Site-specific acetylation of the proteasome activator REGγ directs its heptameric structure and functions*

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Background: REGγ mediates degradation of numerous proteins. How REGγ activity is regulated remains unclear.

Results: REGγ acetylation at lysine 195 promotes its activity by enhancing monomeric interactions and heptameric formation of the REGγ molecules.

Conclusion: Site-specific acetylation of REGγ is important for its structural architecture and enzymatic function.

Significance: Our discovery provides basis for additional venue to intervene the REGγ-proteasome function.

SUMMARY

The proteasome activator REGγ has been reported to promote degradation of Steroid receptor coactivator-3 (SRC-3) and cyclin-dependent kinase inhibitors p21, p16, and p19 in a ubiquitin and ATP independent manner. A recent comparative analysis of REGγ expression in mouse and human tissues reveals a unique pattern of REGγ in specific cell types, suggesting undisclosed functions and biological importance of this molecule. Despite the emerging progress made in REGγ-related studies, how REGγ function is regulated remains to be explored. In this study, we report for the first time that REGγ can be acetylated mostly on its lysine 195 (K195) residue by CREB binding protein (CBP), which can be reversed by Sirtuin 1 (SIRT1) in mammalian cells. Site directed mutagenesis abrogated acetylation at K195 and significantly attenuated the capability of REGγ to degrade its target substrates, p21 and Hepatitis C virus (HCV) core protein. Mechanistically, acetylation at K195 is important for the interactions between REGγ monomers, and ultimately influences REGγ heptamerization. Biological analysis of cells containing REGγ-WT or REGγ-K195R mutant indicates an impact of acetylation on REGγ-mediated regulation of cell proliferation and cell cycle progression. These findings reveal a previously unknown mechanism in the
regulation of REGγ assembly and activity, suggesting a potential venue for the intervention of the ubiquitin-independent REGγ-proteasome activity.

The proteasome activator REGγ (also known as PA28γ, PSME3, Ki antigen) belongs to the REG or 11S family of proteasome activators that have been shown to bind and activate the 20S proteasome (1, 2). REGγ has been reported to promote degradation of some important regulatory proteins, such as SRC-3 and cyclin-dependent kinase inhibitors p21, p16, and p19 in a ubiquitin- and ATP-independent manner(3-5). Moreover, REGγ facilitates the turnover of tumor suppressor p53 by promoting MDM2-mediated p53 ubiquitination (6), and regulating p53 cellular distribution (7). Furthermore, REGγ is overexpressed in some cancers (8, 9), and is linked to multiple cancer-related pathways (10). A unique expression pattern of REGγ in cell specific manner has been documented, suggesting undisclosed functions and biological importance of this molecule (11). Despite recent progress made in this field, how REGγ is regulated in mammalian cells is largely unknown.

Post-translational modification is an important process in regulating protein structures and functions. Acetylation occurs as a co-translational and post-translational modification of histones and non-histone proteins such as p53 and tubulins (12). In fact, proteomic studies have identified thousands of acetylated mammalian proteins (13,14), of which chromatin proteins and enzymes are highly represented. Acetylation commonly occurs at a lysine residue, and can affect protein nuclear localization, stability, transcriptional activity, DNA binding, and interactions with other proteins and cofactors (12, 15), indicating that acetylation has a considerable impact on protein functions. Several studies suggest that acetylation can alter protein structures or protein-protein interactions (16-18). For example, acetylation of KLF5 transcription factor enhances its interaction with Smad4 to promote transcription of target genes (16). Thompson et al. demonstrate that acetylation of p300’s putative inhibitory loop may open the locked gate and activate its acetyltransferase activity (17).

Protein acetylation is a reversible process that is governed by the opposing actions of histone acetyltransferases (HATs) and histone deacetylases (HDACs). CBP and p300 (E1A binding protein p300) possess strong histone-acetyltransferase activity and act on both histone and non-histone proteins (19, 20). HDACs are classified into four classes and two families: classical (classes I, II, and IV) and silent information regulator 2 (Sir2)-related protein (sirtuin) families (class III) (21). Among the seven members of mammalian sirtuins (SIRT1-7), SIRT1 is the most studied and strongly implicated in cellular regulation through its deacetylase activity (22).

In current study, we illustrate that acetylation of REGγ at the lysine 195 residue by CBP is important for the degradation of REGγ substrates, such as p21 and HCV core proteins. However, SIRT1, a deacetylation enzyme, can interact with REGγ and remove acetylation group at K195, attenuating REGγ activity. Further study reveals that blocking acetylation at K195 significantly reduces interactions between REGγ monomers, and ultimately influences the formation of heptamer. Finally, functional analysis in cells containing REGγ-WT or REGγ-K195R mutation has validated the crucial role of acetylation in REGγ-mediated regulation of cell proliferation and cell cycle progression.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**-HEK293/293T, H1299, HeLa and A549 cells were purchased from ATCC and maintained in DMEM (Invitrogen), 10% FBS (Invitrogen), and penicillin/streptomycin (Invitrogen). The HEK293 REGγ inducible cell lines were generated by the Flp-In™ T-REx™ system (Invitrogen). REGγ integration in REGγ-/- MEF (Mouse Embryonic Fibroblast) stable cells were generated by lentivirus infection for 2 days, then selected by puromycin (Invitrogen, 3µg/ml). The antibodies used in this study included anti-REGγ (Invitrogen), anti-Flag, anti-β-actin (Sigma), anti-CBP, anti-p21 (BD Biosciences), anti-HA, anti-AcK (Cell Signaling Technology and Abcam), anti-SIRT1 (Millipore), and anti-Flag M2 Affinity Gel (Sigma). The CBP siRNA Smart-pool was purchased from Dharmaco Inc. Other purchased reagents were proteasome inhibitor MG132 (Sigma), Cycloheximide (Sigma), Trichostatin A (Sigma), Niacinamide (Sigma), Resveratrol (Sigma), BCA Protein Assay Kits (Thermo Scientific), and MTS assay reagents (Promega).
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All the experiments shown in the study were repeated at least three times.

