Identification and characterization of a novel p300-mediated p53 acetylation site, Lysine 305

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Running title: Identification of a novel p300-mediated p53 acetylation site
SUMMARY

Post-translational modifications serve as important regulatory elements in modulating the transcriptional activity of the tumor suppressor protein p53. We have previously reported a tandem mass spectrometry-based method, namely SIT analysis, which can be applied to the identification of phosphopeptides as well as exact mapping of the phosphorylated residues within. In this report, we describe the application of the same strategy for the identification of acetyltransferase p300-mediated acetylation sites on p53. Consistent with the previous finding, lysine residues 370/372/373, 381 and 382 were detected by this modified SIT method as the target sites of p300 in vitro. Moreover, two novel acetylation sites, Lys-292 and Lys-305, were also found. Immunoblotting using anti-acetyl Lys-305 specific antibody confirmed such discovery and demonstrated that Lys-305 could be acetylated by p300 both in vitro and in vivo. We also showed that an alanine or glutamine substitution at Lys-305 site (K305A, or K305Q) suppressed its transcriptional activity, while the arginine mutation (K305R) caused an elevation in transcriptional activity. Thus, p300 may further regulate, through a novel acetylation site Lys-305, the transcriptional activity of p53.
INTRODUCTION

The p53 tumor suppressor protein is a sequence-specific, DNA-binding transcription factor. In response to a wide variety of stress signals, it acts to regulate processes such as cell cycle, cell death, and DNA repair. Loss of p53 activity has been identified in 60% of the human cancers examined, and over 90% of the p53 missense mutations are clustered within the sequence-specific DNA-binding domain (11, 20). The tumor suppressor functions of p53 are directly linked to its ability to mediate transcriptional activation (1).

The regulation of p53 transcriptional activity involves several mechanisms including post-translational modifications such as phosphorylation, acetylation and ubiquitination (2, 10, 13, 14, 19, 22). Most of these modifications occur in the amino- and carboxy-terminal regions of p53. Stress-induced N-terminal phosphorylation increases p53 stability by dissociating MDM2, a negative regulator. For example, Ser-15, Thr-18 and Ser-20, located in the MDM2 binding site, are phosphorylated in response to DNA damage. This phosphorylation may probably be induced by protein kinase such as ATM, ATR, Chk2, CKI, and DNA-PK (28, 33, 34). Acetylation is another type of p53 posttranslational modification shown to be affected by DNA-damage signals (22, 27). Two histone acetyltransferases (HATs), are known to acetylate p53, although on different sites: CREB binding protein (CBP)/p300 acetylates the C-terminus of p53 at Lys-372, 373, 381 and 382, while p300/CBP associated factor (PCAF) acetylates at Lys-320 (10, 22, 27). The acetylated residues of p53 are located in the regulatory domains adjacent to the tetramerization domain (aa. 252-355). Acetylation of p53 may be an important regulatory mechanism of p53 function, since p53 deacetylation by overexpressing histone deacetylase-associated proteins compromise its ability to induce cell cycle arrest and
apoptosis (23). Inhibition of p53 deacetylation also increases the p53 half-life, suggesting that acetylation may play important roles in the turnover of p53 protein (4).

Recently, we have employed a strategy based on LC/MS/MS for identifying residues bearing phosphorylation modifications (32). Based on this technique, moderately phosphorylated peptides could be identified by close examination of specific ion chromatograms (32). This selected ion tracing (SIT) method has a major advantage in analyzing the modified peptides of low abundance in a complex peptide mixture. More importantly, we have proposed that this method could be generally employed to identify peptides of various modifications. Here, we illustrate how this approach is adapted to identify acetylated peptides and to map the modified residues. While we successfully verified the previously reported acetylated residues of p53, we also uncovered two novel acetylation targets using this approach. We also present evidence that acetylation of p53 at Lys-305, like at Lys-382, is elevated by different stress signals. In addition, we found that the acetylation at Lys-305 is important for regulating p53 transcriptional activity.
MATERIALS AND METHODS

