Regulation of Co-repressive Activity of and HDAC Recruitment to RIP140 by Site-specific Phosphorylation*

Pawan Gupta, M. D. Mostaql Huq, Shaukat Ali Khan, Nien-Pei Tsai, and Li-Na Wei‡

Receptor interacting protein 140 (RIP140) is a versatile transcriptional co-repressor that contains several autonomous repressive domains (RDs). The N-terminal RD acts by recruiting histone deacetylases (HDACs). In a comprehensive proteomic analysis of RIP140 by MS, 11 phosphorylation sites of RIP140 are identified; among them five sites are located in the N-terminal RD including Ser\(^{104}\), Thr\(^{202}\), Thr\(^{207}\), Ser\(^{358}\), and Ser\(^{380}\). The role of phosphorylation of RIP140 in regulating its biological activity and the underlying mechanism are examined using a site-directed mutagenesis approach. Mutations mimicking constitutive phosphorylation or dephosphorylation are introduced. The N-terminal RD phosphorylation, mediated by the mitochondrion-activated protein kinase (MAPK), enhances its repressive activity through increased recruitment of HDAC. Mutations mimicking constitutive dephosphorylation at Thr\(^{202}\) or Thr\(^{207}\) significantly impair its repressive activity and HDAC recruitment, whereas mutation at Ser\(^{358}\) only slightly affects its HDAC recruitment and the repressive activity. Consistently, mutations mimicking constitutive phosphorylation at either Thr\(^{202}\) or Thr\(^{207}\) convert RIP140 into a more potent repressor, which is less responsive to a disturbance in the MAPK system. Furthermore, constitutive phosphorylation at both Thr\(^{202}\) and Thr\(^{207}\) residues renders RIP140 fully repressive and strongly interacting with HDAC. The activity of this mutant is resistant to the MAPK inhibitor, indicating an essential role for Thr\(^{202}\) and Thr\(^{207}\) in MAPK-mediated modulation of RIP140 function. The study provides insights into the modulation of RIP140 biological activity through a specific cellular signaling pathway that augments phosphorylation at specific residues of RIP140 molecule and alters its cofactor recruitment. Molecular & Cellular Proteomics 4:1776–1784, 2005.

Environmental factors in the extracellular milieu utilize signal transduction pathways to propagate their cues into gene expression (1–3). Transcriptional factors and their co-regulators are extensively modified at the post-translational level, which usually regulate the critical function and property of the protein (4–9). Deciphering such modifications and relating them to the biological function is referred to as functional proteomics (10). A variety of post-translational modifications have been found that regulate protein functions, including phosphorylation, acetylation, methylation, glycosylation, ubiquitination, and sumoylation, etc. (11–14).

Receptor interacting protein 140 (RIP140)\(^1\) is a co-regulator for many transcription factors including nuclear receptors (15–19). Extensive studies have been conducted to examine the versatile activity of RIP140 in transcriptional regulation. It is known that RIP140 acts, primarily, as a transcriptional corepressor through different mechanisms (20–22). RIP140 is recruited to nuclear receptors through its nine LXLL motifs and a modified motif LXXLL, where X can be any amino acids (23, 24). With respect to its repressive activity, four autonomous repressive domains (RDs) are known. RD1 is located in the amino-terminal region (amino acids 1–495), RD2 and RD3 are located in the central portion (amino acids 336–1006), and RD4 is located in the carboxyl-terminal region (amino acids 977–1161). These domains function through various mechanisms. The amino-terminal RD acts by recruiting histone deacetylases (HDACs) through an HDAC-interacting domain, which has been mapped to amino acids 78–303 (22, 25). The central region interacts with the carboxyl-terminal binding proteins (CtBP1 and CtBP2) (26). In terms of its physiological action, RIP140-null mice exhibit female reproductive defects (15). Further studies of these animals indicate that RIP140 could play an important role in the regulation of fat accumulation in adipose tissues (27).

