Role of Isoprenylcysteine Carboxylmethyltransferase-catalyzed Methylation in Rho Function and Migration*

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A number of proteins that play key roles in biological regulatory events undergo a process of post-translational modifications termed prenylation. The prenylation pathway consists of three enzymatic steps; the final processed protein is isoprenoid-modified and methylated on the C-terminal cysteine. This protein modification pathway plays a significant role in cancer biology because many oncogenic proteins undergo prenylation. Methylation of the C terminus by isoprenylcysteine carboxylmethyltransferase (Icmt) is the final step in the prenylation pathway. Cysmethynil, a specific Icmt inhibitor discovered in our laboratory, is able to inhibit Ras-mediated signaling, cell growth, and oncogenesis. We sought to examine the role of Icmt-mediated methylation on the behaviors of cancer cells associated with metastatic potential. Our results indicate that inhibition of methylation reduces migration of the highly metastatic MDA-MB-231 breast cancer cell line. In addition, cell adhesion and cell spreading are also significantly impacted by cysmethynil. To examine the mechanism of Icmt-dependent migration we focused on RhoA and Rac1, prenylated proteins that are important mediators of cell migration through their control of the actin cytoskeleton. Inhibition of Icmt significantly decreases the activation of both RhoA and Rac1; an increase in Rho GDP-dissociation inhibitor (RhoGDI) binding in the absence of methylation appears to contribute to this effect. Furthermore, in the absence of Icmt activity the addition of exogenous RhoA or Rac1 is able to partially rescue directed and random migration, respectively. These findings establish a role for Icmt-mediated methylation in cell migration and advance our understanding of the biological consequences of Rho methylation.

Post-translational modifications of proteins play vital roles in many aspects of cell biology. Hence, identifying and understanding the biological impact of these processes is crucial to furthering our basic understanding of how cells function. Numerous proteins that control important biological regulatory events undergo a complex series of post-translational modifications that are directed by the presence of a so-called CaaX motif at their C terminus. This post-translational pathway, termed protein prenylation, 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)2 or protein geranylgeranyltransferase-I (GGTase-I), respectively (3). 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. Finally, the prenylated cysteine, which is now located at the C terminus, is methylated by isoprenylcysteine carboxylmethyltransferase (Icmt), another integral ER membrane protein (4, 5). The final result of these modifications is a protein that contains a prenylated and methylated cysteine at its C terminus. 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 (1, 6, 7). Thus, it is clear that CaaX processing is necessary for the biological activities of these proteins.

The prenylation pathway has been targeted for potential anticancer therapy because most members of the Ras superfamily, which contains many known oncogenes, undergo CAAAX processing. The Ras superfamily consists of five large subfamilies; the two most well-characterized are the Ras and Rho subfamilies (8). Both Ras and Rho proteins are processed by the CaaX pathway; Ras family members are farnesylated, while most Rho family members are geranylgeranylated. These monomeric GTPases cycle between a GDP-bound inactive state and a GTP-bound active state. In their active states, Ras and Rho subfamily members control numerous cell signaling pathways that are involved in cell proliferation, differentiation, migration, polarity, and morphology (9).

Abnormally high activity of Ras and Rho signaling pathways contribute to initiation and progression of many types of cancer (10, 11). For example, many breast cancers that are highly metastatic express abnormally high levels of Rho proteins (12). Rho proteins control migration and invasion of cells by tightly coordinating changes in the actin and microtubule cytoskeletons. Acting through their effectors, Rho proteins rearrange the actin

2 The abbreviations used are: FTase, protein farnesyltransferase; GGTase-I, protein geranylgeranyltransferase type I; Rce1, the CaaX protein-specific protease; Icmt, isoprenylcysteine carboxylmethyltransferase; RhoGDI, Rho GDP-dissociation inhibitor; cysmethynil, 2-(5-(3-methylphenyl)-1-octyl-1H-indol-3-yl)acetamide; ER, endoplasmic reticulum; ECM, extracellular matrix; FBS, fetal bovine serum; PBS, phosphate-buffered saline; HA, hemagglutinin; GST, glutathione S-transferase; EGF, epidermal growth factor; DAPI, 4′,6-diamidino-2-phenylindole.

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cytoskeleton to respond to chemo-attractant gradients, polarize cells, and control migration and invasion. While cell migration is necessary for development, leukocyte function, and other normal cell biologies, dysregulation of migration and invasion results in cancer metastasis (13). Metastasis is an important and deadly progression of cancer and understanding the biology of migrating cancer cells is crucial for therapeutic targeting of this aspect of cancer.

Pharmacologic targeting of the enzymes involved in the CaaX-processing pathway has emerged as a promising anticancer strategy. In particular, there has been much effort in designing inhibitors against the protein prenyltransferases, most notably FTase (14, 15). There is also recent evidence that inhibition of geranylgeranylation of Rho proteins also impacts oncogenesis and metastasis (16–18). However, the overall success of the FTase inhibitors (FTIs) in the clinical setting has been somewhat disappointing. One possible reason is a phenomenon termed “alternate prenylation” in which some FTase substrates, most notably K- and N- Ras, are modified by GGTase and escape inhibition by FTIs (19–21). Because the Rce1 protease and Icmt methyltransferase act on all CaaX proteins, problems such as alternate prenylation would not arise if these enzymes were targeted. Hence, while protein prenyltransferase inhibitors still show some promise as anticancer agents, the emerging view that 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.

