RHO methylation matters
A role for isoprenylcysteine carboxylmethyltransferase in cell migration and adhesion

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Numerous proteins involved in diverse aspects of cell biology undergo a process of post-translational modification termed prenylation. The prenylation pathway consists of three enzymatic steps, the final of which is methylation of the carboxyl-terminal prenylcysteine formed in the first two steps by the enzyme isoprenylcysteine carboxylmethyltransferase (Icmt). Due to the prevalence of prenylated proteins in cancer biology and the findings that several of the proteins are involved in processes controlling cell migration and adhesion, we sought to examine the role of Icmt-mediated methylation on cell behavior associated with metastasis. We found that inhibition of methylation reduces migration of the highly metastatic MDA-MB-231 breast cancer cell line. In addition, cell adhesion and cell spreading were also impaired by Icmt inhibition. Further investigation revealed that inhibition of Icmt significantly decreased the activation of both RhoA and Rac1, which are both prenylated proteins. The data obtained were consistent with the decreased activation being due to an increase in Rho GDP-dissociation inhibitor (GDI) binding by both proteins in the absence of their methylation. Importantly, the addition of exogenous RhoA or Rac1 to cells in which Icmt was inhibited was able to partially, but selectively, rescue directed and random migration, respectively. These results establish a role for Icmt-mediated methylation in cell migration, and point to specific prenylated proteins involved in this biology. The prenylation pathway has been targeted for oncogenic therapies, but the role of methylation in cell motility had been largely unexplored until now. The finding that methylation of Rho family members impacts on a specific component of their function provides an additional avenue through which to interrogate the biology of this important class of regulatory proteins.

Post-translational modifications of proteins play vital roles in many aspects of cell biology. Hence, identifying these modifications and determining their impact on functions of the proteins is crucial to advancing our understanding of the biological processes in which the modified proteins participate. One such post-translational pathway is termed protein prenylation. Numerous proteins that control important biological regulatory events undergo a complex series of modifications that are directed by the presence of a so-called CaaX motif at their carboxyl-terminus (Fig. 1). This post-translational pathway is initiated by the attachment of an isoprenoid lipid to an invariant cysteine residue, the “C” of the CaaX motif.1,2 Either a 15-carbon farnesyl or 20-carbon geranylgeranyl isoprenoid is covalently attached to this cysteine by protein farnesyltransferase (FTase) or protein geranylgeranyltransferase-I (GGTase-I), respectively. The prenylation step is followed by cleavage of the three C-terminal amino acids (the -aaX) by an endoplasmic reticulum (ER)-bound protease termed Rce1.4 Finally, the prenylated cysteine, which is now located at the C-terminus, is methylated by isoprenylcysteine carboxylmethyltransferase (Icmt), another integral...
ER membrane protein. The final result of these modifications is a protein that contains a prenylated and methylated cysteine at its carboxyl-terminus. Structural studies and sequence analysis have led to identification of more than 120 probable CaaX proteins, of which more than half have been confirmed as prenyltransferase substrates.

Since CaaX proteins are involved in a myriad of cellular functions, the prenylation pathway has a broad biological impact. One of the most well-known consequences of protein prenylation is increased affinity for cellular membranes. However, numerous studies have demonstrated that this post-translational processing not only facilitates protein association with cellular membranes, but also can play important roles in protein-protein interactions and protein stability. Cell adhesion and motility are complex cell biology behaviors that involve the transmission of extracellular information into the cell and result in coordinated changes in cell morphology, polarity, signaling and movement. Several CaaX proteins are involved in aspects of cell adhesion and migration. Of particular interest, most members of the Ras superfamily are CaaX proteins and undergo this processing. The Ras superfamily consists of five large subfamilies, the two most well-characterized being the Ras subfamily and Rho subfamily. As with all GTP-binding regulatory proteins, Ras and Rho subfamily members cycle between a GDP-bound inactive state and an active GTP-bound state. In the active state, they engage numerous signaling pathways that are involved in cell proliferation, differentiation, migration, polarity and morphology.

Due in large part to the prevalence of abnormal Ras activity in oncogenesis, the prenylation pathway has been targeted for therapeutic design. Constitutively elevated activity of Ras and Rho signaling pathways contribute to the initiation and progression of many types of cancer. In particular, inhibitors of the prenylation enzymes, most notably FTase inhibitors, have been pursued rigorously and have been tested in clinical trials, but to date the clinical responses have been disappointing. The emerging view that a more global attenuation of CaaX protein function may be advantageous in blocking cancer cell growth has increased interest in studying the two downstream enzymes involved in CaaX processing that modify both farnesylated and geranylgeranylated forms of the proteins, in particular, the methyltransferase, Icmt.