**Plasmid Constructs and Site-directed Mutagenesis**—The mammalian expression vector pCDNA5/FRT/TO (Invitrogen) was modified to express REGγ or Flag-tagged REGγ at the N terminus. HA-tagged REGγ and HCV core-173 constructs were generated in pSG5 vector. pCDH-CMV-EF1-REGγ was constructed by inserting a digested PCR fragment into the lentivirus expression vector pCDH-CMV-EF1-Puro. GST-tagged REGγ was generated in pGEX-4T-1 vector. pPAL7- REGγ was constructed into pPAL7 vector. His-SIRT1 was generated in pET28a vector. pCDNA3.1-p21 was generated into the pCDNA3.1 vector. pDNA Flag-CBP was kindly provided by Dr. Qin Feng (Department of Molecular and Cellular Biology, Baylor College of Medicine). pCDNA3 Flag-SIRT1, pCDNA3 SIRT1 and pCDNA3 SIRT1 H363Y were provided by Dr. Qiang Tong (Departments of Pediatrics, Medicine, Molecular Physiology & Biophysics, Baylor College of Medicine). Lysineto-arginine mutations in REGγ or its derivatives with Flag/HA tags were generated by site-directed mutagenesis at residues K6, K14 and K195. All the constructs were verified by DNA sequencing.

**Mass Spectrometry**—The HEK293 Flag-REGγ inducible cells were treated with Doxycycline 1µg/ml for 48h to induce Flag-REGγ highly expressed. The cells were lysed with Lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.5% NP-40). Flag-REGγ was immunoprecipitated from pre-cleared cell lysates by incubation with Anti-Flag M2 Affinity Gel overnight at 4°C. The immunoprecipitates were washed three times with NETN buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% NP-40). The washed beads were boiled with SDS/PAGE loading buffer. Flag-REGγ were resolved by SDS-PAGE and stained with Coomassie Blue. Flag-REGγ protein bands were cut, destained and digested with trypsin or V8 protease overnight in the NH4HCO3 buffer. Mass spectrometry was performed as described previously (5). Recombinant REGγ Protein was purified by Profinity eXact™ Protein Purification System (Bio-Rad). Recombinant REGγ was resolved by SDS-PAGE and stained with Coomassie Blue. After in-gel digestion and peptide extraction, the peptides were analyzed on an Orbitrap Elite spectrometer connected to an EASY-nLC 1000 UPLC system using a nanoelectrospray ion source (Thermo Scientific).

**Immunoprecipitation and Detection of REGγ Acetylation in Cells**—To detect REGγ acetylation in cells, HEK293/A549/HeLa cells were harvested and lysed in Lysis buffer described above. The lysates were centrifuged and the supernatants were incubated with 1µg anti-REGγ antibody overnight at 4°C with 1µg rabbit IgG (Santa Cruz) as control. Each sample was incubated with protein A/G Plus-Agarose beads (Santa Cruz) for 3h. Immunoprecipitated REGγ was resolved on a 10% SDS-PAGE gel and analyzed by Western blotting with anti-AcK antibody. The NC membrane was stripped by the Restore™ Western Blot Stripping Buffer (Thermo), and then probed with anti-REGγ antibody. For the reciprocal immunoprecipitation, HEK293 cell lysate was incubated with anti-AcK antibody overnight at 4°C, then incubated with protein A/G Plus-Agarose beads for 3h. The immunoprecipitates were resolved on a 10% SDS-PAGE gel and analyzed by Western blotting with anti-REGγ antibody. Flag-tagged REGγ was immunoprecipitated from 293 REGγ inducible cells with anti-Flag M2 Affinity Gel. Flag-REGγ was eluted with 200 µg/ml Flag peptide for 1h. Flag-REGγ was incubated with anti-AcK antibody overnight at 4°C in buffer 80 µl (50 mM Tris, 137 mM NaCl, 1 mM EDTA, 10 mM NaF, 0.1mM Na3VO4, 1mM DTT, 10% glycerol, 0.5% NP40, pH7.8), together with 50 mM Sodium Butyrate, 6.6µM TSA, 10mM NAM and protease inhibitors. The mixture was incubated with protein A/G Agarose beads for 3h. The supernatant unbound REGγ and the immunoprecipitated acetylated REGγ was resolved on a SDS-PAGE gel and analyzed by Western blotting with anti-REGγ antibody. For deacetylase inhibition, the HEK293 Flag-REGγ inducible cells were treated with Doxycycline 1µg/ml (for 48h). 6.6µM Trichostatin A (TSA) and 10mM nicotinamide (NAM) for 6h prior to cell harvest. Cells were lysed in Flag lysis buffer (50 mM Tris, 137 mM NaCl, 1 mM EDTA, 10 mM NaF, 0.1mM Na3VO4, 1% NP-40, 1mM DTT, 10% glycerol, pH7.8) containing fresh protease inhibitors (Roche), 6.6µM TSA and 10mM NAM. Cell extracts were immunoprecipitated with anti-Flag M2 Affinity.
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REGγ acetylation was analyzed by Western blotting with a Pan-anti-AcK antibody.

