A Role for Rac3 GTPase in the Regulation of Autophagy*

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The process of autophagy is situated at the intersection of multiple cell signaling pathways, including cell metabolism, growth, and death, and hence is subject to multiple forms of regulation. We previously reported that inhibition of isoprenylcysteine carboxylmethyltransferase (Icmt), which catalyzes the final step in the post-translational prenylation of so-called CAAX proteins, results in the induction of autophagy which enhances cell death in some cancer cells. In this study, using siRNA-mediated knockdown of a group of small GTPases that are predicted Icmt substrates, we identify Rac3 GTPase as a negative regulator of the process of autophagy. Knockdown of Rac3, but not the closely related isoforms Rac1 and Rac2, results in induction of autophagy. Ectopic expression of Rac3, significantly rescues cells from autophagy and cell death induced by Icmt inhibition, strengthening the notion of an isoform-specific autophagy regulatory function of Rac3. This role of Rac3 was observed in multiple cell lines with varying Rac subtype expression profiles, suggesting its broad involvement in the process. The identification of this less-studied Rac member as a novel regulator provides new insight into autophagy and opens opportunities in identifying additional regulatory inputs of the process.

Autophagy (also termed macroautophagy) is a highly conserved cellular process in eukaryotic cells involved in maintaining homeostasis by degrading macromolecules and eliminating unwanted cellular structures (1). In this capacity, autophagy has been closely associated with many cytoprotective functions that include limiting the deposition of aggregate-prone proteins and promoting nutrient recycling (2–4). The dysregulation of autophagy results in numerous pathological conditions (5, 6). Increasingly, autophagy is also being recognized as a participant in programmed cell death; convincing data on this consequence of autophagy has come from studies in development/organogenesis, immune system functions, and recently in cancer therapy (7–12).

Given its role in performing important homeostatic functions, it is not surprising that autophagy is subjected to multiple modes of regulation in response to intra- and extracellular stimuli (13). Many cell signaling processes, including those of nutrient, energy, growth factor and stress response signaling, are integrally involved in autophagy regulation (14–16). Therefore, the identification of regulators in the initiation, progression and regulation of autophagy continues to be an important and evolving field.

Some proteins involved in cellular signaling, including most members of the Ras GTPase superfamily, undergo a series of post-translational modifications initiated by the addition of an isoprenoid lipid at a cysteine residue in a conserved "CAAX motif" at their C terminus (17). Following isoprenoid addition, these proteins undergo further processing by the Rce1 protease and isoprenylcysteine carboxylmethyltransferase (Icmt),2 this entire process is termed the prenylation pathway. These modifications affect the function of the target proteins through impact on subcellular localization, protein–protein interactions and protein stability (18). Inhibitors of the isoprenoid addition step, most notably protein farnesyltransferase (FTase) have garnered much attention due primarily to the dominant roles that Ras proteins play in tumorigenesis (19, 20). Recently, targeting the Icmt methyltransferase has gained attention for several reasons. First, it is recognized that the two prenyltransferases, FTase and protein geranylgeranyltransferase-I (GGTase-I), can substitute for each other’s function for some CAAX protein substrates, making FTase inhibitors less effective (21). Second, the Ras-centric view has given way to the understanding that many other CAAX proteins are important signaling molecules involved in cell survival, proliferation, migration, and other processes associated with tumorigenesis and tumor progression, and functions of these proteins can be affected by C-terminal methylation (22). Because cancer cells may have several different CAAX proteins involved in aberrant signaling, targeting either FTase or GGTase-I may only cover a subpopulation of CAAX proteins involved in the aberrancies of signaling. Third, both genetic and biochemical studies support the importance of carboxymethylation by Icmt on the functions of CAAX proteins in tumorigenesis (23–25).

We recently characterized a specific small molecule inhibitor of Icmt, cysmethinyl, and its impact on cancer cell proliferation and survival. These studies revealed that Icmt inhibition, either by inhibitor or by genetic down-regulation of Icmt, resulted in dramatic elevation of cellular autophagy and autophagy-dependent cell death (11, 12). A critical element of this line of investigation is to identify the substrates of Icmt that serve as mediators of these processes (26, 27).