While the biological consequences of prenylation are fairly well understood, the precise roles of C-terminal methylation in CaaX protein function are still elusive. Depending on the CaaX protein, methylation has been ascribed to roles in localization, protein-protein interactions and protein stability (11). The development of an Icmt knock-out mouse model has furthered our understanding of Icmt function (22, 23). Localization studies conducted in cells with genetically deleted Icmt have shown that methylation is important for proper membrane association of Ras proteins. However, the localization of Rho proteins in the absence of Icmt activity appears to be more complicated and may vary depending on family member and activation status (24–26). Importantly, inhibition of CaaX protein methylation via either genetic or pharmacologic targeting has shown a clear impact on oncogenic transformation and tumor growth (23, 27, 28).

Defining the role of Icmt-mediated methylation in complex cellular behaviors such as migration and invasion is crucial for furthering our understanding of the impact of CaaX protein methylation on the biology of normal and cancer cells. In the current study, we have assessed the impact of Icmt inhibition on cell biological processes associated with the function of Rho proteins, specifically cell adhesion, morphology, and migration. We found that inhibition of Icmt results in a disruption of the actin cytoskeleton and impairs ligand-mediated activation of RhoA and Rac1, a potential consequence of increased RhoGDI binding to both RhoA and Rac1 when their methylation is impaired. Further, we show that the impact of Icmt inhibition on cell migration is due at least in part to impairment of RhoA and Rac1 function. These findings establish a role for Icmt-mediated methylation in cell migration and further elucidate the role that methylation plays in the function of Rho GTPases.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human thrombin was from Enzyme Research Laboratories (South Bend, IN). The human breast cancer line MDA-MB-231 was obtained from the American Type Culture Collection and grown under the recommended conditions. Cysmethylin and biotin S-farnesylcysteine were synthesized by the Duke Small Molecule Synthesis Facility via established methods (28). Cysmethyl analog 1-octyl-mtolyl-1H-indole (J3) was synthesized and characterized previously (27). Cysmethyl and J3 stock solutions were prepared at 30 mM in DMSO and stored at −20 °C. siRNA oligos directed against Icmt and Luciferase were obtained from Ambion and Thermo Scientific Dharmacon, respectively. Recombinant human thrombin was from Enzyme Research Laboratories. Recombinant EGF was obtained from Calbiochem. The antibody recognizing RhoA was obtained from Santa Cruz Biotechnology. Antibodies recognizing Rac1 and RhoGDI were from BD-Transduction Laboratories. Rhodamine-conjugated phallolidin was from Fluka.

siRNA Transfections—MDA-MB 231 cells were plated at 100,000 cells per well in 6-well dishes 24 h prior to transfection. Stock solutions were prepared of Icmt siRNA oligonucleotides (Ambion) or Luciferase siRNA (Thermo Scientific Dharmacon) at 20 μM. Following the manufacturer’s instructions, the oligonucleotides were mixed with Opti-Mem I medium (Invitrogen) and then incubated with oligofectamine (Invitrogen) prior to transfection. Cells were harvested and assayed conducted ~130 h post-transfection.

Methylation Assays—Icmt activity was determined by a modification of the in vitro assay described previously (29, 30). Briefly, cells were harvested in lysis buffer (100 mM Hepes, pH 7.5, 5 mM MgCl2, 1 mM dithiothreitol supplemented with protease inhibitors) and then lysed by sonication. NaCl was added to a final concentration of 150 mM, and then the lysate was precleared by centrifugation 5,000 × g for 5 min. Membranes were isolated by ultracentrifugation 100,000 × g for 30 min, resuspended in lysis buffer plus 150 μM NaCl, and protein concentrations were determined by colorimetric analysis (Bio-Rad Protein Assay™). Icmt activity was assessed by using equal amounts of membrane protein using biotin S-farneslycysteine and S-[3H]adenosylmethionine as substrates.

Adenoviruses—The recombinant adenoviruses were constructed by subcloning human wild-type HA-RhoA, and HA-Rac1, into the Adtrack-CMV vector (gift of Dr. Bert Vogelstein, Johns Hopkins University Medical Center, Baltimore, MD) and then recombining these with pAdEas-1 in the BJ5183 strain of Escherichia coli (Stratagene, La Jolla, CA). The resulting DNA was transfected into HEK 293 cells with Lipofectamine (Invitrogen), and the viruses were purified using Adeno-X™ virus purification kits (BD Biosciences).