DNA constructs and reagents

Plasmid pcDNA3-p53w that contained human wild-type p53 was kindly provided by Dr. M. Y. Lai (National Taiwan University, Taipei, Taiwan). Mammalian expression vector for HA-tagged p300 and bacterial expression vector contained His-tagged HAT domain (amino acids 1195 – 1673) of p300 were gifts from Dr. L. J. Juan (National Health Research Institutes, Taipei, Taiwan). The p50-2 reporter that contained p53-responsive promoter was obtained by Dr. Y. S. Lin (Academia Sinica, Taipei, Taiwan). C-terminal FLAG-tagged p53 was produced by PCR amplification with a pair of primers, 5’-TAATACGACTCACTATAGGG-3’ (forward T7 oligonucleotide) and 5’-TCACTTTATCGTCGTCATCCTTGTAATCGTCTGAGTCAGGCCCTTCTGTCT-3’ (reverse C-FLAG primer); underlined sequence correspond to FLAG tag sequence. The PCR reaction was carried out by 30 cycles of 94°C for 30 s, 47°C for 30 s, and 72°C for 1 min 30 s, which were followed by a 94°C denaturation for 2 min and 72°C elongation for 5 min. The PCR fragment was then cloned into pCR2.1-TOPO plasmid using the TA Cloning kit (Invitrogen). C-terminal FLAG-tagged p53 cDNA was excised by EcoRI digestion and cloned into the corresponding site of pcDNA3 (Invitrogen). C-terminal FLAG-tagged p53 was also subcloned into a baculovirus expression vector pVL1393 (PharMingen). The expression plasmid pVL-p53-FLAG was introduced into Sf21 insect cells using a BaculoGold transfection kit (PharMingen). The p53-FLAG protein was purified by anti-FLAG (M2) immunoaffinity column. His-tagged HAT domain (amino acids 1195 – 1673) of p300 protein (HAT-p300) was expressed in BL21 (Lys) cells transformed with a bacterial expression vector. The protein was purified from the
clarified lysate on Ni-NTA agarose (Qiagen) according to the manufacturer's instructions and then dialyzed against the storage buffer (20 mM Tris – HCl [pH 8.0], 0.5 mM EDTA, 100 mM KCl, 20% glycerol, 0.5 mM DTT, and 0.5 mM PMSF) before storing at -80°C in aliquots.

Site-directed mutagenesis

Mutants of FLAG-tagged p53 were constructed by site-directed mutagenesis by a two-step PCR technique as described (6, 7). Briefly, mutants were prepared by introducing the desired mutation in overlapping oligonucleotide primers. In the first round of PCR, the mutagenic primer and an outside primer are used to amplify a partial fragment of the region where the mutation will be created. This mutagenized fragment is then purified and used as a "megaprimmer," together with the second outside primer, in a second round of PCR to amplify the entire region to be subcloned. Conditions for the first round of PCR were 94°C for 30 s, 47°C for 30 s, and 72°C for 1 min for 30 cycles. Conditions for the second round of PCR was started with a 2 min denaturation step at 94 °C, followed by 30 cycles of 94°C for 30 s, 46.5°C for 30 s, and 72°C for 1 min 30 s. This PCR reaction was ended with an elongation step at 72°C for 10 min.

Plasmid pcDNA3 containing FLAG-tagged p53 was used as the template in the PCR reaction. The outside primers used for all of the mutants in this study were as follows: 5’-TAATACGACTCACTATAGGG-3’ (forward T7 oligonucleotide) and 5’-ATTTAGGTGACACTATAG-3’ (SP6 oligonucleotide). Mutations were introduced into wild type p53 by two-step PCR mutagenesis with oligonucleotides containing base alterations to convert lysine residues 305 and 382 to alanine, argine or glutamine. The oligonucleotides for the mutants were: 5’-AGGGAGCACCTCGGCGAGCACTG-3’
(FP53 –K305R); 5’-GCAGTGCTGCGGAGYGCCTCCCT -3’ (RP53 –K305R);
5’-AGGGAGCAGTCGCGGAGCACTGC -3’ (FP53 –K305A);
5’-GCAGTGCTGCGGAGTCGCTCCCT -3’ (RP53 –K305A);
5’-CCCCCAGGGAGCAGTCGCGGACTGCCCAAC -3’ (FP53 –K305Q);
5’-GTTGGGAGTGCTCGAGTGCTCCCTGCGGGGG -3’ (RP53 –K305Q);
5’-CTCCCGCCATAAAGCGACTCATGT -3’ (FP53 –K382R);
5’-ACATGAGTCCTTTATGCGGGAG -3’ (RP53 –K382R);
5’-CTCCCGCCATAAAGCGACTCATGT -3’ (FP53 –K382A);
5’-ACATGAGTCCTTTATGCGGGAG -3’ (RP53 –K382A);
5’-CCTCCCGCCATAAAACACTCATGTTCAAGACAG -3’ (FP53 –K382Q); and
5’-CTGTCTTGAACATGAGTTGTTATGCGGGAGG -3’ (RP53 –K382Q);
underlined regions correspond to direct amino acid substitutions. The mutated PCR
fragments were cut with BamHI and XhoI and subcloned into the pcDNA3.