REGγ acetylation and deacetylation assays in vitro-Flag-tagged CBP was expressed in HEK293T cells and immunoprecipitated by anti-Flag M2 Affinity Gel. Flag-CBP was eluted with 200 μg/ml Flag peptide (Sigma) for 1h. GST-tagged REGγ was expressed and purified as described previously (5). 5 μg GST-REGγ was incubated with 3 μg Flag-CBP in HAT buffer 30 μl (250 mM Tris-HCl, pH 8.0, 500 μM EDTA, 5mM DTT, 50% glycerol, 50 mM Sodium Butyrate, 6.6μM TSA, 10mM NAM and protease inhibitors) with or without addition of 5mM acetyl-CoA. After 3h at 30°C, samples were resolved on SDS-PAGE and analyzed by Western blot. In vitro deacetylation assays were performed as follows: Flag-REGγ was immunoprecipitated from HEK293 Flag-REGγ inducible cells transfected with CBP, and the enriched REGγ proteins were eluted with Flag peptide. Acetylated Flag-REGγ was incubated with recombinant His-SIRT1 in 50 μl of deacetylation buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 4 mM MgCl2, 0.5mM DTT, 0.1 mM PMSF, 5% glycerol, 0.02% NP40 and protease inhibitors) in the presence or absence of 50 μM NAD+ for 2h at 30°C. The reactions were subjected to SDS-PAGE and analyzed by Western blot.

Transfections and REGγ Activity Detection-Plasmids CBP (1μg) was transiently transfected into HEK293 cells for 32h using FuGENE HD DNA Transfection Reagent (Roche). REGγ acetylation levels were detected as described above. Additional experiments were performed in the HEK293 Flag-REGγ inducible cells by expressing CBP in the presence or absence of SIRT1, followed by determination of REGγ acetylation. In HeLa cells, SIRT1-WT and the deacetylase mutant, SIRT1-H363Y, were ectopically expressed for 32h, and protein expressions were examined by Western blotting using anti-SIRT1, anti-REGγ, and anti-p21. For RNA interference, HEK293 cells were transfected with 60nM ON-TARGET plus small interfering RNA (siRNA) specific for human CBP using Lipofectamine™ 2000 Transfection Reagent (Invitrogen). After 72h of transfection, cells were harvested and lysed for Western blot analysis of CBP, REGγ and p21 protein levels. The p21 and/or HCV core protein levels were measured by Western blotting to estimate REGγ activity. In different cells examined, p21 or HCV-core construct was either co-transfected with a control vector or REGγ-WT, REGγ-K6R, REGγ-K14R, REGγ K195R or REGγ K195Q. HEK293 REGγ inducible cells were incubated with Cycloheximide (CHX) 100mg/ml with or without additional of Doxycycline (1μg/ml, for 48h) at indicated time for p21 protein half-life analysis.

Co-Immunoprecipitation and Protein-Protein Interaction Analysis-HEK293 cells were cultured in the presence of 20μM of MG132 for 8h before harvest. REGγ was immunoprecipitated from pre-cleared cell lysates with 1μg anti-REGγ overnight at 4°C, with 1μg rabbit IgG in the control group. Each sample was incubated with protein A/G Plus-Agarose beads for 3h. The immunoprecipitates were washed three times with IP Washing buffer (50 mM Tris, 137 mM NaCl, 1 mM EDTA, 10 mM NaF, 0.1mM Na3VO4, 1mM DTT, 10% glycerol, pH 7.8), and the co-immunoprecipitated proteins were analyzed by Western blotting with a SIRT1 antibody. The NC membrane was stripped by the Stripping Buffer, then probed with anti-REGγ as references. For the reciprocal co-immunoprecipitation, HEK293 cell lysates were incubated with a SIRT1 antibody followed by detection of REGγ in the immunoprecipitates with Western blot analysis. For interactions between REGγ monomers, HEK293 cells were transfected with HA-tagged and/or Flag-tagged REGγ-WT or K195R mutant for 48h, along with MG132 (20μM for 8h) before harvest. The cell lysates were incubated with anti-Flag M2 Affinity Gel overnight at 4°C. The co-immunoprecipitated HA-REGγ were examined by Western blot with an anti-HA antibody.

Analysis of REGγ Heptamer by Native-PAGE and FPLC-The HEK293 Flag-REGγ-WT or K195R inducible cells were cultured in the presence of Doxycycline (1μg/ml, for 48h), 6.6μM TSA and 10mM NAM (for 6h) prior to harvest. Following cell lysis, the supernatants were prepared in NativePAGE™ Sample Buffer (Invitrogen) and resolved by NativePAGE™ Novex® Bis-Tris Gel System (Invitrogen). Transferred PVDF membranes were stained with Ponceau S to display the NativeMark™ Unstained Protein Standard (Invitrogen). After decolorization, the PVDF membrane was probed with a Flag
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antibody to detect the REGγ heptamer complex. HEK293 cells were transiently transfected with 1µg of Flag-REGγ -WT, K195R, K195Q or K188F (a heptamerization-defective mutant) into 6-well plate for 32h, REGγ heptamer complexes were detected by Native-PAGE as described above. To investigate endogenous REGγ heptamerization, HEK293 Flag-REGγ inducible cells cultured in the presence or absence of TSA, NAM or Resveratrol were fractionated through a Superose 6 column. The elution profiles of REGγ were monitored by γ-modification. To determine whether REGγ is acetylated in mammalian cells.