Preparation of GST Fusion Proteins—The GST-rhotekin-RBD construct was obtained from Robert Lefkowitz (this institution) and the GST-PAK-Crib construct was obtained from Gary Bockoch (Scripps Research Institute, La Jolla, CA). The GST-rhotekin-RBD and GST-PAK-Crib proteins were pro-
duced in the BL21DE3 strain of *E. coli* (Invitrogen). Briefly, starter cultures from a transformed bacterial colony were grown for 16 h and then used to inoculate 1 liter of LB and grown at 37 °C until the optical density reached 0.6–0.8. At this point, the cells were induced with 1 mM isopropyl-β-thiogalactopyranoside (Sigma), and cultures were grown for an additional 4 h at 37 °C. The cells were harvested by centrifugation for 15 min at 6,000 × g at 4 °C, and the resulting pellet was resuspended in 20 ml of rotekin buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, and Complete Mini™, EDTA-free protease inhibitor mixture tablets (Roche Applied Science)) or GST-FISH buffer (25 mM Heps pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 10% glycerol, 10 mM MgCl₂, and protease inhibitor tablets). The cells were then lysed, and lysates were cleared by centrifugation at 100,000 × g for 1 h. The resulting supernatant was incubated with glutathione-Sepharose 4B beads (Amersham Biosciences) equilibrated in buffer rotekin buffer or GST-FISH buffer for 2 h at 4 °C with continuous rocking. Finally, the beads were washed three times in rotekin buffer or GST-FISH buffer. After the final wash, the beads were resuspended in rotekin buffer plus 10% glycerol or GST-FISH buffer and stored in aliquots at −80 °C. Protein concentration was determined by SDS-PAGE with Coomassie Blue staining, using bovine serum albumin as a protein standard.

**Cell Culture and Cysmethinil Treatment**—MDA-MB-231 human breast cancer cells were maintained at 37 °C with 5% CO₂ in DMEM (Cellgro) supplemented with 5% FBS (Atlas), 10 μg/ml insulin (Invitrogen). For all assays, cells were plated 50,000–60,000 cells/ml for cysmethinil treatment and 25,000–30,000 cells/ml for DMSO (control) 18–24 h prior to treatment. Cells were then treated for 72 h with 25 μM cysmethinil (unless otherwise indicated) or control, then placed into starvation medium (DMSO with 0.1% bovine serum albumin, 10 mM HEPES) with 5 μM cysmethinil 18–24 h. To perform assays, cells were washed with PBS and incubated in Cell-Stripper™ (Cellgro), then resuspended in starvation medium. To remove excess Cellstripper, cells were collected by brief centrifugation (1000 × g for 5 min) then resuspended in starvation medium. Then cells were counted with a hemocytometer, and trypan blue (GIBCO) exclusion was utilized to determine viable cells.

**Cell Migration Assay (Transwell)**—For migration assays, transwell chambers (8 μm pore size, polycarbonate filters, 6.5 mm diameter; Costar) were coated with fibronectin (5 μg/ml) for 2 h at 37 °C. Cysmethinil-treated or control (DMSO-treated) MDA-MB-231 cells were starved 18–20 h, then resuspended and counted as described above. Then 2 × 10⁴ cells were plated on top of the transwell and allowed to migrate toward starvation medium or medium containing 5% FBS. After 3 h, cells were stained using the Hema 3 staining kit (Fisher Scientific), and cells on the upper chamber that failed to migrated were removed with cotton swabs. The remaining cells on the bottom chamber were counted under a Nikon TS100 microscope. Four fields using a 20× objective were counted for each transwell, and duplicate transwells were averaged for each data point.

**Cell Migration Assay (Fluorescent Bead)**—To determine individual cell migration utilizing phagocytosis of fluorescent beads the following protocol was adapted from Ref. 31. Glass coverslips (12-mm round Fisherbrand) were coated with 10 μg/ml fibronectin in PBS for 1 h at 37 °C. Then coverslips were coated with 0.025% fluorescent beads (Invitrogen) for 1–2 h at 37 °C. Cells were treated were cysmethinil or the vehicle DMSO, harvested, counted as described above, and then 500 cells/ml (control) or 750 cells/ml (cysmethinil) plated at 1 ml/coverslip in a 24-well dish. Following 1 h of incubation at 37 °C, cells were stimulated with FBS (5% final concentration), allowed to migrate for 15–18 h, and then fixed by addition of 4% paraformaldehyde. The cells were then permeabilized with 0.1% Triton X-100, and rhodamine-conjugated phalloidin (Fluka) was utilized to visualize actin. The coverslips were washed and affixed to slides with mounting medium containing DAPI (Molecular Probes) to visualize nuclei. Images were obtained with a Zeiss Axio Imager fluorescence microscope and analyzed using Metamorph software. To quantitate cell migration, the Metamorph software was used to define cleared regions, and the average area cleared per cell was calculated and plotted as a percentage of untreated cells. To quantitate leading edge morphology and elongated cell morphology, the total number of cells per coverslip was counted, and leading edge morphology assigned to cells displaying movement due to phagocytosis of beads and showing clear polarity as typified by clear tapering of the trailing edge and broadening of the leading edge. Elongated morphology was assigned to cells displaying equal tapering at both ends.