There is now substantial evidence from both pharmacologic and genetic approaches that inhibition of methylation has a major impact on oncogenesis. In the absence of Icmt activity, Ras and B-Raf, a signaling component downstream of Ras, were unable to transform cells. A specific inhibitor of Icmt, termed cysmethynil, dramatically impacted tumorigenic properties of the Ras-driven colon cancer line DKO8 and PC3 prostate cancer cells. Ras-dependent downstream signaling was also disrupted, as demonstrated by a decrease of MAP kinase phosphorylation in the presence of cysmethynil; in addition, Icmt inhibition was shown to induce autophagic cell death. These effects were Icmt specific because overexpression of Icmt restored normal levels of MAP kinase activation in the presence of cysmethynil, and a closely-related analog that lacked Icmt inhibitory activity was ineffective. Genetic and pharmacologic targeting have shown a clear impact on oncogenic transformation and tumor growth in mouse models.

While much of the attention with regard to the impact of C-terminal methylation on prenylated proteins has been on Ras, there have been intriguing results from prior studies on Rho proteins. Permeability studies of pulmonary endothelial cells demonstrated decreased RhoA activity when Icmt activity was inhibited. Inhibition of Icmt-catalyzed methylation with either pharmacologic inhibitors or genetic deletion resulted in increased degradation of Rho. Similar studies have provided evidence that the interaction of Rho proteins with RhoGDI, a chaperone-cum-negative regulator of Rho, was somewhat enhanced when Rho was unmethylated. Similar results for Rac1 and its interaction with RhoGDI were obtained in Icmt null cells, in which Rac1 activation by Tumor Necrosis Factor α was inhibited in the absence of Icmt activity. In another study examining membrane localization and activation states of a number of Rho family members, the role of methylation was seen to vary...
depending on family member and activation. Together, these studies indicate significant complexity with regards to the impact of Icmt inhibition on functions of Rho family members.

Rho proteins are involved in a myriad of cellular activities, including cell proliferation, morphologic changes and differentiation, and a number of studies have implicated elevated Rho activity, particularly that of RhoC, in cancer metastasis. One component of metastasis is enhanced cell migration, a complex biological phenomenon involving coordinated changes in cell morphology and structure in response to extracellular stimuli. Rho GTPases are important players in coordinating changes in the actin cytoskeleton in response to stimuli. In particular RhoA, Cdc42 and Rac1 have been extensively studied and their ability to rearrange actin into, stress fibers, filopodia and lamellipodia, respectively. Despite the interest in the prenylation pathway as a cancer therapy target and the obvious importance of Rho proteins in metastatic behavior, there exists limited knowledge of the role of CaaX protein prenylation in cell adhesion and migration. While it has been long-known that inhibition of isoprenoid biosynthesis by statins results in inhibition cell migration (reviewed in refs. 26–28) methylation in the context of cell motility remains largely unexplored.

We recently demonstrated a role for Rho methylation in modulation of actin cytoskeletal organization and cell migration in the well-studied breast cancer cell line MDA-MB 231. Due to the importance of Rho proteins in cell motility, we focused on determining the biological consequences of Icmt-mediated methylation with respect to cell migration. The impact of Icmt inhibition on cell biological processes associated with Rho function, specifically, cell adhesion, morphology and migration was assessed. Both pharmacological and genetic inhibition of Icmt activity severely impaired both random and directed cell migration assessed using a transwell chamber model and a 2-D model performed on cells adhered to glass coverslips. Icmt inhibition also impaired adhesion and cell spreading upon binding to fibronectin. Examination of only adhered cells in the 2-D model revealed that the inhibition of migration was not solely due to impaired adhesion, which would account for the results seen in the transwell migration model, but that Icmt also resulted in a disruption of the actin cytoskeleton in both adhering and migrating cells. In adhering cells in which Icmt was inhibited, the actin cytoskeleton remained unorganized and lacked peripheral actin filaments and stress fiber formation in the interior of the cell, although eventually organized actin cytoskeletal structure would arise. This finding suggested that the cellular machinery is still present to rearrange the actin cytoskeleton, but due to Icmt impairment the process took longer. Although technically difficult, it would be interesting to determine whether the level of Icmt inhibition directly corresponds to the time necessary to complete the various cell biological functions.