**RESULTS**

REGγ is Acetylated in Mammalian Cells-

Recent studies reveal a variety of physiological functions of REGγ in growth, cell proliferation, and cancer progression (9-11, 23, 24). How REGγ function is regulated remains to be elucidated. Our previous proteomic analysis (5) indicates REGγ as a protein with different post-translational modification. To determine whether REGγ could be an acetylated protein, we carried out immunoprecipitation (IP) of REGγ in lysates from different mammalian cells followed by Western blot analysis using a general anti-acetyl-lysine (anti-AcK) antibody. In human embryonic kidney cells (HEK293), endogenous REGγ is clearly recognized in the IP enriched sample by the anti-AcK antibody (Fig. 1A). Similarly, in human cancer cell lines including A549 and HeLa, REGγ protein acetylation can also be detected (Fig. 1B and 1C). Alternatively, we performed a reciprocal IP analysis with the anti-AcK antibody followed by Western blotting using anti-REGγ. The result showed a single band at the molecular weight identical to the size of REGγ, indicative of an acetylated REGγ (Fig. 1D). To further test our observation, we treated cells with HDACs inhibitors TSA and NAM (25, 26) to enhance cellular levels of acetylated proteins. In the doxycycline induced Flag-REGγ over-expressing HEK293 cells, we found a notable increase in the acetylation of Flag-REGγ with TSA/NAM treatment (Fig. 1E). Taken together, these results suggest that REGγ can be acetylated in mammalian cells.

K195 is a Major Acetylation Site in REGγ-

To identify the potential acetylated residues, doxycycline induced Flag-REGγ in HEK293 cells was immunoprecipitated with Flag beads, resolved by SDS-PAGE and analyzed by MS/MS (LC-MS/MS). MS/MS spectrum showed acetylation at three lysine residues at positions 6 (K6), 14 (K14) and 195 (K195) respectively (Fig. 2A and data not shown). This was partially endorsed by a different mass spectrometry study with identification of acetylation at K195 (13). The mass score (Score Diff. Ave.) for REGγ acetylation at 195 is above the mean average of nearly 2000 acetylated proteins identified (13), reflecting relative abundance of this site specific acetylation. As a control, the recombinant REGγ protein purified from E. coli was resolved by SDS-PAGE and cut out for LC-MS/MS analysis. To our surprise, bacterially generated recombinant REGγ was also acetylated at K195 (Fig. 2B). Sequence analysis of REGγ from multiple species reveals that the K195 site and surrounding residues are highly conserved across the animal kingdom (Fig. 2A, lower panel), with K6 and K14 slightly less conserved among vertebrates (Fig. 2A, upper panel). In order to define the ratio of acetylated REGγ in total molecules, we purified Flag-tagged REGγ from 293 REGγ inducible cells followed by the reciprocal immunoprecipitation with anti-AcK antibody. We found that around half of the REGγ molecules are acetylated (Fig. 2C lane 4 and lane 5) compared with the unbound REGγ protein (Fig. 2C lane 2 and lane 3). To analyze acetylation, normally Lys→Gln (KQ) substitution is used to mimic lysine acetylation, whereas Lys→Arg (KR) substitution is to eliminate acetylation target site without neutralization of the positive charges (27-29). Therefore, we generated doxycycline-
inducible REGγ expressing HEK293 cells, including acetylation-defective mutations of K6R, K14R or K195R in Flag-REGγ. In these stable cell lines, the K195R mutation significantly reduced REGγ acetylation level, while K6R and K14R mutations had little impact on the overall acetylation in REGγ (Fig. 2D). Similar result was found in HEK293 cells transiently expressing Flag-REGγ-K195R mutant, which was poorly recognized by the anti-AcK antibody compared with Flag-REGγ-WT (Fig. 2E). These results illuminate that K195 is a major acetylation site in REGγ, reflecting a potentially important role of this lysine residue in modulating REGγ activity.

**Blocking Acetylation at K195 Attenuates REGγ Activity** To understand the biological consequence of REGγ acetylation, expressions of p21 and a truncated HCV core-173 protein, well-known substrates of REGγ-proteasome (3, 4, 30), are utilized to evaluate REGγ activity. We analyzed the ability of REGγ acetylation-defective mutants K6R, K14R, and K195R to promote degradation of p21 and HCV core-173 in HEK293 cells as well as the human lung cancer cell line H1299. The latter cell line lacks p53 expression and therefore avoids REGγ’s impact on p21 degradation through down-regulation of p53 (6). While REGγ mutations at K6 or K14 had minor effect to attenuate REGγ-mediated degradation of p21 protein (data not shown), REGγ mutation at K195 significantly reduced the capacity of REGγ to degrade HCV core-173 and p21 proteins in H1299 cells (Fig. 3A lane 3 and Fig. 3B lane 3). As controls, REGγ-WT retained the activity to promote degradation of HCV core-173 and p21 proteins (Fig. 3A lane 2 and Fig 3B lane 2). In the REGγ inducible HEK293 cells, we also obtained results similar to what we observed in H1299 cells (data not shown), indicating that acetylation of REGγ occurs in different cell types. Next, we generated an acetylation-mimetic REGγ-K195Q mutant, which is capable of accelerating p21 degradation in comparison with REGγ K195R in H1299 cells, but to a less extent compared to REGγ-WT (Fig. 3B). To understand the action of endogenous REGγ acetylation mutants in cells without wild type REGγ, we generated REGγ derivatives in lentivirus, including REGγ-WT, K195R, K195Q, along with a vector control, and stably integrated these constructs in REGγ −/− MEF cells. Despite that stable integration of REGγ-WT in REGγ −/− MEF cells does not function efficiently in its degradation of endogenous p21 probably due to compensation mechanisms, the stable cells expressing REGγ-WT and REGγ-K195Q in the parental REGγ −/− MEFs had comparable effect on p21 degradation, whereas REGγ-K195R showed a remarkable inhibition of p21 protein degradation (Fig. 3C). Even though the Lys→Gln substitution does not always faithfully mimic the acetylation status of the lysine residue (29, 31), our data indicate that acetylation at K195 is crucial for maintaining REGγ activity in its degradation of target proteins.