**Adhesion and Cell Spreading Assays**—To perform adhesion assays MDA-MB 231 cells were treated for 4 days with cysmethinil or vehicle (DMSO). Cells were harvested as described above then 25,000 cells/well were then dispensed into 96-well plates coated with fibronectin (10 μg/ml) and allowed to adhere for 1 h. Non-adherent cells were removed by washing with medium. The cells were then fixed and stained with crystal violet. After washing and drying, the amount of adhered cells was quantitated by eluting the crystal violet with methanol and reading the absorbance at 550 nm. Cell spreading assays were conducted by treating MDA-MB-231 cells with cysmethinil or DMSO as described above. Following starvation, cells were harvested, dispensed onto coverslips coated with fibronectin (10 μg/ml), and allowed to adhere. After an additional 1.5 h, coverslips were washed with PBS to remove non-adherent cells, then the remaining cells were fixed with paraformaldehyde. Actin was visualized with rhodamine phalloidin.

**RhoA and Rac1 Activation Assays**—Following cysmethinil treatment and serum starvation as indicated in the appropriate figure legend, MDA-MB-231 cells were stimulated with agonist (1 unit/ml thrombin (Enzyme Research Laboratories) for 15 min for RhoA activation or 100 ng/ml EGF (Calbiochem) for 15 min for Rac1 activation). Following stimulation, cells were washed with PBS, harvested in MLB buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10 mM MgCl₂, 1 mM EDTA, 2% glycerol) or GST-FISH buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 10% glycerol, 10 mM MgCl₂) respectively, then incubated for 5 min to ensure complete lysis. The lysates were then
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cleared by centrifugation (15,000 \( \times g \) for 5 min). Rho activation assays were performed as previously described (32). Rho-GTP in cell lysates was precipitated using the GST fusion of the activated Rho-binding domain Rhotekin and detected by separation on SDS-PAGE followed by immunoblot analysis with a monoclonal Rho-specific antibody (Santa Cruz Biotechnology). Rac1 activation assays were similarly performed; however GST-FISH buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 10% glycerol, 10 mM MgCl2) was utilized, and Rac1-GTP was precipitated from cell lysate using a GST fusion of the activated Rac1-binding domain Pak-Crib. Rac1 was detected by immunoblot analysis with a monoclonal antibody specific to Rac1 (BD-Transduction Labs).

**RhoGDI Binding Assay**—MDA-MB-231 cells were treated for 4 days with 25 \( \mu M \) cysmethylin or vehicle (DMSO). Adenovirus expressing HA-tagged RhoA, Rac1, or GFP (vector) was incubated with the cells the final 24 h of treatment. Following cysmethylin treatment, cells were washed with PBS, harvested in lysis buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10 mM MgCl2, 1 mM EDTA, 2% glycerol), then incubated for 5 min. The lysates were then cleared by centrifugation (15000 \( \times g \) for 5 min), and protein concentrations determined by colorimetric analysis (Bio-Rad Protein AssayTM). Co-immunoprecipitation of the HA-tagged GTPase and RhoGDI were performed utilizing anti-HA agarose to bind the expressed HA-RhoA or Rac1. Levels of bound RhoGDI were determined by separation with SDS-PAGE followed by immunoblot analysis using a monoclonal antibody specific to RhoGDI (BD-Transduction Labs). Immunoblot analysis and quantitation was performed using the Odyssey System (LICOR, Lincoln, Nebraska) according to the manufacturer’s instructions.

**RESULTS**

**Icmt Inhibition Impairs Cell Migration in a Dose-dependent Manner**—To assess the role of Icmt-mediated CaaX protein methylation in cell migration, we chose the well-studied MDA-MB 231 cancer cell line. Typical of many breast cancers, MDA-MB 231 cells exhibit high levels of RhoA expression and are very motile, making them ideal for studying the impact of RhoGTPase methylation on migration. To determine whether Icmt-mediated methylation is necessary for cell migration, we initially conducted transwell migration assays. Following starvation, cells were harvested, and equal numbers placed in the upper wells of transwell chambers. Cysmethylin-mediated inhibition of Icmt impairs cell growth and at higher doses results in cell death (data not shown). Because of the long halflife of Rho proteins (23), we chose to treat the cells for at least 72 h to maximize the pool of unmethylated Rho proteins. To ensure that only live cells were assayed we chose time points and concentrations of cysmethylin that resulted in minimal cell death and trypan blue dye exclusion was a criteria for counting. To assess random migration, starvation medium was placed on both sides of the transwell, while for directed migration, medium containing FBS was placed in the lower chamber creating a chemoattractant gradient. Stimulation with FBS resulted in an approximate 6-fold increase of migration as compared with random migration (not shown). Cysmethylin treatment of the MDA-MB-231 cells significantly impacted both random and directed migration in a dose-dependent manner (Fig. 1, A and B). Random migration (Fig. 1A) was slightly more sensitive than directed migration (Fig. 1B), with an \( \sim 70\% \) decrease in random migration observed in the presence of 15 and 20 \( \mu M \) cysmethylin as compared with the control; treatment with 25 \( \mu M \) cysmethylin resulted in an 80% decrease in random migration. Directed migration was also clearly inhibited in a dose-dependent manner (Fig. 1B), with a 60, 80, or 90% decrease in cell migration with 15, 20, and 25 \( \mu M \) cysmethylin treatment, respectively, compared with control cells. To ensure that the effects of cysmethylin were mechanism-based, a structurally similar, inactive analog, J3 (27), was used as a control. Both random and directed cell migration were minimally reduced in the presence of 25 \( \mu M \) J3 (Fig. 1, A and B), indicating mechanism-based action of cysmethylin.