In cells that were allowed to adhere overnight, those in which Icmt was inhibited looked similar to mock-treated cells under starvation conditions. However, upon stimulation with fetal bovine serum, there was no reorganization of actin into filopodia and polarization of the cells into a clear leading and trailing edge. Rather, the cells in which Icmt was inhibited resembled unstimulated cells, with elongated morphology and actin localized to both ends of the cell; we interpreted this to indicate a failure in transmitting extracellular signals that affect actin reorganization and polarization. To explore the mechanism underlying this impairment in Rho-driven biology, ligand-mediated activation of RhoA and Rac1 was examined and a significant decrease in activation upon stimulation with thrombin or EGF, respectively, was observed. In agreement with studies noted above, increased RhoGDI binding to both RhoA and Rac1 was observed in the cells treated with the Icmt inhibitor. This increase in RhoGDI binding may explain the impairment of Rho and Rac activation under conditions in which Icmt activity is suppressed.

An important component of the study examining the impact of Icmt inhibition on cell migration was the finding that the negative impact of Icmt inhibition was due, at least in part, to impairment of RhoA and Rac1 function. Ectopic expression of RhoA and Rac1 in inhibitor-treated cells was able to partially rescue directed and random migration, respectively. Given the number of Icmt substrates potentially involved in cell motility, it is significant that these types of experimental approaches can be used to ascribe at least some, if not all, of the effects to a specific subset of the proteins. Attempts were made to further rescue migration by ectopic expression of RhoA and Rac1 together, and also with RhoC, but we could not increase the rescue significantly. It remains quite possible that other Rho proteins such as CDC42 are also involved. In spite of being able to completely classify the CaaX proteins involved, these findings established a role for Icmt-mediated methylation in cell migration and enhanced our understanding of the role that methylation plays in the function of Rho GTPases.

Based on our results and those of others, it has become clear that affinity of RhoGDI for RhoA and Rac1 is affected by the methylation state of Rho proteins, specifically that the unmethylated GTPase binds with higher affinity to RhoGDI resulting in impaired activation of the Rho GTPases (Fig. 2). The interaction of RhoGDI with Rho proteins is complex, dynamic and has several biological ramifications. In addition to activation status, interactions with RhoGDI play a role in the stability of Rho proteins, and in cellular localization of Rho via the ability of RhoGDI to sequester the hydrophobic C-terminus and thereby prevent Rho membrane association. As noted above, there is evidence that at least the former parameter of Rho function is impacted by methylation status. Hence, by affecting the affinity of the RhoGDI interaction, methylation of Rho proteins could play a multiple roles in their regulation. In the known structures of Rho GTPases bound to RhoGDI, the C-terminal prenylcysteine moiety is buried into a hydrophobic pocket on RhoGDI. There is some evidence that manipulating the charge of residues near the C-terminus of Rho GTPases can impact the interaction between RhoGDI. In one such study, phosphorylation of Ser188 of RhoA, which is located two residues from the C-terminal modified cysteine, was found to increase binding to RhoGDI.
It would be interesting to determine if the phosphorylation status of Rho plays a role in RhoGDI binding in the presence and absence of C-terminal methylation. While there are no basic residues in the region of RhoGDI where the C-terminus of the Rho protein binds, there are potential hydrogen bonds that could be formed by the negative charge that results from the lack of methylation of the prenylated cysteine.29 Clearly, further investigation needs to be conducted to fully understand the role methylation plays in RhoGDI interactions with RhoGTPases.

Other than RhoGDI, additional methylation-dependent interactions have yet to be described for Rho proteins. While it is clear that lack of methylation of Rho proteins can negatively impact their ability to be activated by increasing binding to RhoGDI, the potential impact of the lack of methylation on binding downstream effectors has yet to be examined. Since the primary impact of loss of methylation appears to be loss of activation, it would be interesting to see if expression of dominant-active forms of Rho proteins abrogates the effects of Icmt inhibition on Rho-dependent functions. While CAAX modifications have been shown to affect the interaction of some Rho proteins and their exchange factors,32,33 a careful assessment of the contributions of each stage of modification has not been made. To fully address these questions, biochemical studies with purified proteins will be required. Finally the role of methylation for other CaaX proteins such as Rap1 has yet to be examined in the context of cell migration. With the increasing interest in targeting Icmt in cancer therapeutic strategies, our findings have highlighted the need for further investigation into the role of Icmt-catalyzed methylation in cancer cell metastasis.

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