Post-translational Modification of Nuclear Co-repressor Receptor-interacting Protein 140 by Acetylation*

M. D. Mostaqul Huq and Li-Na Wei‡

Receptor-interacting protein 140 (RIP140) is a versatile co-regulator for nuclear receptors and many transcription factors and contains several autonomous repressive domains. RIP140 can be acetylated, and acetylation affects its biological activity. In this study, a comprehensive proteomic analysis using liquid chromatography-tandem mass spectroscopy was conducted to identify the in vivo acetylation sites on RIP140 purified from Sf21 insect cells. Eight acetylation sites were found within the amino-terminal and the central regions, including Lys111, Lys158, Lys287, Lys311, Lys482, Lys529, Lys597, and Lys932. Reporter assays were conducted to examine the effects of acetylation on various domains of RIP140. Green fluorescent protein-tagged fusion proteins were used to demonstrate the effect on nuclear translocation of these domains. A general inhibitor of reversible protein deacetylation was used to enrich the acetylated population of RIP140. The amino-terminal region (amino acids (aa) 1–495) was more repressive and accumulated more in the nuclei under hyperacetylated conditions, whereas hyperacetylation reduced the repressive activity and nuclear translocation of the central region (aa 336–1006). The deacetylase inhibitor had no effect on the carboxyl-terminal region (aa 977–1161) where no acetylation sites were found. Hyperacetylation also enhanced the repressive activity of the full-length protein but triggered its export into the cytosol in a small population of cells. This study revealed differential effects of post-translational modification on various domains of RIP140 through acetylation, including its effects on repressive activity and nuclear translocation of the full-length protein and its subdomains. Molecular & Cellular Proteomics 4:975–983, 2005.

Environmental factors in the extracellular milieu utilize signal transduction pathways to propagate their cues into gene expression (1–3). Often the proteins involved in such signaling pathways undergo post-translational modification. A variety of post-translational modifications, including phosphorylation, acetylation, methylation, and glycosylation, regulate protein functions (4–7). The study of protein function by identification of proteins along with their post-translational modification has been referred to as “functional proteomics” and is an important step in delineating signal transduction pathways. One major challenge is to identify post-translational modifications on these proteins in vivo (8).

Receptor-interacting protein 140 (RIP140)† is a co-regulator for many transcription factors (9). Nuclear receptors represent the largest group of transcription factors that interact with RIP140 (10–13). Human RIP140 was initially characterized as a ligand-dependent co-activator for a chimeric estrogen receptor (10). However, the mouse RIP140 cloned in our laboratory with the ligand-binding domain of an orphan nuclear receptor TR2 as the bait was shown to be a potent co-repressor for TR2 in the absence of putative ligand (14). Later many researchers including our group reported RIP140 as a suppressor for nuclear hormone receptors and many other transcription factors (15–17). RIP140 is recruited to nuclear receptors through its nine LXXLL motifs and a modified motif of LXXLM where X can be any amino acid (14, 18). RIP140 also contains four autonomous repressive domains (RDs). RD1 is located in the amino-terminal region (aa 1–495), RD2 and RD3 are located in the central portion (aa 336–1006), and RD4 is located in the carboxyl-terminal region (aa 977–1161). These domains function through various mechanisms. The amino-terminal region recruits histone deacetylases (HDACs) (17), whereas the central region interacts with carboxyl-terminal binding proteins (CtBP1 and CtBP2) (19). In terms of its physiological action, RIP140-null mice showed female reproductive defects (9). Studies of these animals revealed that RIP140 could play an important role in the regulation of fat accumulation in adipose tissues (20).

Post-translational modifications of transcription factors including nuclear receptors and their co-regulators have attracted much attention (21–26). However, few studies have examined modifications of these proteins in vivo due to technical difficulties in expressing and purifying these nuclear proteins from eukaryotic cells. Recently we were able to ex-

† The abbreviations used are: RIP40, receptor-interacting protein 140; GFP, green fluorescent protein; aa, amino acids; RD, repressive domain; HDAC, histone deacetylase; CtBP, carboxyl-terminal binding protein; GST, glutathione S-transferase; IDA, information-dependent acquisition; NLS, nuclear localization signal; TR2, testicular receptor 2.