To confirm the role of Icmt in cell migration, a genetic inhibition strategy was performed by using siRNA oligonucleotides targeted to Icmt. Icmt activity was reduced \(~ 50\%\) in MDA-MB-231 cells treated with the siRNA (Fig. 1C), and an impact similar to that of cysmethylin treatment was observed with random migration inhibited by 45% (Fig. 1D) and directed migration by 35% (Fig. 1E) compared with control cells transfected with control oligonucleotides. Because the transwell migration assay also depends on the ability of cells to adhere to the upper chamber, we utilized a fluorocescent bead assay to directly examine cell movement. Cells were plated onto glass coverslips that had been coated with fluorescently labeled beads. After the cells adhered, they were stimulated with FBS to induce chemokinesis. As the cells move, they phagocytose the fluorescent beads, leaving cleared paths tracing their movement. Quantitation of the bead migration assay can be accomplished by measuring the area cleared by moving cells. The advantage of this assay is that only cells that have adhered are assessed, so cell movement is measured independently of the initial adhesion step. DMSO-treated control cells showed clear movement, while following treatment with 25 \( \mu M \) cysmethylin, stimulated cell movement was greatly reduced (Fig. 1F). Quantitation of the data revealed that, in the presence of cysmethylin migration was inhibited by 60% as compared with control cells (Fig. 1G). These results, along with those of the transwell migration assays, demonstrate that inhibition of Icmt with cysmethylin severely impairs both random and directed cell movement.

**Inhibition of Icmt Impairs Cell Adhesion and Disrupts the Actin Cytoskeleton**—Cell migration is a complex cellular process that requires multiple coordinated steps involving interactions with the extracellular matrix (ECM) and subsequent changes in the actin cytoskeleton. One important step is cell adhesion to ECM proteins. To determine if Icmt activity is necessary for this type of cell adhesion, we assessed the ability of MDA-MB 231 cells to bind to fibronectin following cysmethylin treatment. Inhibition of Icmt resulted in an \(~ 40\%\) loss of adhesion to fibronectin as compared with vehicle-treated cells (Fig. 2A). Upon cell binding to ECM proteins, integrins cluster and multiple signaling pathways are activated in cells. Rho GTPases are activated subsequent to adhesion and the actin cytoskeleton is rearranged, causing a change in cell morphology from a rounded shape to a more flattened shape, in a process.
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FIGURE 1. Icmt inhibition impairs cell migration. A and B, migration of the breast cancer cell line MDA-MB-231 in the presence of the Icmt inhibitor cysmethynil (Cysmeth). Cells were treated for 3 days with indicated concentrations of cysmethynil, an inactive structural analog (J3), or vehicle (Cont.) as indicated, and then starved in serum-free medium containing 5 μM Cysmeth, J3 or vehicle for 18 h. Cells were harvested and transwell migration assays were conducted as described under “Experimental Procedures.” Either starvation medium (panel A, random migration) or medium containing 5% FBS (panel B, directed migration) was placed in the bottom chamber to assess the two types of migration. Data are presented as mean ± S.E. of duplicate transwells from a single experiment that is representative of three such experiments. C, siRNA-mediated knockdown of Icmt. MDA-MB-231 cells were transfected with siRNA oligonucleotides directed against Icmt or control oligonucleotides. On the 6th day, cells were harvested, membranes isolated, and Icmt activity determined as described under “Experimental Procedures.” Data shown are the mean ± S.E. of four determinations for each condition. D and E, cell migration in the presence of siRNA-mediated knockdown of Icmt. MDA-MB-231 cells were transfected with siRNA or control oligonucleotides as described above. Five days after transfection, the cells were starved in serum-free medium for 18 h, harvested, and transwell migration assays were conducted as described above. F and G, stimulated cell migration in the absence or presence of Icmt inhibition. MDA-MB-231 cells were treated 3 days with 25 μM cysmethynil (Cysmeth) or the vehicle DMSO (Cont.). On the 3rd day, cells were harvested in serum-free medium for 18 h, and on the 5th day, cells were plated onto coverslips coated with fibronectin and fluorescein beads as described under “Experimental Procedures.” After allowing to adhere for 1 h, cells were stimulated by adding FBS (final conc. 5%) and then incubated for an additional 16 h. Cells were then fixed with paraformaldehyde and processed for immunofluorescence. Representative images are showing cleared paths formed by cell migration (F). Quantitative data (G) is presented as the mean ± S.E. of the area cleared per cell, n = 20 for each condition.

F. Fluorescent-bead assay

G. Quantitation

remained cells were in the process of spreading (arrow 2). In contrast, most of the cysmethynil-treated cells (Fig. 2C) were still completely rounded (arrow 3). In the presence of cysmethynil, fewer cells spread subsequent to fibronectin binding, suggesting an inhibition of downstream signaling impacting on actin cytoskeletal rearrangement.