Recently, we initiated a functional proteomic study of RIP140 expressed and purified from insect cells. Through extensive mass spectrometric analyses, we found RIP140 are extensively phosphorylated (28) and acetylated (29). To continue the functional proteomic endeavor, we took a systematic mutagenesis approach to uncover the function and the mechanism of actions of specifically modified residues of RIP140. This study reports our systematic studies of the functional role of phosphorylation on RIP140, specifically with respect to its regulation of fat accumulation in adipose tissues (27).

\(^{1}\) The abbreviations used are: RIP140, receptor interacting protein 140; RD, repressive domain; HDAC, histone deacetylase; CtBP, carboxyl-terminal binding protein; MAPK, mitogen activated protein kinase; WT, wild type; Mut, mutant; DCC, dextran charcoal; PKC, protein kinase C; CaCal, calcium-calmodulin-dependent protein kinase II; RLU, relative luciferase unit; CN, constitutive negative; CP, constitutive positive.
role in the HDAC-mediated repressive activity of RD1.

We previously reported the identification of 10 phosphorylation sites on RIP140 purified from insect cells by LC-ESI-MS/MS analysis (28). These residues included Ser104, Thr207, Ser358, Ser380, Ser486, Ser519, Ser531, Ser543, Ser572, and Ser1003. One residue, Thr202, appeared ambiguous in our initial analysis, and a reanalysis of the MS/MS data enabled us to verify Thr202 as an additional phosphorylation site on RIP140. We then determined the effects of phosphorylation on the biological activity of RIP140 and explored the mechanism underlying such an effect in this current study. RIP140 employs multiple repressive pathways including HDAC- and CtBP-triggered events for RD1 and RD2/3, respectively. We focus this initial functional proteomic study on the HDAC-mediated RD1 activity. We identified mitogen-activated protein kinase (MAPK)-mediated phosphorylation as the critical pathway for RD1-mediated repressive activity, which was caused by enhanced recruitment of HDAC by phosphorylated RIP140. Mutations mimicking either constitutive phosphorylation or dephosphorylation were generated to determine whether the effects were caused by altered charges brought about by phosphorylation on specific residues or the amino acid residues per se. With this systemic approach, we identified two residues, Thr202 and Thr207, crucial for the repressive activity of RD1 and its ability to recruit HDACs. Furthermore, increased negative charge caused by phosphorylation on these two specific residues is essential.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors—Constructs of RIP140 full length/N-terminal fused to GAL4-BD (20), RIP140 full length fused to His-epitope (28), FLAG-epitope (22), and GAL4-tk-luciferase reporter (23) have been described previously. To construct pAD-GAL4-HDAC3, coding regions of HDAC3 were released from pG4-hRPD3-2B (22) by EcoRI/XbaI and ligated to the EcoRI/XbaI site of pVP16 (AD-GAL4).

Purification and Mass Spectrometric Analysis of RIP140 —To identify the phosphorylation sites on RIP140, we expressed the His-tagged RIP140 in insect cells as a eukaryotic host for mammalian protein expression. The protein was purified by affinity column over Talon resin with 95% homogeneity. The details of the procedure for RIP140 purification was described previously (28). The purified RIP140 was subjected to tryptic digestion, and LC-ESI-MS/MS analysis was conducted as already described to identify the modified residues (28).