From the Department of Pharmacology, University of Minnesota Medical School, Minneapolis, Minnesota 55455

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press and purify RIP140 from insect cells, a model system widely used for expressing eukaryotic proteins. We found 10 phosphorylation sites on RIP140 purified from insect cells (27) that appeared to play a role in its repressive activity (not shown). Although RIP140 could be acetylated (28), this modification was identified on the in vitro modified protein at Lys\textsuperscript{446} using p300 and p300/CBP-associating factor/p300 as the enzyme. However, point mutation of this particular residue could not prevent RIP140 acetylation, suggesting that RIP140 could also be acetylated at other residues. We therefore conducted a comprehensive proteomic analysis of the acetylation pattern of eukaryotically expressed RIP140. For comparison, RIP140 expressed in \textit{Escherichia coli} was examined in parallel. LC-ESI-MS/MS technique identified eight acetylated residues in the amino-terminal and the central regions of RIP140 from insect cells.

We also determined the effects of acetylation on the biological activity of RIP140 and explored the mechanism by which such activity was initiated. Reporter assays were conducted for each dissected domain by using a general inhibitor of reversible protein deacetylation. In addition GFP-tagged proteins were used to examine the effect of acetylation on the nuclear translocation of each domain. It was found that hyperacetylation enhanced both the repressive activity and the nuclear translocation of the amino-terminal region. In contrast, hyperacetylation abolished the repressive activity of the central region and diminished its nuclear translocation. No effects were found in the carboxyl-terminal region where no acetylation sites were identified.

**EXPERIMENTAL PROCEDURES**

\textbf{Construction of His-tagged RIP140 and Purification of RIP140 from Sf21 Insect Cells—} The full-length mouse RIP140 (14, 27) was tagged with a His epitope by replacing the carboxyl-terminal domain of RIP140 with a PCR-amplified His-tagged fragment. The full-length His-tagged RIP140 cDNA was cloned into an insect expression vector, pVL1392 (Invitrogen), at the BglII and KpnI sites. The expression and purification of RIP140 was performed as described previously (27). Briefly Sf21 insect cells were infected with the recombinant baculovirus vector. RIP140 was purified from the nuclear extract by affinity chromatography on Talon resin. The protein was concentrated through a 10-kDa Centricon filter. The glutathione S-transferase (GST)-RIP140 protein from \textit{E. coli} was prepared as described previously (14, 27).

\textbf{Mass Spectrometric Analysis of RIP140—} Detailed experimental procedures for mass spectral analysis of RIP140 protein samples were described previously (27). Purified His-RIP140 protein from insect cells and GST-RIP140 from \textit{E. coli} were resolved by SDS-PAGE. Gel slices containing RIP140 were subjected to overnight in-gel trypsin digestion (29, 30). The samples were analyzed by MALDI-TOF MS (QSTAR XL, Applied Biosystems, Inc., Foster City, CA) using \textit{a}-cyano-4-hydroxycinnamic acid as a matrix in a positive ion reflection mode. For LC-MS, an LC Packings (Dionex, Sunnyvale, CA) Famos autosampler and an LC Packings Switchos pump were used to concentrate and desalt the sample on an LC Packings C\textsubscript{18} precolumn. The precolumn was connected in-line with a capillary column (100-\textmu m inner diameter, packed with 5-\textmu m, 200-A pore size C\textsubscript{18} particles), and peptides were eluted using an LC Packings Ultimate LC system.

The LC system was on line with an Applied Biosystems QSTAR Pulsar quadrupole TOF mass spectrometer equipped with a Protana nanoelectrospray source. As peptides were eluted from the column, they were focused into the mass spectrometer. Information-dependent acquisition (IDA) was used to acquire MS/MS. The IDA mode was set to measure continuous cycles of three full scan TOF MS from 400–550, 550–750, and 750–1200 m/z plus three product ion scans from 50 to 4000 m/z. Data from the IDA experiments were analyzed using the MASCOT MS/MS data search (www.matrixscience.com) of the NCBI data bank. The mass tolerance of both precursor ions and the MS/MS fragment ions was set at ±0.1 Da, and carbamidomethylcysteine was specified as a static modification. Acetylated lysine and oxidized methionines were specified as variable modifications. All MS/MS spectra were manually checked to verify sequence assignments. Peaks with a minimum height of 3% relative to the base peak were considered, and a 100-ppm tolerance was used to establish matches with the theoretical b and y ions that were predicted with the help of Bioanalyst software (Applied Biosystems).