Because migration and cell spreading are dependent on actin cytoskeletal rearrangement, we next wanted to examine the effect of Icmt inhibition on the actin cytoskeleton in both adhering and migrating cells. To examine adhering cells, MDA-MB-231 cells were treated with cysmethynil or DMSO, incubated on fibronectin-coated coverslips for 90 min, and then the actin cytoskeleton was visualized with rhodamine phalloidin. Under these conditions, control DMSO-treated cells show organized actin cytoskeletal structures, with actin filaments around the periphery of the cell and the presence of long organized fibers in the interior (Fig. 3A). In contrast, the majority of cysmethynil-treated cells show a lack of actin organization, with unstructured actin staining in the interior of the cells (Fig. 3B; indicating that Icmt inhibition impairs actin organization subsequent to ECM adhesion. To more directly investigate the status of the actin cytoskeleton during migration, we utilized the UV-bead assay described above. Cells were incubated for ~16 h on glass coverslips that had been coated first with fibronectin and then with fluorescently labeled beads (blue spheres). Cells were visualized by staining actin with rhodamine-phalloidin (red). In non-stimulated control cells, essentially no movement of the cells is observed (Fig. 3C). In contrast, DMSO-treated cells stimulated with FBS showed clear movement, indicated by phagocytosis of beads (Fig. 3D), while cysmethynil-treated cells did not respond to FBS stimulation, since no cleared paths through the fluorescent beads were observed (Fig. 3E). The absence of movement in FBS-stimulated cysmethynil-treated cells is similar to that observed in non-stimulated control cells (Fig. 3C). To examine actin cytoskeletal structure, higher magnification images of the cells in the insets (white boxes) were
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In the absence of stimulation, cells displayed an elongated morphology and strong actin staining at both ends of the cell (Fig. 3F). During stimulation induced migration, actin morphology showed extended filopodia at the leading surface and organized actin along the cell periphery (Fig. 3G). Cysmethynil-treated cells failed to respond to stimulation, and the actin cytoskeleton remained elongated and resembled unstimulated control cells (Fig. 3H). Quantification of leading edge morphology (see Fig. 3G) under stimulated conditions revealed that there were approximately twice as many control cells displaying leading edge morphology as compared with cysmethynil-treated cells (Fig. 3I). Conversely, quantification of the elongated morphology seen in the cells in Fig. 3F and H revealed that 59% of cysmethynil-treated cells were elongated as compared with 11% of control cells (Fig. 3F). These observations indicate that in both adhering cells and migrating cells, signaling pathways that result in actin reorganization are impaired when Icmt activity is inhibited.

Icmt Inhibition Impairs RhoGTPase Activation—Having established that cysmethynil-mediated disruption of actin cytoskeletal function impacts cell migration, adhesion, and spreading, we sought to determine a mechanism for these consequences of Icmt inhibition. Because an established role of Rho GTPases is remodeling of the actin cytoskeleton, and these proteins are subject to processing by Icmt, they were attractive candidates to pursue. To determine if methylation of Rho and Rac could influence their activation status, cells were first treated with cysmethynil to inhibit Icmt and then stimulated with thrombin to activate Rho or epidermal growth factor (EGF) to activate Rac1. GTP-bound RhoA was then selectively precipitated from cell lysates using the GST fusion of the Rho binding domain (RBD) of rhotekin, while the GST-PAKcrib fusion was utilized to precipitate activated Rac1. As shown in Fig. 4A, cysmethynil treatment dramatically impaired thrombin-mediated RhoA activation. Similar results were observed when ectopic expression of dominant-active Ga12 was used to stimulate Rho activation (data not shown), indicating that the impairment of thrombin signaling was not due to lack of methylation of receptor-coupled heterotrimeric G proteins. Rac activation was also impaired in cells in which Icmt was inhibited (Fig. 4B). In DMSO-treated cells, EGF stimulation increased the levels of Rac1-GTP by 2–3-fold, while cysmethynil treatment completely eliminated EGF-mediated activation of Rac1. These data indicate that C-terminal methylation is required for optimal Rho and Rac activation, thus providing insight into the mechanism of disruption of the actin cytoskeletal rearrangements in cysmethynil-treated cells.