Analysis of Biological (Trans-repressive) Activity of RIP140 —The technique for culturing COS-1 cells, transfection experiments, and luciferase and lacZ assay were as described previously (23). Cells were transiently transfected by the calcium phosphate precipitation method with a mixture of pBd-GAL4-RIP140 full length/N-terminal, wild-type (WT)/mutant (Mut) (0.1 μg) or pBd-GAL4 (0.1 μg), GAL4-tk-luciferase (0.5 μg) reporter, and a CMV-lacZ internal control (0.05 μg). Forty h post-transfection, cells were exchanged with fresh medium containing dextran charcoal (DCC)-treated serum. At 48 h post-transfection, cells were harvested, and RIP140 full length was immunoprecipitated by anti-FLAG antibody (Sigma) on protein G-agarose (Sigma) beads (22). The partially purified FLAG-RIP140 fusion protein was incubated with [35S]methionine-labeled HDAC3 protein for pull-down reaction as described previously (23). The specific bound proteins were released by resuspending beads in 20 μl of SDS loading buffer, divided in equal amounts, and resolved by SDS-PAGE. One gel was subjected to Western blot analysis probed with anti-FLAG antibody followed by horseradish peroxidase-conjugated secondary antibody and then washed extensively with extraction buffer followed by binding buffer and then tested for interaction with [35S]methionine-labeled HDAC3 protein. For input, Western blot analysis was probed with anti-His antibody (Upstate Biotechnology).

In Vivo Protein-Protein Interaction Assays (Mammalian Two Hybrid Test)—COS-1 cells were maintained as described previously (23). To test the interaction of RIP140 full-length/N-terminal, WT/Mut constructs with HDAC3, cells were co-transfected with pBd-GAL4-RIP140 full-length/N-terminal, WT/Mut (0.1 μg) as bait and pAD-GAL4-HDAC3 (0.1 μg) as prey. All co-transfections included GAL4-tk-luciferase (0.5 μg) reporter and a CMV-lacZ internal control (0.05 μg). Forty h post-transfection, cells were exposed to either 1 μM MAPK activator (anisomycin) or 3 μM MAPK inhibitor (PD98059) for 8 h in Dulbecco’s modified Eagle’s medium containing DCC serum. At 48 h post-transfection, cells were harvested, and RIP140 full length was immunoprecipitated by anti-FLAG antibody (Sigma) on protein G-agarose (Sigma) beads (22). The partially purified FLAG-RIP140 fusion protein was incubated with [35S]methionine-labeled HDAC3 protein for pull-down reaction as described previously (23). The specific bound proteins were released by resuspending beads in 20 μl of SDS loading buffer, divided in equal amounts, and resolved by SDS-PAGE. One gel was subjected to Western blot analysis probed with anti-FLAG antibody followed by horseradish peroxidase-conjugated secondary antibody and then washed extensively with extraction buffer followed by binding buffer and then tested for interaction with [35S]methionine-labeled HDAC3 protein. For input, Western blot analysis was probed with anti-His antibody (Upstate Biotechnology).

Effect of RIP140 Phosphorylation

Site-directed Mutagenesis—Constructive negative/positive, point/sequential mutations involving residues Ser104, Thr202, Thr207, Ser315, Ser358, and Ser380 in WT pBd-GAL4-RIP140 full-length/N-terminal WT and pAD-GAL4-HDAC3 co-transfection. Reported values are an average of three experiments with triplicate measurement taken in each experiment.
Effect of RIP140 Phosphorylation

primers employed to generate mutant constructs are: S104A: 5'-dGGAAGAGGCTGGCTGATGCCATCGTGAATTTAAAC-3' (sense), 5'-dGTTTAAATTCACGATGGCATCAGCCAGCCTCTTCCG-3' (antisense); T202A: 5'-dGAAAAGTCAGATCCCGCCCTCCCTGACGTG-3' (sense), 5'-dCACGTCAGGGAGGGCGGGATCTGACTTTTC-3' (antisense); T207A: 5'-dCTCCCTGACGTGGACCAAACCTTATC-3' (sense), 5'-dGATAAGGTTGGAGCCACGTCAGGGAG-3' (antisense); S315A: 5'-dAGAAGGACGTGGGCGCTTCGCAGCTCTCC-3' (sense), 5'-dGGAGA-GCTGAGCCAGCGCTCTTCTG-3' (antisense); S358A: 5'-dGTGTTGTCCCTTCCGCCCCCAAAAACACGAGC-3' (sense), 5'-dCGTGTTTTTGGGTTCGGAAAGGGACAAC-3' (antisense); S380A: 5'-dGTTGTCCCTTCCGAACCCAAAAACACG-3' (sense), 5'-dCGTGTTTTTGGGTTCGGAAAGGGACAAC-3' (antisense). The positive clones were verified by DNA sequencing.