\textbf{Cell Culture, Transfection, and Reporter and Translocation Assays—} For reporter gene assays, the amino-terminal region (aa 1–495, for RD1), central region (aa 336–1006, for RD2 and RD3), carboxy-terminal region (aa 977–1161, for RD4), and the full-length RIP140 (aa 1–1161) were cloned into pBD-GAL4 vector (Stratagene, La Jolla, CA) as described previously (14). The luciferase reporter construct for the trans-repressive activity was made by placing five copies of the GAL4 binding sites (5'-CGGAGGACAGTACTCCG-3') upstream of the thymidine kinase-luciferase (TK-luc) reporter (14). GFP-tagged fusion proteins of the repressive domains and full-length RIP140 were also constructed by placing each domain (14) into a pEGFP-C1 vector (Clontech). Both the amino-terminal and the central regions encode a nuclear localization signal (NLS). The carboxy-terminal region, however, is devoid of a NLS. Therefore, a NLS derived from TR2 nuclear receptor (31) was fused to its 3'-end prior to fusion with GFP, ensuring its nuclear accumulation for subsequent assays on the effects of inhibitory drugs. The assays were conducted in COS-1 cells maintained at 37 °C in a CO\textsubscript{2} incubator in Dulbecco’s modified essential medium supplemented with 10% fetal bovine serum. Transfection experiments and luciferase and \beta-galactosidase gene (\textit{lacZ}) assays were done as described previously (14). Sodium butyrate (Upstate Biotechnology, Lake Placid, NY) was used as a general protein deacetylase inhibitor.

**RESULTS**

\textbf{Expression, Purification, and Mass Spectral Analysis of Tryptic RIP140—} Prior studies validated the effectiveness of expressing recombinant proteins in insect cells to generate large quantities of low abundance proteins such as transcription factors (32, 33). Although stoichiometry appears variable, post-translational site occupancy of such proteins expressed in insect cells is the same as that seen in proteins from mammalian cells (34). Therefore, we expressed RIP140 protein in insect cells for further purification and analyses. To confirm that modification by acetylation occurs specifically in RIP140 expressed in insect cells, we also expressed and purified GST-tagged RIP140 from \textit{E. coli} and analyzed them in parallel by mass spectroscopy. Using affinity chromatography under denaturing conditions, we were able to purify the recombinant protein to over 95% homogeneity (27). The proteins were subjected to trypsin digestion, and the tryptic peptides were first analyzed by MALDI-TOF MS to identify RIP140. The MALDI-TOF MS data were subjected to a MAS-
COT search at the NCBI data bank. The MASCOT search results confirmed the identity of the protein with sequence coverage to over 60% of the total protein (data not shown). LC-ESI-MS/MS was used to identify post-translational modification sites on RIP140. We recorded three independent full scan ion chromatograms from m/z 400 to 550, 550 to 750, and 750 to 1200. IDA was used to acquire MS/MS data. The MS/MS data were analyzed manually to confirm the sequences of the modified peptides. However, careful analysis of the MS/MS data confirmed that this additional 1-unit mass shift was due to the deamidation of either glutamine or asparagine residues present in the acetylated peptides. The MS/MS spectra of the modified peptides were always compared with the unmodified peptides (data not shown) for fingerprinting purposes.