To explore whether acetylation of REGγ at K195 affects its function in the turnover of substrate protein p21, we used REGγ inducible HEK293 cells treated with Cycloheximide (CHX) for indicated time periods. As expected, p21 degradation was expedited when overexpressing REGγ WT (Fig. 3D). However, induced overexpression of REGγ K195R mutant had no significant impact on the decay rate of p21 (Fig. 3E). In a parallel experiment, induced overexpression of REGγ K195Q could effectively decrease p21 half-life as REGγ WT did (Fig. 3F). Taken together, these results indicate that acetylation at K195 is critical for retaining REGγ activity.

**CBP and SIRT1 Reversely Regulate REGγ Acetylation and Activity** CBP and p300 are transcriptional coactivators with intrinsic histone-acetyltransferase activity (32-36) to regulate gene expression. To test whether REGγ could be acetylated by these HATs, CBP was transiently expressed into HEK293 cells. We found significantly increased acetylation level of REGγ with CBP co-transfection (Fig 4A). In addition, the recombinant GST-tagged REGγ protein could be acetylated by Flag-tagged CBP in the presence of acetyl-CoA in vitro (Fig. 4B). Next, we investigated whether REGγ is a substrate of the Sir2 families in mammalian cells. Among SIRT1-SIRT7, Flag-tagged SIRT1 showed strong interaction with GFP-tagged REGγ (to be published elsewhere). Furthermore, we found robust interactions between endogenous SIRT1 and REGγ in HEK293 cells by IP analysis using the anti-REGγ antibody (Fig. 4C). Similarly, a reciprocal IP with anti-SIRT1 antibody detected endogenous co-IP of REGγ and SIRT1 in HEK293 cells (Fig. 4D), indicating that REGγ is a potential
target of SIRT1. In the inducible Flag-REGγ-WT expressing HEK293 cells, augmented CBP expression enhanced acetylation in the Flag-REGγ, whereas co-expressing CBP and SIRT1 blocked the effect by CBP, reflecting a causal relation between SIRT1 and REGγ deacetylation (Fig. 4E left panel). In contrast, transient overexpressing CBP failed to significantly enhance REGγ acetylation in the Flag-REGγ-K195R inducible HEK293 cells and co-expressing SIRT1 and CBP further diminished REGγ acetylation (Fig. 4E right panel). In addition, we found an obviously increased acetylation level of REGγ after SIRT1 knocking down in 293T cells (Fig.4F). As expected, when acetylated Flag-REGγ was incubated with recombinant His-SIRT1 and NAD⁺, REGγ acetylation level was obviously reduced in vitro (Fig. 4G). These results strongly suggest that CBP and SIRT1 mainly target K195 for acetylation/deacetylation in REGγ, although we can not exclude their regulation in other residues in REGγ.

Furthermore, we determined whether the influence of SIRT1 on REGγ activity depends on its deacetylase activity. Transient overexpression of SIRT1-WT in HeLa cells significantly increased p21 protein level, while exogenously expressed SIRT1-H363Y, a deacetylase-defective mutant (37), failed to inhibit REGγ-dependent degradation of p21 (Fig. 4H). Consistently, we also found that silencing CBP in HEK293 cells enhanced p21 protein level (Fig. 4I). Taken together, these results suggest that CBP and SIRT1 can regulate REGγ activity through acetylation and deacetylation in REGγ at specific sites.

Acetylation at K195 is Crucial for REGγ’s Monomeric Interactions and Overall Structure—Based on the discovery that acetylation in REGγ influences its activity, we intended to address how this could be achieved. Previous studies demonstrate that functional REGγ exists as a heptameric ring in cells (8, 38, 39). We tested if acetylation is involved in regulation of REGγ’s overall structure. In Flag-REGγ-WT overexpressing HEK293 cells, REGγ heptamers were easily detected by Native-PAGE followed by membrane transferring and antibody blotting (Fig. 5A lane 1). Interestingly, enhancing REGγ acetylation by TSA and NAM treatment greatly increased the amount of REGγ heptamer complexes (Fig. 5A lane 2). In Flag-REGγ-K195R overexpressing HEK293 cells, REGγ heptamer formation was dramatically suppressed even in the presence of TSA and NAM treatment (Fig. 5A lane 3 and lane 4). Furthermore, we transiently expressed Flag-REGγ-WT and corresponding acetylation mutants in HEK293 cells to examine REGγ heptamerization by Native-PAGE. The data clearly showed that Flag-REGγ-WT and the acetylation-mimetic Flag-REGγ-K195Q mutant formed heptamers in cells (Fig. 5B lane 2 and lane 4). In contrast, the acetylation-defective Flag-REGγ-K195R mutant and the heptamerization-defective Flag-REGγ-K188F mutant (38) had poor REGγ heptamer formation (Fig. 5B lane 3 and lane 5). The crystal structure of heptameric REGα (40), which is highly homologous to REGγ, suggests a parallel intermolecular interactions between helix 2 of one monomer with the helix 4 of the neighboring molecule. We then questioned whether acetylation affects association between REGγ monomers. By transiently expressing a Flag-tagged REGγ and an HA-tagged REGγ construct or corresponding mutant constructs in HEK293 cells followed by IP and Western blot analysis (Fig. 5C), we found that REGγ-WT interacted better with each other while REGγ-K195R monomeric interactions were compromised, suggesting a role of acetylation at this position in REGγ protein-protein interactions. To further test the impact of acetylation on REGγ monomeric interactions, we extended part of the above experiments described in Fig. 5C with additional treatment of either TSA/NAM or SIRT1 co-transfection, alone or in combination. As expected, TSA/NAM treatment enhanced the interactions between Flag-REGγ and HA-REGγ (Fig. 5D compare lane5 with lane 6), whereas SIRT1 attenuated their association in the presence or absence of TSA/NAM (Fig. 5D, compare lanes 5 vs. 7; lanes 6 vs. 8; and lanes 7 vs. 8). Moreover, we performed size exclusion chromatography using lysates from the inducible Flag-REGγ-WT and Flag-REGγ-K195R HEK293 cells. Based on the molecular standard, we found that majority of the Flag-REGγ-WT formed heptamers with a peak at ~ 230 KD (Fig. 5E upper panel). Upon TSA/NAM treatment and increased REGγ acetylation, a change in fraction-pattern occurred with a shift toward the peak fraction, indicating an increase in REGγ heptamerization (Fig. 5E middle panel). On the contrary, Flag-REGγ-K195R
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expressing cells produced a stretched elution pattern ranging from monomers (~30KD), various degrees of oligomers, and reduced higher molecular weight fractions (heptamers) (Fig. 5E lower panel). Similar results were obtained following REGγ deacetylation by Resveratrol in the induced Flag-REGγ-WT overexpressing cells (data not shown). Based on the results that acetylation-defective mutant significantly impaired its heptamerization and monomers association (Fig. 5A, Fig. 5B and Fig. 5C), we examined whether REGγ-K195R mutant is unstable which may be targeted for degradation or association with other protein complexes. Consequently, we found that acetylation-defective mutant REGγ-K195R degrades faster compared with REGγ-WT (Fig. 5F). Collectively, these results demonstrate acetylation at K195 is crucial for REGγ monomeric interactions and assembly of REGγ heptameric complexes.