RESULTS

Verification of Phosphorylation at Thr202 of RIP140—A MS/MS analysis of tryptic phosphopeptides was carried out to identify the modified residues. Previously, we reported 10 phosphorylation sites including Ser104, Thr207, Ser358, Ser380, Ser488, Ser519, Ser531, Ser543, Ser672, and Ser1003 (28). Phosphorylation on Thr202 appeared ambiguous according to the initial analysis of the MS spectrum. To clarify the ambiguity, we reanalyzed the data to verify Thr202 phosphorylation. In the total ion chromatogram, the tryptic monophosphopeptide spanning residues 199–212 (SGPTLPDVTPNLIR) eluted as a doubly charged precursor ion at m/z 780.38 (precursor mass 1558.76 Da) at 47.36 min. Product ion analysis of this precursor analysis suggested that some species of the peptide were phosphorylated at Thr202, whereas some were phosphorylated at Thr207. The MS/MS data clearly showed an 80-amu delta mass shift at y6**, through y9** showed a 80-amu shift, which clearly suggested Thr207 phosphorylation. The product ion at m/z 1024.56 was attributable to the singly charged y9 ions of the unmodified peptide, suggesting in some species of the precursor ion (m/z 780.38), Thr207 was not modified. On the other hand, the doubly charged a8 ion showing a 40-amu delta mass shift suggested Thr202 phosphorylation.

![Fig. 1. Identification of Thr202 phosphorylation by MS/MS analysis of the precursor ion m/z 780.38. The product ions for Thr202 were indicated as y** and b** ions, whereas the ions due to Thr207 phosphorylation were marked as y* and b* ions. The doubly charged ions are indicated as underlined bold characters over the spectrum. The y ions y6** through y9** showed a 80-amu shift, which clearly suggested Thr207 phosphorylation. The product ion at m/z 1024.56 was attributable to the singly charged y9 ions of the unmodified peptide, suggesting in some species of the precursor ion (m/z 780.38), Thr207 was not modified. On the other hand, the doubly charged a8 ion showing a 40-amu delta mass shift suggested Thr202 phosphorylation.](image-url)
Eleven phosphorylation sites of RIP140 were identified by LC-ESI-MS/MS analysis (28). Sequences to these sites were compared to consensus sites of all known protein kinase (34). Correspondence of these sequences to the consensus motifs suggest kinases having a potential role to phosphorylate these residues. The residues have been segregated on the basis of RDs of RIP140.

**Phosphorylation Sites and Corresponding Kinases**—All the 11 MS-confirmed phosphorylation sites of RIP140 were compared with the consensus motifs of all known protein kinases (31) as listed in Table I. The N-terminal RD contains five phosphorylation sites. Thr202 and Thr207 are potential sites for MAPK phosphorylation, Ser358 can be phosphorylated either by MAPK or PKC, and the other two at Ser104 and Ser380 were unmatched to consensus sequences of known kinases. The remaining six sites in the central and C-terminal RD are potential targets for MAPK, PKC, or CaCal. We then explored the role of these kinases in regulating the repressive activity of RIP140.