The peptide spanning residues 101–112 (precursor molecular weight, 1371.75) displayed a doubly charged ion at m/z 686.88 in the total ion chromatogram (Fig. 1A). The precursor molecular mass showed a 43-amu shift due to acetylation of a lysine and deamidation of an asparagine residue (Table I). The MS/MS spectrum of the modified peptide showed a y1 ion at m/z 147.1 and a y1'' ion at m/z 129.1 caused by loss of H$_2$O, identical to the unmodified peptide. In contrast, the y2 ion appeared at m/z 317.2 instead of m/z 275.2, suggesting acetylation of Lys$^{111}$. This was confirmed by shifts of subsequent y ions (y2 to y8) caused by the modified lysine residue. Incidentally the y ions starting from y4 showed a 43-amu shift instead of 42, suggesting deamidation of Asn$^{109}$. The triplicate charged precursor ion at m/z 620.98 of the modified peptide spanning residues 155–170 (molecular weight, 1859.91) showed a 43-amu shift that could be accounted for by acetylation at the lysine residue and deamidation of a glutamine residue (Table I). The singly charged y ions from y1 to y9 and b ions from b1 to b3 were identical to those of the unmodified peptide (Fig. 1B). The doubly charged y12 ion at m/z 681.3 corresponded to the unmodified peptide deamidated at Gln$^{160}$. The singly charged b5 ion at m/z 628.4 and the b6 and b7 ions were attributed to the acetylated Lys$^{158}$. The MS/MS spectrum of the modified peptide spanning residues 283–298 exhibited a y9 ion at m/z 1011.5 and a b4 ion at m/z 451.2, identical to the unmodified peptide (Fig. 1C). However, the intense b5 ion at m/z 621.3 displayed a 43-amu shift, suggesting acetylation at Lys$^{297}$. Similarly a 42-amu shift in the molecular mass was observed for the doubly charged precursor ion at m/z 880.93 of the peptide spanning residues...
FIG. 1. CID MS/MS spectra and associated peptide sequences of precursor ions of specific acetylated peptides derived from RIP140. The presence of the immonium ion at \( m/z \) 126 specific for acetylated lysine is indicated in the figure. The \( y^* \) or \( b^* \) ions represent \( y \) or \( b \) ions caused by loss of ammonia. The \( y^o \) or \( b^o \) ions represent \( y \) or \( b \) ion caused by loss of \( \text{H}_2\text{O} \). The mass values for doubly charged ions in the peptide sequence (shown over each MS/MS spectra) are underlined. A, residues 101–112, \( m/z \) 686.88. The \( y^2 \) ion shows a 42-amu shift, suggesting Lys\(^{111}\) acetylation. B, residues 155–170, \( m/z \) 620.98. The \( b^5 \) ion at \( m/z \) 628.4 signifies Lys\(^{158}\) acetylation. C, residues 283–298, \( m/z \) 621.31. The \( b^5 \) ion of the modified peptide appears at \( m/z \) 621.3 instead of \( m/z \) 579.3 due to acetylation of Lys\(^{287}\). D, residues 305–320, \( m/z \) 880.93. The doubly charged \( y^16 \) ion at \( m/z \) 859.4 caused by loss of an acetyl group was considered evidence of Lys\(^{311}\) acetylation. E, residues 476–492, \( m/z \) 945.43. The \( y^{10} \) and the \( a^6 \) ions suggest acetylation of Lys\(^{482}\). This is confirmed by the \( b^{11} \) ion showing a 42-amu shift and the doubly charged \( y^16 \) and \( y^17 \) ions caused by loss of an acetyl group. F, residues 517–537, \( m/z \) 802.69. The doubly charged \( y^9 \) and \( y^{10} \) ions, along with the marker ion at \( m/z \) 126, confirmed acetylation of Lys\(^{529}\). G, residues 606–630, \( m/z \) 850.74. The \( b^2 \) ion at \( m/z \) appears at \( m/z \) 228 instead of \( m/z \) 186, indicating Lys\(^{507}\) acetylation. H, residues 930–938, \( m/z \) 547.79. The \( y^7 \) ion at \( m/z \) 877.5 displays a 43-amu shift, likely due to Lys\(^{502}\) acetylation and Asn\(^{505}\) deamidation. rel., relative.
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305–320 (molecular weight, 1759.84) (Table I). In the MS/MS spectrum (Fig. 1D), the y9 ion at \( m/z \) 920.5 corresponded to that seen in the unmodified peptide. The b5 ion at \( m/z \) 542.3 and the b6 ion at \( m/z \) 652.30 were also identical in the native peptide. Taken together, these data suggested the acetylation at Lys529. This suggested an acetylation site between b11 and y8. Thus, the presence of this acetylation site was substantiated by the presence of doubly charged y16 ion at \( m/z \) 859.4 caused by loss of an acetyl group.