Acetylation-defective Mutant REGγ Impairs Cell Growth and Cell Cycle Progression—As a broad-acting cyclin-dependent kinase inhibitor, p21 plays a central role in cell cycle regulation in many cells types (41, 42). Recent studies indicate that REGγ influences cell cycle through degradation of several cell-cycle regulators including p21, p16, and p19 (3, 4). Thus, we reasoned that blocking REGγ acetylation at K195 may also affects cell proliferation and cell cycle progression. Using the HEK293 cells inducibly expressing Flag-REGγ-WT and Flag-REGγ-K195R for the cell proliferation assay, we observed that cells overexpressing Flag-REGγ-K195R had significantly reduced growth rate at day 2 and all the way to day 5 (Fig. 6A). Next, these cells were also subjected to flow cytometry analysis to evaluate the impact of acetylation defect on cell cycle progression. Compared to Flag-REGγ-WT expressing cells, Flag-REGγ-K195R expressing cells had an increased population at the G0/G1 phase, and a significantly decreased proportion of S phase cells (Fig. 6B), indicating a reduced cell cycle progression from G0/G1 to S phase transition. Taken together, our results further substantiate an important role for REGγ acetylation in cell growth and cell cycle regulation.

DISCUSSION

Emerging evidence leads to a renewed attention to the ubiquitin-independent REGγ-proteasome pathway. With the discovery of more and more substrates in this protein degradation pathway (3-6, 30, 43, 44), REGγ is being recognized as an important regulator. Yet the regulatory input that may alter the biological function of REGγ remains poorly understood. In this study, we demonstrate that site specific acetylation of REGγ at K195 is crucial for its monomeric interactions and formation of a functional heptameric complex. Modulating acetylation status at this lysine residue has profound impact on REGγ’s activity in substrate protein degradation. Our results provide the first biochemical evidence for a role of acetylation in structural and functional regulation of the REGγ-proteasome complex.

In eukaryotic cells, acetylation is among the most common covalent modifications and ranks among the most important master switches similar to phosphorylation (45). It is now clear that prokaryotes have the capacity to acetylate both the α-amino groups of N-terminal residues and the ε-amino groups of lysine side chains, suggesting that acetylation appears to be an ancient reversible modification like phosphorylation (46). Reversible protein acetylation provides key regulatory switches for cell signaling pathways, which has been shown to affect a diverse array of biochemical properties including protein activity, protein stability, DNA/protein-protein interactions and intracellular localization (12, 15). We have defined lysine 195 in REGγ as the mostly affected residue for acetylation/deacetylation mediated by CBP/SIRT1. The REGγ K195R mutant that can no longer be acetylated failed to promote its substrates degradation, whereas the K195Q mutant that mimics a constitutively acetylated state retained the capacity to degrade target proteins, which further correlates with REGγ-dependent regulation in cell growth and cell cycle progression. We also demonstrated reversible acetylation-deacetylation modification at the K195 sites. Our results by no means exclude the possibility of weak acetylation on other sites or regulation by other HATs/HDACs. In fact, K6 and K14 in REGγ can also be acetylated based on mass spectrometry and bioinformatics prediction. We have previously demonstrated that SUMOylation of REGγ can be enhanced in the
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presence of PIAS1 at K6, K12, and K14, which results in cytoplasmic distribution and stabilization of REGγ (47). It is likely that competition by SUMOylation at K6 and K14 attenuates acetylation at these sites.

Although we failed to generate a K195 specific Ac-antibody, the Pan-AcK antibody successfully detected acetylation at K195 in REGγ-WT but not in REGγ-K195R mutant, suggesting high prevalence of this site-specific acetylation in mammalian cells. Based on our comparative analysis of Score Diff. Ave. (which is a standard for estimation of modification signal) for ~2000 of the acetylated proteins (13), we found the score for acetylation of REGγ at K195 is above the average score, indicating that acetylation of REGγ at K195 is around average levels compared to all proteins examined so far. In addition, our IP-Western results with Pan-acetylation antibody suggest that around half of the molecules are acetylated. Since the REGγ molecules form heptamers, it is likely that such an acetylation fraction may be enough for enhancing protein-protein interactions.