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2 The abbreviations used are: Icmt, isoprenylcysteine carboxylmethyltransferase; FTase, protein farnesyltransferase, GGTase-I, protein geranylgeranyltransferase-I.
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ators in Icmt inhibitor-induced autophagy. Here we report the identification of a specific Ras family member, Rac3, as a negative regulator of autophagy and present evidence for the impact of Rac3 function on cancer cell survival through its modulation of autophagy.

EXPERIMENTAL PROCEDURES

Materials—Cysmethynil was synthesized by the Duke Small Molecule Synthesis Facility via established methods (24). Antibodies recognizing human GAPDH, p62 were from Cell Signaling Technology (Danver, MA), HA and Myc antibody were from Upstate (Waltham, MA), GFP antibody was from Fitzgerald Industries Intl (Concord, MA), and LC3 antibody was from Abgent (San Diego, CA). All cell lines used in the study were obtained originally from American Type Culture Collection.

Screening and Statistical Analysis—Standard analyses were employed to determine significance of findings and to select CAAX proteins for further exploration. In the primary screen to identify targets, two siRNAs were employed for each target transcript. The mean value of the ratio of LC3-II/GAPDH for each target was determined, and that of Rac3 was significantly above the mean of the entire group. Further confirmation of Rac3 being a regulator of autophagy was carried out with independent knockdown of the individual Rac isoforms, Rac1, -2, and -3. For the secondary screening using fluorescent microscopy imaging of HeLa cells expressing GFP-LC3, image analysis using Metamorph® software (Molecular Devices Inc., Sunnyvale) to quantify the LC3-positive punctae in control (luciferase), Rac1, Rac2, and Rac3 siRNA knockdown cells.

Cell Culture, Drug Treatment, and Viability Assays—Cells were maintained at 37 °C with 5% CO2 in DMEM (Sigma-Aldrich) supplemented with 10% FBS (Hyclone, Novato, CA), 50 units/ml penicillin and 50 μg/ml streptomycin (Invitrogen, North Andover, MA). Cysmethynil treatment of cells was performed with concentrations of 18–25 μM according to the sensitivity of different cell lines; exact conditions are noted in the respective figure legend. Cell viability was determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI).

Immunoblot and Immunofluorescence Analysis—Immunoblot analysis was carried out by standard established procedures. Immunofluorescence analysis was also performed by standard paraformaldehyde procedure followed by primary antibody and conjugated-fluorescent secondary antibody protocol. Imaging data were analyzed by MetaMorph® Analysis Software. Colocalization efficiency of mRFP to that of GFP fluorescent signals was measured using ImageJ software.

Transfection of siRNA or Expression Vectors—siRNA duplexes targeting Rac1, 2 and 3 were: 5′-ACA AGCC TCT TCT AAA GCC TTA-3′, 5′-AAC TACT TCA GCC AAT GTG ATG-3′, and 5′-CGC GCC CAT GCA GCC CAT CAA-3′. HA-Rac3, Myc-Rac1, Myc-Rac2, and Myc-Rac3 were cloned into pcDNA3.1 for overexpression. Tandem fluorescent mRFP-LC3 is a gift from Dr. Tamotsu Yoshimori (26). Transfections were done using LipofectamineTM 2000 (Invitrogen, Carlsbad) based on manufacturer’s protocol; the transfected cells were subjected to desired treatment 24–48 h after transfection.

Quantitative PCR—Total RNA used was extracted using Trizol-based reagent (Invitrogen), according to the manufacturer’s instructions. Subsequently, first-strand cDNA was synthesized under standard conditions with the Superscript First-strand Synthesis System (Invitrogen). Quantitative PCR was carried out with iCyler iQ5 Real-time Detection (Bio-Rad). The quantification of the transcripts was performed using a standard curve with 10-fold serial dilution of a cloned plasmid of the corresponding genes. Rac isoform specific PCR reactions were verified by both melt-curve analysis and sequencing analysis. The quantity of the transcript was normalized to the level of ribosomal proteins 18S.