**Effects of MAPK/PKC/CaCal Inhibitors on Trans-repressive Activity of Full-length RIP140**—To evaluate the intrinsic repressive activity of RIP140, a standard trans-repressive assay was conducted where the full-length RIP140 was fused to a GAL4-BD and analyzed using a GAL4-responsive reporter in a standard cellular system, the COS1 cells. Inhibitors of MAPK, PKC, and CaCal were used to determine the effects of these kinase pathways on the trans-repressive activity of RIP140. The fold relative luciferase activity was evaluated to determine the effect on trans-repression. As shown in Fig. 2, both PKC and CaCal inhibitors have no effect on the trans-repressive activity of RIP140, whereas the specific MAPK inhibitor significantly relieves the repression, suggesting a role for MAPK-induced phosphorylation in mediating the repressive activity of RIP140. Our previous study has identified HDAC recruitment as the primary repressive mechanism of RIP140 through RD1 (22, 25). Therefore we further examined the effects of MAPK-induced phosphorylation on the recruitment of HDAC by RIP140.

**Effect of Phosphorylation on the Recruitment of HDAC**—To examine the direct effect of phosphorylation on HDAC recruitment, we employed an *in vitro* pull-down assay that directly monitors protein-protein interaction. *In vivo* phosphorylated or hypophosphorylated RIP140 was prepared by transfecting FLAG-RIP140 into a mammalian cell line that has a full capacity for MAPK signaling, 3T3, followed by an 8-h pulse of 1 μM chelethrin chloride (PKC inhibitor), 0.5 μM KN-93 (CaCal inhibitor), 3 μM PD 98059 (MAPK inhibitor), and compared with control (endogenous condition). MAPK inhibitor abolished the trans-repressive activity of RIP140. This suggests MAPK-mediated phosphorylation potentiates the repressive activity of RIP140.
Effect of RIP140 Phosphorylation

Fig. 3. Effect of phosphorylation status of RIP140 on recruitment of HDAC3. In the FLAG pull-down assay (A), the phosphorylated (lane b) and hypophosphorylated (lane c) state of RIP140 was ensured by in vivo treatment with MAPK activator (1 μM) and MAPK inhibitor (3 μM), respectively. The endogenous condition is represented in lane a. Whereas phosphorylated RIP140 demonstrated efficient interaction with HDAC, the hypophosphorylated state showed dramatically reduced ability to interact with HDAC. In the His pull-down assay (B), the phosphorylated state is ensured by purification of RIP140 from insect cells in the presence of phosphatase inhibitor (lane a) and phosphorylation sites were confirmed by MS, whereas the dephosphorylated state (lane b) is obtained in vitro by treatment with alkaline phosphatase. Dephosphorylated state was less effective in recruitment of HDAC.

Fig. 4. Demonstration of trans-repressive activity of RIP140 N-terminal RD (WT/Mut) constructs in presence of MAPK activator or inhibitor. Trans-repression by RIP140 N-terminal WT and CN Mut constructs of GAL4-tk-luciferase reporter are compared in presence of MAPK activator or inhibitor (A). Expression profiles of all relevant WT and Mut constructs at the transcription and translational level are shown in B. Abrogation of site-specific negative charge at Thr202 and Thr207 as mimicked by Thr-Ala mutations abolished the trans-repressive potential of RIP140. (Statistics: I, † versus * (p < 0.04); || versus I (p < 0.01); double curved bars with horizontal bar versus || (p > 0.05).)

Efficiency of RIP140 with HDAC, whereas the MAPK inhibitor drastically reduces the level of HDAC3 recruited to RIP140 (3-fold), suggesting hypophosphorylation of RIP140 impairs its HDAC-recruiting ability. The marginal effect of the MAPK activator on RIP140 prepared from this cell line with regards to its HDAC recruitment is consistent with the notion that the MAPK system is constitutively active in the 3T3 cellular background.

To provide further direct evidence, phospho-RIP140 (His-tagged) was purified from insect cells under a condition that stabilizes protein phosphorylation (28). The dephosphorylated state was achieved in vitro by treating this protein preparation with alkaline phosphatase. These two protein preparations were analyzed also in a His pull-down assay by incubating in vitro-translated, 35S-labeled HDAC3. As shown in Fig. 3B, ~2.5-folds more HDAC was recruited to the phospho-RIP140 than to the dephosphorylated protein. The input was also monitored as.
shown at the bottom of this figure. From these two protein-protein interaction experiments, it can be concluded that phosphorylated RIP140 more effectively recruits HDAC.