Given that REGγ is highly homologous to REGα, the predicted location of K195 in REGγ should be at the very C-terminus of the Helix3 based on the structure of REGα (48). Facing the substrate interaction surface, K195 should be easily accessed by enzymes such as SIRT1 to dynamically regulate REGγ’s disassembly. If interactions between REGγ monomers only occur between helix 2 and 4, we believe K195 acetylation may induce favorable structure to facilitate this interaction. Acetylation in REGγ remarkably enhances monomeric interactions and heptameric formation, which is consistent with previous reports that acetylation can enhance protein-protein interactions (49-51). Whether acetylation of REGγ at K195 is a default or translationally coupled process remains to be investigated. Future crystal structure analysis of REGγ may enable us to understand how this site specific acetylation facilitates its protein-protein interactions.

Interestingly, the endogenously expressed acetylation-defective REGγ-K195R mutant dramatically impaired the heptameric complex, resulting in an elution pattern ranged from monomers, various degrees of oligomers, and reduced amount of heptamers (Fig. 5E). The results suggest that REGγ indeed has intrinsic properties in self association (48). It is likely that acetylation may accelerate the oligomerization processes of intracellular REGγ, which may be otherwise targeted for degradation or association with other protein complexes. In support of this idea, we found that acetylated REGγ is more stable while acetylation defective mutant degrades faster (Fig. 5F). Despite that cells stably or transiently expressing REGγ-K195R also have significant amount of endogenous wild type REGγ, we still observe significant impact of K195R mutant on protein-protein interaction and proteolytic functions (Fig. 3, 5).

To summarize our findings in this study, CBP acetylates REGγ at K195, which promotes its heptamerization and increases REGγ activity in the degradation of targets proteins. In contrast, SIRT1 can bind with REGγ and deacetylates REGγ, which inhibits heptamerization or trigger disassembly of REGγ, leading to inactivation of REGγ capacity (Fig. 7). Our results provide a novel mechanism for the reciprocal regulation of REGγ homeostasis by acetylation. As a potential drugable target, REGγ activity may be modulated by HAT/SIRT inhibitors or activators.

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FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. REGγ is acetylated in mammalian cells. Endogenous REGγ in HEK293 (A), A549 (B) or HeLa cells (C) were immunoprecipitated with anti-REGγ antibody. Acetylation of REGγ was detected by immunoblotting with an anti-AcK antibody. D. A reciprocal immunoprecipitation was performed with the anti-AcK antibody using lysates from HEK293 cells and immunoprecipitated REGγ was examined by immunoblotting with anti-REGγ antibody. E. HEK293 cells inducibly expressing Flag-REGγ were cultured in the presence of doxycycline (1µg/ml) for 48h, with 6.6 µM TSA and 10 mM NAM for 6h before harvesting the cells. Flag-REGγ was immunoprecipitated by Flag M2 Affinity Gel. REGγ acetylation was examined as in A-C. Asterisk (⁎) refers to non-specific bands.

FIGURE 2. K195 is a major acetylation site in REGγ. A. Sequence analysis of REGγ from multiple species indicates that K195 is a highly conserved acetylation site, with a slightly less conservation at K6 and K14 among vertebrates, consistent with the results of Mass Spectrometry. B. LC-MS/MS spectrum of IAK(ac)YPHVEDYR identified from recombinant REGγ protein expressed in E. coli. The search result was opened by Viewer in MaxQuant package. The precursor ion m/z showed a mass shift of 42.01Da, b3, b4, b7-10 and y3-7, y9, y10 fragment ions were found in MS/MS spectrum. The acetylated peptide hits were filtered by 1% FDR at protein, peptide and site level. C. Flag-tagged REGγ was purified from 293 REGγ inducible cells by Flag peptide. Acetylated REGγ was immunoprecipitated by anti-AcK antibody. The unbound REGγ and bound acetylated REGγ were examined by immunoblotting with anti-Flag antibody. D. HEK293 cells inducibly expressing Flag-REGγ were treated with 1µg/ml of doxycycline for 48h. Flag-REGγ WT and each mutants were immunoprecipitated with anti-Flag M2 Affinity Gel and their acetylation status were examined by immunoblotting with anti-AcK antibody. E. In HEK293 cells, transiently expressed Flag-REGγ-WT and Flag-REGγ K195R were immunoprecipitated with anti-Flag M2 Affinity Gel and their acetylations were determined by immunoblotting with anti-AcK antibody.

FIGURE 3. Blocking acetylation at K195 attenuates REGγ activity. A. H1299 cells were co-transfected with HA-HCV core-173 and REGγ plasmids for 32h. The HA-HCV core protein levels were determined by immunoblotting. B. REGγ-WT, K195R and K195Q were co-transfected with p21 for 32h in H1299 cells and p21 levels were determined by immunoblotting. C. Endogenous p21 protein levels were examined by immunoblotting in cells stably integrated REGγ derivatives in REGγ-/- MEF stable cells as indicated. Stability of endogenous p21 was examined by CHX (100mg/ml) treatment for indicated time in HEK293 inducible cells overexpressing REGγ WT (D), REGγ K195R (E), or K195Q (F). Asterisk (⁎) means non-specific bands.
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FIGURE 4. CBP and SIRT1 regulate REGγ acetylation and activity. A. HEK293 cells were transfected with CBP for 32h and acetylation of endogenous REGγ was examined by immunoblotting with anti-AcK antibody. B. The recombinant GST-REGγ (5 µg) were incubated with Flag-CBP (3 µg) in HAT buffer 30 µl for 3h at 30°C. C. Endogenous REGγ protein in HEK293 cells was immunoprecipitated with anti-REGγ antibody, and IgG as a control. D. Endogenous SIRT1 protein in HEK293 cells was immunoprecipitated with an anti-SIRT1 antibody, the immunoprecipitated REGγ protein levels were determined by immunoblotting with anti-REGγ antibody. E. CBP and SIRT1 were transfected into HEK293 cells inducibly expressing Flag-REGγ-WT or Flag-REGγ-K195R as indicated. Acetylation levels of REGγ were examined by immunoblotting with anti-AcK antibody after immunoprecipitation of Flag-REGγ with anti-Flag M2 Affinity Gel. F. Endogenous REGγ acetylation levels were examined in SIRT1 knocking down 293T cells by immunoblotting with anti-REGγ antibody. G. Acetylated Flag-REGγ was incubated with recombinant His-SIRT1 in the deacetylase buffer 50 µl for 2h at 30°C. REGγ acetylation level was analyzed by immunoblotting. H. HeLa cells were transfected with SIRT1 or SIRT1-H363Y mutant for 32h. Endogenous p21 and REGγ protein levels were analyzed by immunoblotting with anti-p21 or anti-REGγ antibody. I. HEK293 cells were transiently transfected with siRNA against CBP for 72h, CBP knockdown efficiency and REGγ, p21 protein levels were examined by immunoblotting.