The MS/MS spectrum of the modified peptide spanning residues 476–492 showed a b11 ion at \( m/z \) 1252.6, consisting of an intact acetyl moiety (Fig. 1E). The presence of an acetylated lysine residue in the peptide was demonstrated by the presence of the immonium marker ion at \( m/z \) 126 and the doubly charged y16 and y17 ions appearing at \( m/z \) 867.4 and 923.9, respectively, due to loss of an acetyl group. The y10 ion at \( m/z \) 1124.5, b5 ion at \( m/z \) 482.3, and the a6 ion at \( m/z \) 567.4 were identical to those of the native peptide. This provided evidence that Lys482 was the acetylation site.

The modified peptide spanning residues 606–630 (precursor molecular weight, 2549.19) appeared as a triply charged y16 ion at \( m/z \) 850.74 (Fig. 1G), whereas that of the unmodified peptide appeared at \( m/z \) 836.4 (precursor molecular weight, 2506.17). The 43-amu difference between the two indicated the modification by acetylation along with deamidation of either a glutamine or an asparagine residue. The singly charged y12 ion at \( m/z \) 1168.6 and the doubly charged y21 and y22 ions at \( m/z \) 1053.5 and \( m/z \) 1097.0, respectively, of the modified peptide corresponded to those seen in the unmodified peptide. This indicated an acetylation site at Lys607. This was confirmed by the observation of the singly charged b2 ion at \( m/z \) 228.1, the b3 ion at \( m/z \) 357.2, and the b4 ion at \( m/z \) 444.2.

The modified peptide spanning residues 930–938 showed a 43-amu shift (Table I). This indicated that the peptide was modified either by acetylation along with deamidation or carbamylation. However, the intense immonium ion signal at \( m/z \) 126 indicated the shift was more likely due to an acetylated lysine (Fig. 1H). The modified peptide showed a y6 ion at \( m/z \) 707.4, corresponding to deamidation of Asn953 of the unmodified peptide. The y7 ion at \( m/z \) 877.5 exhibited a 43-amu shift, suggesting acetylation of Lys932. This was supported by the a3 ion at \( m/z \) 359.2 and the b4 ion at \( m/z \) 474.2, both of which showed a 42-unit mass shift due to acetylation on Lys932.

Role of Acetylation on Repressive Activity of RIP140—A trans-repressive assay was conducted using the GAL4 reporter system in COS-1 cells. However, RIP140 contains multiple RDs, and multiple acetylation sites were found in these various domains. These experiments were therefore performed on both the full-length protein and its subdomains fused to the same DNA-binding domain of GAL4. Hyperacetylation was induced by blocking protein deacetylases with sodium butyrate. In the presence of this deacetylase inhibitor, the amino-terminal region (aa 1–495) was more repressive than the untreated control (Fig. 2). In contrast, the repressive activity of the central region (aa 336–1006) was significantly reduced under the same conditions. Deacetylase inhibitors exerted no effect on the carboxyl-terminal region (aa 977–1161) of RIP140 where no acetylation was identified. As observed for the amino-terminal region, full-length RIP140 had greater repressive activity when it was hyperacetylated, suggesting that the repressive activity of the amino-terminal domain probably dominated over regulation by the rest of this molecule.
Effect of Acetylation on Translocation of RIP140—