Effects of Phosphorylation on the Trans-repressive Activity of RIP140-N-terminal RD—Because the HDAC-mediated repression involved, primarily, the N-terminal RD1 domain, we thus focused our attention on the RD1 to dissect the roles of specifically phosphorylated residues within this domain. A systematic approach was taken by mutating the five residues independently and in combination within this domain that can be phosphorylated. Furthermore, to test whether the residue itself or the increased site-specific negative charge (30) brought about by phosphorylation on that particular residue triggered the effects on the repressive activity of RD1, both dominant negative and constitutive positive mutations were made in the context of the GAL4 fusion that can be analyzed readily using the GAL4 reporter system. The dominant negative mutations (CN) were made by changing the residue to Ala, whereas the constitutive positive (CP) mutations were made by changing the residue to Glu.

To dissect the effects specifically on the property of RD1, the first series of CN mutants were examined in the context of GAL4-RD1 fusion. As shown in Fig. 4A, in the presence of the MAPK inhibitor (thus the hypophosphorylated state) the repressive activity of WT RD1 is reversed (from 0.2 to 0.7 relative trans-activation). A single mutation at either Thr202 or Thr207 renders RD1 much less repressive in the presence of the MAPK activator, whereas the single mutant, at either Ser104 or Ser358, behaves almost like the WT. The repressive activity of the Ser358 single mutant is also slightly affected, although to a much lesser extent than the Thr202 or Thr207 single mutant. Importantly, double mutation at both Thr202 and Thr207 completely abrogates the MAPK-mediated trans-repression because the same level of activity is detected in the presence of the MAPK activator or inhibitor. The triple mutant at Thr202/Thr207/Ser358 behaves essentially like the Thr202/Thr207 mutant. A mock mutation at residue Ser315, serving as a negative control, has no effect on the repressive activity as compared with the WT. This result suggests that Thr202 and Thr207 are crucial for the MAPK-enhanced trans-repressive activity of RD1 and that Ser358 also modulates the RD1 activity, but to a lesser extent. To monitor the efficiency of the production of these Mut proteins, the expression of transfected expression vector was examined using RT-PCR (to monitor RNA production) and Western blot (to monitor protein production) as shown in Fig. 4B, which verifies the production of a comparable level of mutant proteins.

Effects of Phosphorylation on Interaction of RD1 with HDAC3—To provide a mechanistic insight, the effects of these mutations were evaluated for their effects on the interaction between N-terminal RD and HDAC3 in both phosphorylated and hypophosphorylated conditions using a two-hybrid interaction test as shown in Fig. 5. As predicted, interaction between the WT RD1 and HDAC3 occurs effectively in the presence of the MAPK activator, whereas the MAPK inhibitor abrogates almost completely this interaction. Single mutation at either Ser104 or Ser380 exerts little effect. Interestingly, single mutation at either Thr202 or Thr207 dramatically affects the interaction in the presence of the MAPK activator. Consistent with the effect seen in the repressive activity of the Ser358 mutant (Fig. 4), interaction of this mutant with HDAC is also less effective than the WT. More dramatically, double mutation at Thr202/Thr207 as mimicked by Thr-Ala mutations reduces the ability to interact with HDAC.

(Statistics: † versus * (p < 0.01); ‖ versus † (p < 0.02); double curved bars with horizontal bar versus ‖ (p > 0.05).)
however, this residue is less critical than either Thr\textsuperscript{202} or Thr\textsuperscript{207}.