FIGURE 5. Acetylation at K195 is crucial for REGγ heptamerization and interactions between REGγ monomers. A. HEK293 cells inducibly expressing Flag-REGγ were cultured with 1µg/ml of doxycycline for 48h, in the presence or absence of TSA and NAM for 6h before cells were harvested. REGγ heptamerization was determined by Native-PAGE Gel system with anti-Flag antibody. B. Flag-REGγ-WT, K195R, K195Q or K188F was transfected into HEK293 cells for 32h. Cell lysates were analyzed by Native-PAGE Gel system to examine REGγ heptamerization. C. Flag-REGγ and HA-REGγ constructs were transfected into HEK293 cells as indicated. Cell lysates was immunoprecipitated by Flag M2 Affinity Gel. Co-immunoprecipitated HA-REGγ was detected by immunoblotting with an anti-HA antibody. D. HEK293 cells were treated with TSA/NAM or TSA/NAM along with transient expression of SIRT1 as indicated, together with co-expression of different tagged REGγ derivatives. Co-immunoprecipitated HA-REGγ was detected by immunoprecipitating Flag-REGγ and immunoblotting with indicated antibodies. E. Cell lysates from the Flag-REGγ inducible HEK293 cells were subjected to size exclusion chromatography for FPLC analysis. F. Stability of Flag-REGγ WT or K195R mutant was examined by CHX treatment for indicated time in HEK293 inducible cells overexpressing Flag-REGγ WT or K195R.

FIGURE 6. Acetylation mutation at K195 in REGγ reduces cell proliferation and mitigates cell cycle progression. A. HEK293 cells inducibly expressing Flag-REGγ were treated with doxycycline after cells were seeded in 96-well plates for 24 hours. Absorbance was measured at indicated times. Data were analyzed as means ± SD of spectrometric absorbance of three independent experiments. * P<0.05, ** P<0.01, and *** P<0.001 represent the statistic comparisons between growth in HEK293 with Flag-REGγ-WT and HEK293 with Flag-REGγ-K195R. B. The HEK293 with Flag-REGγ-WT and HEK293 with Flag-REGγ-K195R were treated with doxycycline for 48h and DNA contents of the inducible cells in different cell cycles were analyzed by flow cytometry. Each bar indicates the distribution of the cell cycles. Data are reported as means ± SD of three independent experiments. * P<0.05, ** P<0.01, and *** P<0.001 indicate statistic differences between indicated groups.

FIGURE 7. A working model simplify the influences of acetylation on REGγ assembly and function. CBP acetylates REGγ at K195, which promotes REGγ to form heptamer, resulting in augmented REGγ activity. When SIRT1 binds with REGγ, it deacetylates REGγ and inhibits REGγ heptamerization, releasing monomers from REGγ dis-assembly.
Acetylation regulates REGγ activity by facilitating its heptamerization

Figure 1

A

B

C

D

E

HEK293 cells

A549 cells

HeLa cells

HEK293 cells

Doxycycline inducible Flag-REGγ HEK293 cells
Acetylation regulates REGγ activity by facilitating its heptamerization
Acetylation regulates REGγ activity by facilitating its heptamerization
Acetylation regulates REGγ activity by facilitating its heptamerization

Figure 4

A

Input

CBP

- +

250

25

α-CBP

α-REGγ

IP: REGγ

25

α-AcK

α-REGγ

B

CoA

- +

Flag-CBP

+ +

GST-REGγ

+ +

α-CBP

α-AcK

α-REGγ

C

IP

IP: IgG

IP: REGγ

100

100

100

α-SIRT1

α-REGγ

α-REGγ

D

IP

IP: IgG

IP: SIRT1

100

100

100

α-SIRT1

α-REGγ

α-REGγ

E

DOX

- + + + + + +

CBP

- - + + + + +

SIRT1

- - + + + + +

α-CBP

α-SIRT1

α-Flag

α-AcK

α-Flag

Input

Input

Flag-REGγ WT

Flag-REGγ K195R

250

250

100

100

25

25

5 6 7 8

35

35

F

Input

Input

α-SIRT1

α-REGγ

α-actin

α-AcK

α-REGγ

G

NAM

- - - +

NAD+

- - + +

His-SIRT1

- + + +

Ac-Flag-REGγ

1 2 3 4 5

- + + + +

α-SIRT1

α-AcK

α-Flag

H

SIRT1

Ctrl

WT

H363Y

1 2 3 4 5

130

25

25

α-SIRT1

α-Act

α-REGγ

α-p21

α-actin

I

si Ctrl

si CBP

250

25

25

37

α-CBP

α-RFγ

α-p21

α-actin
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Acetylation regulates REGγ activity by facilitating its heptamerization.
Site-specific acetylation of the proteasome activator REGγ directs its heptameric structure and functions
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