Data Analysis—Statistical differences were assessed by Student’s t test. All experimental data are presented as mean ± S.D. Differences were considered statistically significant at p < 0.05.

RESULTS

Characterization of Cysmethynil-induced Autophagy in Cancer Cells—In our previous studies, we observed the induction of autophagy by both pharmacologic and genetic inhibition of Icmt in a number of cancer cell lines (11, 12). As the initial step of conducting the loss-of-function screen to identify Icmt substrates involved in this process, we characterized autophagy induced by the Icmt inhibitor in HeLa cells, which were employed for the screen together with the previously described PC3 cells (11). Upon treatment with cysmethynil, HeLa cells exhibited a marked elevation of LC3-II protein levels, and a selected line stably expressing GFP-LC3 showed increased autophagosome formation (Fig. 1, A and B); these results are quite similar to those obtained in PC3 cells in our prior study (11). Image analysis provided further quantitative information on autophagy induced by cysmethynil in these cells; there were significant increases in the number of LC3-II-positive vesicle per cell and the quantity of LC3-II protein on the vesicles as determined by fluorescence intensity (Fig. 1C). Similar results were observed in PC3 cells when endogenous LC3-II was visualized by immunofluorescence of this protein (11).

To investigate whether cysmethynil-induced elevation of LC3-II was the result of increased initiation and progression of autophagy and not the inhibition of autophagosome-lysosome fusion or the inhibition of proteolysis, the autophagy flux was assessed by using the co-localization analysis of tandem fluorescent mRFP–GFP-LC3 expression vector (26). RFP and GFP have different chemical properties; GFP fluorescence is attenuated and subsequently proteolysed at acidic pH while RFP is much more stable under similar conditions. In autophagosomes, both GFP and RFP fluorescence is colocalized. In autophagolysosomes, which are much more acidic, the green fluorescence weakens and eventually disappears; progression from autophagosomes to autophagolysosomes, therefore, presents as less colocalization and stronger red fluorescence.

In DMSO-treated control cells, the lower basal level of autophagy presents as low intensity fluorescence but poorly localized (Fig. 1D), since autophagosomes progress to merge with acidic compartment. In baflomycin-treated cells, the inhibition of fusion of autophagosomes with lysosomes results in the accumulation of RFP–GFP–LC3 on autophagosomes, therefore high level of GFP and RFP fluorescence and their co-local-
Cysmethynil treatment induces autophagy in HeLa cells. A, immunoblot analysis of LC3-II levels in lysates from HeLa cells treated with either control (DMSO) or cysmethynil (20 μM) for 24 and 48 h. GAPDH levels were determined as control. B, microscopy analysis of LC3-II localization in cysmethynil-treated cells. HeLa cells stably expressing GFP-LC3 were treated similarly as in A. Following 48 h of treatment with cysmethynil or vehicle, the cells were analyzed by confocal microscopy. C, analysis of the images from the experiment shown in panel B using MetaMorph software to determine the average number of GFP-positive particles per cell and average particle GFP fluorescent intensities in control and cysmethynil-treated cells. **, p < 0.01. D, assessment of autophagy flux in HeLa cells treated with DMSO (control), 20 μM of cysmethynil, 25 μM of bafilomycin or 200 nM of rapamycin. mRFP-GFP-LC3 tandem-tagged fluorescent protein (tf-LC3) construct was used to transfect HeLa cells. Cells were subjected to treatment 24 h after transfection. After 48 h of treatment, cells were examined using confocal microscopy for mRFP and GFP fluorescence. Colocalization of mRFP and GFP, an indicator of autophagy flux, was analyzed using ImageJ software. Representative dotplots depicting extent of colocalization appears under each corresponding image; the horizontal axis measures the intensity of red fluorescence while the vertical axis measure that of the green fluorescence. E, quantitative summary of colocalization. Pearson Coefficients are plotted for each treatment condition; >100 cells were analyzed for each population. **, p < 0.01.
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FIGURE 2. siRNA screening to identify negative regulators of autophagy. A, feasibility analysis. PC3 cells were transfected with siRNAs targeting a group of CAAX proteins; cell lysates were prepared 96 h after transfection and LC3 and GAPDH levels assessed by immunoblot analysis. The upper portion shows the immunoblots, while the lower portion presents the normalized LC3-II levels for each target knockdown. As positive control, the response to cysmethynil (cysmeth) was determined in parallel. The three horizontal lines mark the mean, and one and two S.D. above the mean, of the LC3-II/GAPDH ratio of the group, excluding the cysmethynil-treated sample. Shown is a representative experiment which has been repeated twice with similar results. B, screening. A panel of siRNAs targeting selected Icmt substrates (see supplemental Table S1) was obtained; two siRNAs for each target were employed. The standard deviation of GAPDH corrected LC3 II levels was plotted against each target. Cell lysates were prepared 96 h after transfection for standard SDS-PAGE and immunoblot analysis, as detailed under “Experimental Procedures.” The LC3-II value was normalized to GAPDH for each sample, and the mean and standard deviation of the LC3-II/GAPDH ratio determined and plotted. Zero on the y axis represents the mean of the ratio of the entire panel of siRNAs tested, and the values above and below zero represent positive and negative deviation from the mean. The two circled points represent the values from Rac3 and Rheb knockdown as indicated. The data presented in the graph are the composite results from four separate knockdown points, i.e. two experiments each with the two siRNAs against individual target in each experiment.