Icmt Inhibition Increases RhoGDI Binding to RhoA—The finding that Icmt-mediated carboxyl methylation plays a significant role in Rho and Rac activation prompted us to explore potential mechanisms that could underlie the impaired activation in the absence of methylation. Numerous proteins control activation of RhoGTPases. RhoGDIs bind Rho proteins in the GDP-bound inactive state. While mostly characterized for their ability to facilitate the removal of Rho proteins from cellular membranes, RhoGDIs also sequester Rho proteins away from their GEFs and hence maintain them in the GDP-bound state (33). A previous study utilizing cells in which the Icmt gene had been disrupted showed that, in the absence of methylation, Rac association with RhoGDI was enhanced (34). This observation led us to hypothesize that the decrease in Rho activation may be due to increased binding of RhoGDI in the absence of Rho methylation. To test this hypothesis, we performed co-immunoprecipitation experiments employing HA-tagged Rho proteins. Cells were treated with cysmethynil and then transduced with adenovirus carrying either HA-tagged RhoA and GFP, or only GFP. The cells were lysed and RhoA was immunoprecipitated with an anti-HA antibody, bound proteins separated by SDS-PAGE, and the presence of RhoGDI in the immunoprecipitates was determined by immunoblotting with a specific antibody. Through this approach, we found that inhibition of Icmt-catalyzed methylation increased RhoGDI binding to RhoA (Fig. 5A). In the absence of HA-tagged RhoA, no RhoGDI was immunoprecipitated with the anti-HA antibody as demonstrated by the GFP control (Fig. 5A). In the vehicle control RhoGDI bound to RhoA, while treatment with cysmethynil resulted in increased binding of RhoGDI to RhoA (Fig. 5A). Quantification of band intensities for two separate experiments revealed that treatment with cysmethynil resulted in a 50% increase in RhoGDI binding to RhoA as compared with DMSO treatment (Fig. 5A). Similar, although less dramatic results were observed with Rac1 (Fig. 5B), cysmethynil treatment resulted in a 30% increase in its binding to RhoGDI. Because RhoGDI has been shown to inhibit activation of Rho proteins, these results indicate a potential mechanism for the impact of Icmt inhibition on RhoGTPase activation. In the absence of carboxyl methylation, RhoGDI binds more tightly to RhoA and Rac1, inhibiting their interactions with, and subsequent activation by particular GEFs.
Overexpression of RhoGTPases Partially Rescues Migration Impaired by Icmt Inhibition—The finding that inhibition of Icmt increased RhoGDI binding to Rho GTPases and inhibited Rho activation, led us to hypothesize that increased expression of Rho proteins may overcome this inhibition and rescue the migration phenotype in the presence of cysmethynil. To assess this scenario, we again utilized recombinant adenovirus to overexpress wild-type Rho proteins in MDA-MB 231 cells, and then measured cell migration to determine if the overexpression of RhoGTPases could protect cells from the effect of Icmt inhibition on migration. Cells were treated with cysmethynil or DMSO and infected with virus expressing RhoA, Rac1 or GFP (as a control). Overexpression of RhoA had no effect on random migration (Fig. 6A), but was able to markedly rescue directed migration in the presence of cysmethynil (Fig. 6B). Elevating RhoA level in this manner resulted in a 3-fold increase of directed cell migration in the presence of cysmethynil as compared with cells expressing only GFP. Interestingly, a converse effect was observed upon overexpression of Rac1; elevating the level of this Rho GTPase resulted in a 3-fold increase of random migration in cysmethynil-treated cells (Fig. 6C), while directed migration remained unaffected (Fig. 6D). Attempts to increase the amount of rescue by increasing protein expression resulted in impaired migration (data not shown), indicating the importance of the balance of protein levels and regulation of activity between the Rho family members. These results demonstrate that increased expression of Rho GTPases can partially rescue migration in the absence of Icmt activity, and support the hypothesis that impaired activation of Rho GTPases is an important component in the impairment of cell migration observed upon Icmt inhibition.

DISCUSSION

Inhibition of the enzyme responsible for post-translational methylation of CaaX proteins, Icmt, has emerged as a promising target for cancer therapy. The prenylation pathway has been an attractive target for many years due to the prominent role that aberrant activation of Ras plays in...
mediated activation of RhoA. Cells were treated with thrombin (DMSO (Cont.) or with the vehicle DMSO (−) as indicated. During the final 24 h of treatment, recombinant adenovirus carrying HA-tagged RhoA or GFP was incubated with the cells as indicated. Cells were harvested, lysed, and then co-immunoprecipitation performed utilizing anti-HA-agarose to precipitate HA-RhoA. Levels of bound RhoGDI were determined by immunoblot analysis using a RhoGDI antibody. Quantitative representation of binding is presented in the lower panel. A, RhoGDI binding to RhoA. MDA-MB-231 cells were treated 4 days with 25 μM cysmethynil (Cysmeth) (−) or with the vehicle DMSO (−) as above for 4 days. During the final 24 h of treatment, recombinant adenovirus carrying HA-tagged Rac1 or GFP was incubated with the cells as indicated. Cells were harvested, lysed, and then co-immunoprecipitation performed utilizing anti-HA-agarose to precipitate HA-Rac1. Levels of bound RhoGDI were determined as above. Quantitative representation of binding is presented in the lower panel. B, RhoGDI binding to Rac1. MDA-MB-231 cells were treated with 25 μM cysmethynil (−) or DMSO (−) as above for 4 days. During the final 24 h of treatment, recombinant adenovirus carrying HA-tagged Rac1 or GFP was incubated with the cells as indicated. Cells were harvested, lysed, and then co-immunoprecipitation performed utilizing anti-HA-agarose to precipitate HA-Rac1. Levels of bound RhoGDI were determined as above. Quantitative representation of binding is presented in the lower panel. For both panels, quantitative data are presented as mean ± S.E. from two independent experiments.

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Oncogenesis. Recent work by our laboratory and others has highlighted the potential of Icmt as a therapeutic target. However, many basic biological questions concerning C-terminal methylation of CaaX proteins remain unanswered. For instance, while Ras oncogenes have been the primary focus of those developing prenylation pathway inhibitors, there is evidence that Icmt inhibition may inhibit oncogenesis by impairing Rho proteins (23). Hence, we felt it was important to examine the role Rho methylation plays in the behavior of cancer cells, particular behavior relevant to metastasis given the increasing evidence for a role for Rho GTPases in this process.