Transcription activities of nuclear receptors and transcription factors are partially regulated by their nuclear translocation. We determined whether acetylation affected the nuclear translocation of RIP140 and if the effect was the same for different domains of RIP140. We used a GFP tag strategy to follow the cellular distribution of RIP140 domains under normal and hyperacetylated conditions. According to amino acid sequences, both the amino-terminal and the central regions encode a NLS and therefore could be enriched in the nuclei. The carboxyl-terminal domain contained no NLS but could be accumulated in the nuclei by fusing to a NLS (Fig. 3c). As expected, the full-length protein is exclusively nuclear (Fig. 3d). Treatment with deacetylase inhibitor enhanced nuclear localization of the amino-terminal region (Fig. 3, compare a and e). In control cells, it was restricted to the nuclei of ~26% of cells, whereas hyperacetylation dramatically enhanced its translocation to the nucleus in 74% of cells (Fig. 3f). In contrast, sodium butyrate caused the central region to be evenly distributed between the cytoplasm and the nucleus, suggesting a reduction in its nuclear accumulation (Fig. 3f). Approximately 73% of treated cells exhibited an even distribution pattern in the cytoplasm and the nucleus, whereas only 38% of the untreated cells had the even distribution pattern (Fig. 3i). No significant effect on translocation was detected for the carboxyl-terminal domain of RIP140 under the same conditions (Fig. 3, c and g). This is consistent with the results of the reporter assays where sodium butyrate had no effect on the repressive activity of this region. Interestingly, although hyper-acetylation rendered the full-length RIP140 more repressive in the reporter assay (Fig. 2), this condition triggered the full-length RIP140 to be translocated to the cytosol in a portion of the cell population. This suggests that effects of acetylation extend to some as yet undetermined function of RIP140 that contributes to its repressive activity in addition to its nuclear accumulation.

In summary, acetylation exerted differential effects on the various domains of RIP140 with respect to their contribution to the overall biological activity of RIP140 as a transcription co-repressor. Nuclear translocation of both the amino-terminal and the central regions was affected by acetylation of lysine residues, which correlated with the effects on their
repressive activity. The activity of the carboxyl-terminal region, which contains no acetylated residues, was not affected by treatment that affected the level of lysine acetylation.

**DISCUSSION**

Post-translational protein modification plays an important role in multiple cellular processes including DNA repair, protein stability, nuclear translocation, and protein–protein interactions and in cellular proliferation, differentiation, and apoptosis (21). One major challenge is the identification of post-translational modifications of these proteins in vivo. Characterization of proteins by mass spectrometry has become an important tool in the postgenomic era (37). In this report, we focused on the acetylation sites of RIP140, a well-known co-regulator for many transcription factors including nuclear receptors. We identified eight acetylated lysine residues specifically on RIP140 from insect cells: Lys$^{111}$, Lys$^{158}$, Lys$^{287}$, Lys$^{311}$, Lys$^{382}$, Lys$^{529}$, Lys$^{607}$, and Lys$^{932}$. No acetylation sites on RIP140 expressed in E. coli were detected, attesting to the specificity of acetylation of RIP140 in a eukaryotic environment and suggesting that in vivo RIP140 acetylation requires a complex eukaryotic cellular process. Whether acetylation identified in insect cells mimicked that in mammalian cells remains to be examined. Nevertheless, studies of other proteins showed variation only in stoichiometry but not the post-translational site occupancy (32–34). Therefore, the modified residues identified in insect RIP140 could also be the sites of modification in the mammalian cells.

Acetylation has been observed on other nuclear receptors such as estrogen receptor and androgen receptor as well as transcription factors such as p53 and NF-κB (21, 38, 39). Acetylation of nuclear receptors can affect ligand sensitivity and antagonist response (40–41). The activities of many transcription factors under cellular stress can also be regulated by acetylation (38, 39). However, the role of post-translational modification of RIP140 in gene regulation has remained a mystery. Our ability to express and purify this protein in both prokaryotic and eukaryotic cells presented an opportunity to address this problem. This study has identified residues of RIP140 acetylated in vivo and demonstrated the effects of acetylation on the biological activity of this important transcription co-regulator. More importantly, we demonstrated the differential effects of acetylation on distinct autonomous repressive domains of RIP140, suggesting complicated mechanisms mediated by acetylation in the repressive activity of RIP140. Recent investigation showed that nuclear co-regulator steroid receptor co-activator-3, a known oncogenic factor in breast and prostate cancer, was regulated by phosphorylation. The phosphorylated steroid receptor co-activator-3 was eventually more oncogenic than the unmodified form (34). We also reported the complete map of phosphorylation sites on RIP140 (27) and found phosphorylation of RIP140 also affected its repressive activity. It is likely the regulatory activities of RIP140 are subjected to modulation by extensive protein modification.