We then investigated whether the regulatory role of Rac3 in autophagy extended beyond HeLa and PC3 cells. Analysis of Rac isoform expression in HCT116 colon cancer and MDA-MB231 breast cancer cell lines was performed, along with HeLa and PC3 lines; these four cell lines represented a range of Rac1, Rac2, and Rac3 expression profiles (Fig. 4A). For example, HCT116 exhibited highest Rac3 expression with low levels of Rac1 and Rac2, while PC3 and MDA-MB231 cells expressed much lower level of Rac3. Despite their differing ratios of Rac isoform expression, Rac3 knockdown in all these cells induced autophagy significantly, while knockdown of Rac1 and Rac2 did not (Fig. 4B). We speculate that the reason the induction of LC3-II is not as robust in HCT116 cells by Rac3 siRNA knockdown may be due to its high Rac3 expression level. These data, in addition to suggesting a broad role in autophagy regulation by Rac3, provide increased confidence in the Rac3 subtype-specific regulatory role of this process.

To investigate the impact of Rac3 function on autophagic flux, we used two approaches. First we assessed RFP/GFP colocalization in MDA-MB231 cells stably expressing the aforementioned tandem fluorescent fused LC3 (mRFP-GFP-LC3); this was the only cell line in which we were successful in achieving stable expression of this construct. Using this stable cell line, the Rac3 knockdown cells developed more LC3 positive puncta (not shown) similar to that observed in Fig. 3C. In addition, image analysis of GFP and RFP fluorescence demonstrated a small but significant decrease in colocalization, indicating more attenuation and proteolytic degradation of GFP upon down-regulation of Rac3 (Fig. 4C). The substantial increase in total LC3-II positive punctae, and continued autophagy flux as measured by the colocalization of GFP and RFP, indicates up-regulated initiation and progression of the autophagy process (26, 32). In the second approach, we took advantage of the property of free GFP production from the LC3 fluorescent fusion protein as an indicator of autophagy flux; this approach assesses the autophagy-related proteolysis directly (32, 33). In the same experiment using Rac3 knockdown in MDA-MB231 cells stably expressing mRFP-GFP-LC3 as shown in Fig. 4G, immunoblot analysis was performed with cell lysates 72 h and 96 h after siRNA transfection. The analysis showed both an increased endogenous LCII level, and increased GFP-LC3 cleavage to generate free GFP, in Rac3 knockdown cells compared with mock knockdown cells (Fig. 4D), again indicating increased autophagy progression with Rac3 knockdown.

