor blocked PMA-induced expression of the hyposialylated glycoform, whereas the phosphoinositide 3-kinase
PKC/Ras/ERK Regulation of β1 Integrin Sialylation/Function

FIGURE 1. A PKC/Ras/ERK signaling cascade regulates integrin sialylation and function. A, cells were pretreated with R031-8220 (R031), manumycin A (man A), PD98059 (PD98), or wortmannin (wort) and then further incubated with the respective inhibitor plus PMA for 15 h. 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, the precursor β1 integrin isoform. Cont, control. 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 and then Western-blotted for β1 integrins. Note that only the mature integrin species is precipitated by SNA, because 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 acids. C, cells treated as above were subjected to Western blot analysis to determine levels of ST6Gal-I. D, cells 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 described previously and then seeded onto fibronectin-coated tissue culture dishes. Cell adhesion was quantified using a standard crystal violet staining method. Values represent the means and S.E. for three independent experiments performed in duplicate.

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 and 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.

Down-regulation of ST6Gal-I Is Mediated by PKC/Ras/ERK—Given that PMA induces down-regulation 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 down-regulation in ST6Gal-I expression could be 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-terminating 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 down-regulated 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 down-regulation 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 uncpped 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 down-regulation and hyposialylated integrin expression, we anticipated that inhibitors of this pathway would block integrin-dependent cell adhesion to β1 substrates. Thus, cells were preincubated 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 β1 integrins in tandem with down-regulation of ST6Gal-I (Fig. 2, A 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.

α2-6 Sialylation Directly Regulates α5β1 Binding to Fibronectin—To further establish that α2-6-linked sialic acids play a causal role in regulating integrin function, we manipulated the sialylation of purified α5β1 integrins and then monitored integrin binding to fibronectin using a modified ELISA. Consistent with our prior results (5), the enzymatic desialylation of purified α5β1 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 α2-6 sialic acid residues back onto desialylated α5β1 integrins. These data provide
strong evidence that α2-6-linked sialic acids directly regulate α5β1 ligand binding activity. Also important, the behavior of purified α5β1 integrins recapitulates the behavior of α5β1 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 α5β1 integrin. As shown in Fig. 3B, sialidase treatment of α5β1 led to significantly reduced SNA reactivity, suggesting that the sialidase was very effective in removing α2-6-linked sialic acids. The subsequent incubation of desialylated α5β1 integrins with recombinant ST6Gal-I restored SNA reactivity to base-line levels, indicating re-addition of α2-6-linked sialic acids. Treatment of control α5β1 integrins, which are already heavily sialylated, with ST6Gal-I slightly increased α2-6 sialylation, although this did not appear to affect ligand binding activity (Fig. 3A).

**β1 Integrins Are Glycosylated on 10 of 12 of the Asparagine Residues That Have the Appropriate Consensus Sequence for N-Glycosylation, Including Three 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 determine the relative contributions of N-glycosylation to integrin structure/function.
approach to identify the sites carrying N-linked glycans. Specifically, the asparagine residues within the NX(S/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. 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 section as a representative example. Mutants N564Q (right section) and N74Q (not shown) did not show mobility shifts, suggesting that these sites are not normally glycosylated.

Ten of twelve sites appear to carry N-linked glycans, including three sites within the I-like domain. PSI, plexin-semaphorin-integrin; I-EGF, integrin-epidermal growth factor.

FIGURE 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-1, 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 section as a representative example. Mutants N564Q (right section) 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 of twelve sites appear to carry N-linked glycans, including three sites within the I-like domain. PSI, plexin-semaphorin-integrin; I-EGF, integrin-epidermal growth factor.

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-1 expression in epithelial cells (7) and fibroblasts (22–25). However, the current study describes regulation of ST6Gal-1 by an endoge-
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, cytotrophoblasts, T lymphocytes, and several kinds of epithelial cells (reviewed in Refs. 30 and 31). Importantly, the altered β1 glycosylation described in most of these studies occurred in response to physiologic events or stimuli and 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.

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