The adenoviral transforming protein E1A possesses a conserved PXDLSXXK motif (where X can be any amino acid) for its interaction with CtBP. The lysine residue in this motif was found to be acetylated (42). RIP140 has the same consensus sequence ($^{440}$PIDLSCK$^{449}$), and antibody specific for acetylated lysine detected acetylation of Lys$^{446}$ of RIP140 modified in vitro (28). However, a point mutation at this residue could not prevent RIP140 acetylation in vitro or in vivo. This suggested that RIP140 could be acetylated at other sites. Intriguingly Lys$^{446}$ was not detected in our comprehensive mapping of in vivo acetylation sites on RIP140. We speculate that this site was not detected because of the location of Lys$^{446}$ in the large theoretical tryptic peptides aa 403–446 (4811 Da) and aa 338–446 (6589 Da), considering zero and one missed cleavage sites for tryptic digestion, respectively. Peptides of molecular mass greater that 4000 Da were not covered in the mass range of full scan ion chromatograms for RIP140. Alternatively this could be due to technical difficulties in obtaining sufficient quantities of these relatively large peptides to analyze. The pattern of theoretical digestion of the above peptides with other proteinases such as chymotrypsin or proteinase K suggests that the use of such proteases would be unlikely to help to identify Lys$^{446}$ acetylation in RIP140. In the current study, we could detect as little as 2% acetylated peptides with an average molecular mass no greater than 3000 Da by mass spectral analysis (Table I). Moreover the acetylated lysine residues were identified in one or two missed cleaved tryptic peptides but never on terminal lysine residues of the tryptic peptides (Table I). This suggests that Lys$^{446}$, if acetylated, is resistant to tryptic digestion. Alternatively the discrepancy could be due merely to the different methods used in the in vitro versus the in vivo studies.

RIP140 contains four autonomous repressive domains, defined as RD1 in the amino-terminal (aa 1–333), RD2 (aa 400–800) and RD3 (aa 644–916) in the central, and RD4 (aa 927–1158) in the carboxy-terminal regions (19). RD1 is responsible for recruitment of HDACs, whereas RD2 interacts with CtBP1 and CtBP2. However, the mechanism of RD3- and RD4-mediated repression remains unknown. Our results suggest that the repressive activity of RIP140 is regulated by protein acetylation. Among the acetylation sites identified on RIP140, five of them (Lys$^{111}$, Lys$^{158}$, Lys$^{287}$, Lys$^{311}$, and Lys$^{482}$) were located in the amino-terminal region (RD1). Four (Lys$^{529}$, Lys$^{607}$, and Lys$^{932}$) were present in the central region, covering RD2 (aa 333–800) and RD3 (aa 644–916). No acetylation sites were identified in the carboxy-terminal region (aa 977–1161), which represented the RD4 (aa 927–1158). A general protein deacetylase inhibitor, sodium butyrate, was used to enrich acetylation of RIP140. With this inhibitor, the amino-terminal region of RIP140 was more repressive, but the central region became less so. This effect was specific as the same inhibitor exerted no effect on the trans-repressive ac-
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Acetylation of RIP140 likely depends upon its stoichiometry and the relative abundance of other involved components in the cellular environment (such as HDACs and CtBP) and the nuclear translocation machinery under specific conditions.

Previously we reported the complete map of phosphorylation sites on RIP140 (27) and found phosphorylation of RIP140 also affected its repressive activity. It is likely the regulatory activities of RIP140 are subjected to modulation by extensive protein modification. Indeed in the complex cellular environment, a particular protein might be subjected to a wide range of post-translational modifications, producing a heterogeneous population. Such a protein might well be diversified to interact with many different factors. It is tempting to speculate that RIP140 modification by acetylation at different lysine residues could contribute to its varied effects on the regulation of gene expression. The current study represents the first step in the detailed mapping of potential acetylation sites of RIP140. Such a map will be useful in the generation of acetylated peptide-specific antibodies, which may then be used to map acetylation sites on RIP140 in mammalian cells. Future studies are needed to determine the specific residues responsible for the effects of RIP140 on the expression of various genes.

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

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