Pharmacologic and genetic inhibition of Icmt activity greatly impaired cell migration of MDA-MB-231 cells (Fig. 1), a highly metastatic breast cancer cell line. A decrease in cell adhesion and cell spreading was also observed when Icmt activity was impaired (Fig. 2), and these effects were accompanied by a disruption of actin cytoskeleton structure in both adhering and migrating cells (Fig. 3). While untreated migrating cells showed a clear actin arrangement, with filopodia protruding from the leading edge and strong actin filaments anchoring the rear of the cell, cysmethynil-treated cells that were stimulated with serum looked very similar to control cells in the absence of stimulation. These results indicated a loss of ability of the cells to detect signaling inputs and initiate migration.

Cell migration requires the coordinated signaling and activity of numerous proteins and pathways. While investigating possible mechanisms for cysmethynil inhibition of migration, we observed that inhibition of Icmt markedly impaired the ability of Rho and Rac activation by thrombin and EGF, respectively (Fig. 4). Previous studies have also seen a link between Icmt activity and RhoGTPase function (34, 35), and some of these effects have been ascribed to a reduced stability of Rho proteins when their methylation is impaired (23). In genetically null cells, derived from Icmt knock-out mice, lower levels of Rho protein are observed (23). In agreement with those studies, we also see a decrease in Rho protein levels after 5–6 days of treatment with cysmethynil (data not shown). However, we observed an inhibition of cell migration at doses and treatment times of the Icmt inhibitor in which no significant effect on Rho levels was seen, indicating that simply decreasing the pool of Rho proteins is not the primary mechanism for the reduction in Rho signaling. Rather, our data suggest that, at least in the MDA-MB-231 cell model, the inhibition of Rho and Rac activation is due to increased RhoGDI binding in the absence of Rho GTPase methylation (Fig. 5). This assessment is also consistent with a previous report showing an increase in Rac binding to Rho GDI in Icmt-deficient MEFs (34). Interestingly, there is evidence that phosphorylation of Ser-188 of RhoA increases its binding to RhoGDI (36). Because Ser-188 is located two res-
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FIGURE 6. Rho proteins partially rescue cell migration impaired by inhibition of Icmt. A and B, RhoA rescues directed migration. MDA-MB-231 cells were treated for 3 days with 25 μM cysmethinyl (Cysmeth) or equivalent volume of the vehicle DMSO (Cont) and then starved in serum-free medium containing 5 μM Cysmeth or vehicle for 18 h. Cells were incubated with recombinant adenovirus carrying HA-tagged RhoA or GFP as indicated, 24 h prior to starvation. Cells were harvested and transwell migration assays were conducted as described under “Experimental Procedures.” Either starvation or random migration (Cysmeth) was placed in the bottom chamber to assess the two types of migration. C and D, Rac1 rescues random migration. MDA-MB-231 cells were treated as described above. Cells were incubated with recombinant adenovirus carrying HA-tagged Rac1 or GFP as indicated, 24 h prior to starvation. Then cells were harvested and transwell migration assays were conducted as described under “Experimental Procedures.” Random migration (C) and directed migration (D) were assessed as described above. Data are presented as mean ± S.E. of duplicate transwells from a single experiment that is representative of three such experiments.

idues from the C-terminal-modified cysteine, it is possible that the negative charge that results from the lack of methylation of the prenylated cysteine mimics phosphorylated Ser-188 and increases binding to Rho GDI. Clearly, further investigation needs to be conducted to fully understand the role methylation plays in RhoGDI interactions with RhoGTPases. Interest in RhoGDI involvement in oncogenic progression has increased recently; this study provides a mechanistic link between the prenylation pathway and RhoGDI regulation of Rho family members during cell migration.

An important component of the current study was the demonstration of a partial rescue of both directed and random migration impaired by Icmt inhibition by expressing exogenous RhoA and Rac1, respectively (Fig. 6). Notably, the rescue in migration was seen with wild-type RhoA and Rac1, suggesting that a sufficient amount of protein is able to escape capture by RhoGDI and be activated upon stimulation. The specific rescue of random migration by Rac1 is consistent with a previously study which linked Rac1 function to random migration (37). In addition to RhoA and Rac1, several other CaaX proteins, including other Rho family members, are involved in coordinating cell migration. The fact that we were unable to completely rescue migration with RhoA, Rac1, or combinations of both (data not shown) indicate that other CaaX proteins involved are likely also affected when their methylation is impaired. However, the fact that we were able to rescue specific types of cell migration by expressing different Rho GTPases demonstrates the ability to examine the biological effects of methylation for specific CaaX proteins. This study reveals a role for Icmt in metastatic behavior and further provides insight into the biological function of Rho GTPase methylation. Because Icmt inhibition shows promise as a strategy for oncogenic therapy, the ability to examine the biological consequences of methylation for specific oncogenes will be extremely valuable in future studies.

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