Ectopic Expression of Rac3, but Not Rac1 or Rac2, Confers Resistance to the Increased Autophagy and Cell Death Induced by Icmt Inhibition—To provide further evidence for the suppressive role of Rac3 on autophagy, we assessed the impact of Rac protein overexpression on the ability of the Icmt inhibitor cysmethynil to induce autophagy. HeLa cells transiently transfected with Myc-Rac1, Myc-Rac2, or Myc-Rac3 were subjected to cysmethynil treatment, and autophagy scored by quantitation of GFP-LC3-positive particles as described above. Cells overexpressing Rac3 exhibited much lower level of autophagy, indicating a resistance to cysmethynil-induced autophagy; while those cells expressing Rac1 or Rac2 had similar levels of autophagy induction as parental cells (Fig. 5). We then probed several cancer cell lines for Rac3 transcript and basal autophagy levels. Interestingly we observed a trend that the higher the Rac3 expression, the lower the basal autophagy as monitored by expression of LC3-II (Fig. 6A). For example, HCT116 had the highest level of Rac3 transcripts and the lowest basal levels of LC3-II, while MDA-MB231 and PC3 have low Rac3 but high LC3-II.

To further investigate the inhibitory effect of Rac3 on autophagy, we transfected PC3 cells with pcDNA3.1-HA-Rac3 treated with control siRNA or with siRNA targeting Rac1 or Rac2 (Fig. 3D).
and selected cells stably expressing HA-Rac3. When subsequently subjected to both DMSO control and cysmethynil treatment, PC3 cells overexpressing Rac3 have lower basal and cysmethynil-induced autophagy compared with cells stably transfected with vector, evidenced by both lower levels of LC3-II and higher levels of p62 (Fig. 6B). Similar results were obtained with HeLa cells expressing Rac3 (supplemental Fig. S1). Given our previous findings that excessive induction of autophagy in cancer cells by Icmt inhibition resulted in cell death (11, 12), we also examined whether Rac3 overexpression...
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**DISCUSSION**

The Rac subfamily of GTPases is comprised of three isoforms, Rac1, Rac2, and Rac3, which are more than 90% homologous (34). These small G-proteins, usually prototyped by Rac1, are among the most well studied signaling proteins. Rac proteins are post-translational modified at their C terminus via the prenylation process. Inhibition of GGTase-I, the first enzyme in the C-terminal modification of these proteins, i.e., preceding proteolysis by Rce1 and caboxymethylation by Icmt, impacts the cellular functions of Rac1 and Rac3 (35). As a group there is substantial evidence for the involvement of Rac proteins in multiple cellular processes. These include cytoskeletal organization, stress response pathways, NADPH oxidase function, and translational control; in addition, Rac protein dysregulation has been associated with numerous pathophysiological (36–39).

Although mostly studied as a group of proteins with similar functions, there are indications that Rac isoforms play different roles. For example, Rac1 and Rac3 were reported to localize to plasma membrane and endomembrane, respectively, under activating conditions (37); these two proteins were also reported to have opposing functions in cell adhesion and differentiation (40). We report here, through the use of subtype specific siRNAs to selectively down-regulate each family member, that Rac3, but not Rac1 or Rac2, plays a negative role in autophagy regulation. Aberrant activation of Rac3 has been recognized to be important in tumor proliferation in both breast cancer (41) and prostate cancer (42), and potentially in additional malignancies. This evidence of subtype-specific involvement of a Rac protein in pathological conditions supports the notion that different Rac family members play distinct roles in biology, despite of the lack of understanding of the exact processes in which these Rac proteins are involved. It will be interesting to investigate the mechanistic and therapeutic importance of specifically perturbing the function of Rac3 and its downstream effectors.