Role of Site-specific Phosphorylation on the Trans-repressive Activity and HDAC Recruitment of the Full-length RIP140—The CN mutation studies reveal the essential functional role of two specific residues, Thr\textsuperscript{202} and Thr\textsuperscript{207}, in regulating the trans-repressive activity of RD1 and its ability to recruit HDAC. However, two relevant questions remain. First, this result does not allow conclusion to be made as to whether the effects are due to phosphorylation or because of changes in amino acid residues. Second, it is unknown if the effects of these RD1 domain mutations remain effective in the context of the full-length RIP140. One primary effect of protein phosphorylation is to impart the site-specific negative charge of the protein, which may subsequently alter its behavior such as interaction with other proteins or its enzyme activity (30). To test whether an increase in the site-specific negative charge of these residues, as predicted from phosphorylation, was responsible for the effects on trans-repressive activity and HDAC recruitment, we generated CP mutations by changing residues Thr\textsuperscript{202}, Thr\textsuperscript{207}, and Ser\textsuperscript{358} singly or in combination into Glu.

![Graph showing trans-repressive activity of RIP140 full-length (WT/Mut) constructs](image)

**Fig. 6.** Demonstration of trans-repressive activity of RIP140 full-length (WT/Mut) constructs in presence of MAPK activator or inhibitor. Trans-repression by RIP140 full-length WT or Mut (CP or CN) constructs of GAL4-tk-luciferase reporter are compared in presence of MAPK activator or inhibitor (A). Expression profiles of all relevant WT and Mut constructs at the transcription and translational level are shown in B. A site-specific negative charge imparted at Thr\textsuperscript{202} and Thr\textsuperscript{207} as mimicked by Thr-Glu mutations is essential for trans-repressive activity.

(A) (B)

Effect of RIP140 Phosphorylation
Effect of RIP140 Phosphorylation

Fig. 7. Interaction of HDAC3 with RIP140 full-length (WT/Mut) constructs. A mammalian version of the two-hybrid system was used to examine in vivo interaction between HDAC3 and RIP140 full-length (WT and CP or CN Mut) constructs in COS-1 cells. The GAL4-BD-RIP140 full-length fusion and AD-GAL4-HDAC3 fusions were co-transfected pair-wise in COS-1 cells along with the reporter and a CMV-lacZ internal control. Luciferase units of mutant interaction with HDAC were normalized to WT interaction with HDAC. A site-specific negative charge imparted at Thr202 and Thr207 as mimicked by Thr-Glu mutations seems to be crucial for recruitment of HDAC. (Statistics: I versus * (p < 0.03); † versus * (p > 0.05); II versus I (p < 0.03); double curved bars with horizontal bar versus || (p > 0.05)).

action as predicted. Whereas all the single CP mutants retain their HDAC interaction potency in the presence of the MAPK activator (open bars) or without drug treatment (data not shown), their ability to interact with HDAC is affected by the MAPK inhibitor, but to a lesser extent than the WT RIP140. Importantly, the double CP mutant at Thr202/Thr207 and the triple CP mutant at Thr202/Thr207/Thr358 constitutively interact with HDAC even in the presence of the MAPK inhibitor, further supporting the notion that HDAC interaction is also critically regulated by the site-specific negative charge of these residues on RD1 of RIP140, i.e. Thr202 and Thr207. Hence, the MAPK-elicited phosphorylation on these two residues increases the site-specific negative charge of RD1, thereby enhancing its ability to recruit HDAC to RIP140. As predicted, the triple CN mutant at Thr202/Thr207/Thr358 can no longer interact effectively with HDAC even in the presence of the MAPK activator.

DISCUSSION

RIP140 is extensively modified post-translationally. Among 11 phosphorylated residues that are confirmed by the mass spectrometric study (28), 5 are located in the N-terminal RD that encodes an intrinsic repressive activity through recruiting HDACs. This study demonstrates for the first time that RD1 activity of RIP140 is regulated by MAPK-elicited phosphorylation on specific residues of RD1, i.e. Thr202 and Thr207. Furthermore, the phosphorylation-elicited site-specific negative charge on these residues plays a critical role in both recruiting HDACs and conferring a repressive activity. Phosphorylation on Ser104 or Ser380 has no effect in these regards, whereas phosphorylation at residue Ser358 has a marginal effect.

In our assays conducted in the cellular background of 3T3 and COS-1, the repressive potential remains essentially the same in the presence or absence of MAPK activator. Therefore, the MAPK pathway is constitutively active in these cell lines. Hence, the basal (without drug treatment) repressive activity resembles the repressive activity in the presence of the MAPK activator, whereas the repressive activity is dramatically affected by the addition of the MAPK inhibitor.

The HDAC-interacting surface of RIP140 is mapped to its N-terminal domain between amino acids 78–303 (22, 25). In this domain, Thr202 and Thr207 are directly involved in the enhanced recruitment of HDAC3 and the repressive activity. Presumably, negative charges brought about by phosphorylation on these residues play a critical role for RIP140 to make a direct contact with HDAC. For instance, charges on these two residues may directly control the binding affinity between RIP140 and HDAC. An interesting finding is with Ser358. This residue may not be directly involved in interaction with HDAC; however, its phosphorylation probably facilitates the interaction with HDAC by inducing a conformational change in the tertiary structure of this protein because of the increased negative charge on this site. However, this effect is only apparent in the context of the dissected RD1 (Figs. 4 and 5) because mutation at Ser358 in the context of the full-length RIP140 exerts no significant effect on either HDAC recruitment or the repressive activity of the full-length protein (Figs. 6 and 7). Conceivably, the conformation of RD1 may be slightly different from its configuration in the context of the intact protein. It remains to be further examined whether Ser358 phosphorylation has an effect on other aspects of RIP140. Similarly, mutation at either residue Ser104 or Ser380 has no effect on the HDAC-mediated repressive potential of RIP140. Whether phosphorylation at these residues plays a role in other aspects of RIP140 also remains to be determined. Alternative, we can not rule out the possibility that these sites can be nonspecifically modified by low affinity kinases in insect cells (28).

The MAPK/PKC/CaCal sites in the RD2 and RD3 domains do not seem to have any effect on HDAC-mediated RD1 activity because mutations at phosphorylated sites within RD1 alone can fully account for the MAPK-sensitive, HDAC-mediated repression of RIP140. Therefore, the role of phosphorylation in RD2 and RD3 remains to be examined.

Protein phosphorylation is known to be one of the most important intracellular signal transduction events (1–3, 32). It has been estimated that approximately one-third of the mammalian proteins contain covalently bound phosphate groups, and many are subjected to regulation by multiple phosphorylation events (33, 34). Recent investigation showed that nuclear co-regulator SRC-3, a known oncogenic factor in
breast and prostate cancer, is regulated by phosphorylation. The phosphorylated SRC-3 is eventually more oncogenic than the unmodified form (30). RIP140 knock-out studies have shown that RIP140, though not involved in the process of adipogenesis, regulates fat accumulation (27, 35). RIP140 is also known to have a role in follicular development and is essential for oocyte release during ovulation (35). The functional proteomic studies presented here and the CP and CN mutants of RIP140 generated from these studies would provide powerful tools for further studies of the functional role of RIP140 in these biological systems where the second messenger systems such as the MAPK pathway could play an important role.

Acknowledgments—We thank the staffs of the Mass Spectrometry Consortium for the Life Sciences, University of Minnesota, Department of Biochemistry, Molecular Biology and Biophysics.

* This work was supported by National Institutes of Health Grants DA111190, DA11806, DK54733, DK60521, and K02-DA13926 (to L.-N. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Pharmacology, University of Minnesota Medical School, 6–120 Jackson Hall, 321 Church St SE, Minneapolis, MN 55455-0217. Tel.: 612–625–9402; Fax: 612–625–8408; E-mail: weixx009@umn.edu.

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