Autophagy has garnered increasing attention as a regulated physiological process. It is involved in the cell response to its nutritional environment, one of the most cardinal external factors to which the cell needs to adapt for survival, and autophagy is now recognized to be involved in cell growth and survival (1). Misregulation of autophagy has been recognized to result in numerous pathological conditions; hence manipulation of autophagy has important therapeutic implications (5). Although many players have been identified in the autophagy process and its regulation, a complete picture of autophagy regulation remains blurred. A pressing task is to identify novel regulators of autophagy and to connect these players to other vital cellular signaling networks. The identification of Rac3 as a regulator of autophagy opens a new window to better understanding this process. It is particularly interesting that in the cell lines we surveyed there was a consistent pattern that the higher the expression of Rac3 protein, the lower the basal autophagy; we also observed that cells with lower basal autophagy are more prone to cysmethynil induced autophagy and cell death, while cells with higher level of autophagy, such as MDA-MB231 cells, are more resistant to the cytotoxic effect of cysmethynil. A similar observation has recently been made that cells with high basal autophagy, such as most pancreatic cancer cells, are more vulnerable to the autophagy suppressive effect of chloroquine (43). Our data and that of others suggest

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**FIGURE 5.** Enforced expression of Rac3 protects HeLa cells from autophagy induced by cysmethynil treatment. HeLa cells stably expressing GFP-LC3 were transfected with vectors expressing Myc-Rac1 (panel A), Myc-Rac2 (panel B), and Myc-Rac3 (panel C), and then treated with cysmethynil (20 μM) for 48 h prior to analysis by fluorescent microscopy. GFP-LC3 was visualized directly, and rhodamine conjugated secondary antibody was used to identify the cells transfected with Myc fusions of Rac1, -2, and -3. The numbers of GFP-LC3-positive particles were determined in both Myc-Rac transfected and untransfected cells following treatment with cysmethynil. The median values of GFP-LC3-positive particles per cell, for untransfected, Myc-Rac1, Myc-Rac2, and Myc-Rac3-transfected cells, are plotted as relative values, i.e., median (non-Rac-expressing cells) versus median (Rac-expressing cells), such that the non-Rac-expressing cells have a value of 1.0 by definition. In the case of Rac1 and Rac2, no significant difference was observed in the median number of GFP-LC3-positive particles between Myc-Rac-transfected and untransfected cells, while in cells overexpressing Rac3, there were significantly fewer GFP-LC3-positive particles. ***, p < 0.01.**
that significant deviations from physiological set point of basal autophagy, either higher or lower, can lead to cytotoxicity which can be exploited by specific therapeutic intervention.

The observations, that some cancer cells are sensitive to induction of autophagy over their set basal level while others that may have physiological need for copious autophagy are more sensitive to autophagy suppression, underscores the need to identify regulators of the autophagy process in the pursuit of tumor specific targeting. In this regard, the screening for CAA proteins that play regulatory roles in the autophagy process, e.g. the identification of Rac3, is significant not only for understanding Rac isoform specific functions but also in the identification of therapeutic targets. In addition, further screening among Icmt substrates may identify more autophagy regulators among these CAA proteins, since ectopic expression of Rac3 only partially reversed the effect of cysmethynil treatment in inducing autophagy and cell death (Fig. 6, B and C), and Rac3 knockdown did not generate the same level of autophagy induction and autophagic cell death as cysmethynil treatment (not shown).

Given the many connections of Rac signaling, it will likely require extensive studies to delineate the complete path of Rac3 signaling in exerting control of autophagy; most likely this will involve some proteins already linked to the autophagy process, but it may also implicate new players or new roles of known proteins which transmit or mediate the impact of Rac3 function on autophagy.

The findings that similar effects were observed with Rac3 knockdown and with cysmethynil treatment, and that ectopic expression of Rac3 can rescue cells from excess autophagy and cell death induced by Icmt inhibition, not only supports the conclusion that Rac3 plays a key role in regulating autophagy, but also underscores the importance of Icmt-catalyzed methylation on Rac3 function. In this regard, pharmacological inhibition of Icmt has potential therapeutic utility in manipulating cellular autophagy by impacting Rac3 function.

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