**Cell culture, transient transfections, and reporter assays**

H1299 (the human lung carcinoma cells) and MCF-7 (human breast cancer cells)
were maintained in Dulbecco’s modified Eagle's medium (DMEM) supplemented with
10% fetal calf serum (HyClone) at 37°C in a 5% CO₂-humidified atmosphere.
Approximately 1 × 10⁶ cells were seeded in each 100-mm culture dish 12-24 h before
transfection. Calcium phosphate-mediated DNA transfection was performed as
described previously (8). DNA was prepared by the Clontech procedure and adjusted to
12 µg per transfection with pcDNA3 plasmid. Chloramphenicol acetyltransferase (CAT)
assays were performed with two-thirds of total cell extract as described earlier (6, 31).
The *p50-2* that contains two copies of p53 binding motif in its promoter region was used
as p53 responsive CAT reporter plasmid (12). A 1 µg aliquot of a RSV-β-Gal vector was included as internal control in all transfections. Quantification of the acetylated [14C] chloramphenicol was determined with a PhosphorImager (Molecular Dynamics). The β-galactosidase assays were performed according to protocols from the manufacturer (Promega). A total of 10 µg of the plasmid was used for each experiment.

**Antibodies**

Antisera against acetylated p53 peptides were generated in rabbits using KLH-conjugated acetylated peptides (p53300-310: PPGSTK*RALPN, acetylated at Lys-305; p53376-387: STSRHK*LMFKT, acetylated at Lys-382) (29). The antisera from immunized rabbits were affinity purified successively with non-acetylated and acetylated peptides columns (28). DO-1 monoclonal antibody was obtained from Oncogene Research Products. Anti-FLAG monoclonal antibody, M2 antibody, was obtained from Sigma.

**Western blot and Dot blot analysis**

To verify the antibody specificity, we used a dot-blots analysis as previously described (36). Equal amount of peptides (20 µg) were spotted on strips of Hybond-C membrane (Amersham Biosciences). After blocking by 5% nonfat milk in PBS and 0.1% Tween 20 buffer, membranes were probed with the purified rabbit polyclonal antibodies. Blots were then incubated with a HRP-conjugated goat anti-rabbit IgG.
(Sigma). The blots were visualized using the enhanced chemiluminescence system (ECL; Amersham Biosciences).

Western blot was performed as previously described (24). Briefly, equal amounts of cell extracts were separated on a 10% SDS-polyacrylamide gel and blotted onto a membrane. The membranes were blocked by 5% nonfat milk in PBS and 0.1% Tween 20 buffer and probed with anti-p53 antibody (DO-1), anti-FLAG, anti-acetylated K305 (Ab-165) antibody, or anti-acetylated K382 (Ab-166) antibody.

**In vitro acetylation assay**

Acetylation assays were carried out primarily according to the published method (11, 13). Four micrograms of recombinant FLAG-p53 was acetylated in vitro by incubating with indicated amount of HAT-p300 in the presence of a reaction mixture containing 50 mM Tris-HCl (pH 8.0), 10% glycerol (v/v), 0.1 mM EDTA, 1 mM dithiothreitol, and 18 µM [14C]acetyl-CoA (Amersham Pharmacia Biotech). The mixture was incubated at 30 °C for 60 min and analyzed by SDS polyacrylamide gel electrophoresis followed by Coomassie blue staining. The [14C] labeled p53 was detected by autoradiography and quantified by a PhosphorImager.

**In-gel digestion**

The gel piece containing p53 polypeptides was first reduced and pyridylethylated as described previously (32). Up to 0.2 μg of the enzyme, Arg-C (Roche) or Asp-N (Roche), was added to the dried gel. After overnight incubation, the supernatant was removed and the gel was extracted twice with adequate amount of 0.1% formic acid. The supernatant and the extracts were combined together and dried in a Speed-Vac. The
digests were kept at –20°C and suspended in 0.1% formic acid immediately before use.

**LC/MS/MS analysis**

Electrospray mass spectrometry was performed using a Finnigan Mat LCQ ion trap mass spectrometer interfaced with an ABI 140D HPLC (Perkin-Elmer). A 150 x 0.5 mm PE Brownlee C18 column (Perkin-Elmer) (5 mm particle diameter, 300 Å pore size) with mobile phases of A (0.1% formic acid in water) and B (0.085% formic acid in acetonitrile) were used. The peptides were eluted using the acetonitrile gradient and analyzed by “triple-play” experiment as described (32). The samples were analyzed by two runs of LC/MS/MS experiments. In the first analysis, the most abundant ion in an MS spectrum was selected for collision-induced dissociation (CID) experiment; in the later analysis, only the ions with m/z values corresponding to the potential acetylated peptides were selected for CID experiment. The acquired CID spectra were interpreted using a Finnigan Corporation software package, the SEQUEST Browser, which correlated the MS/MS spectrum with the amino acid sequence of human p53 protein. Enzyme was not specified in the search parameters, which increased the confidence of identification when the matched peptides had appropriate cleavage sites. A 105.14-Da mass tag was constitutively assigned to the Cys residue, which was modified with 4-vinylpyridine in experiments. Those MS/MS scans that matched the peptide sequences with proper cleavage sites were considered significant and also were subjected to manual evaluation to confirm the SEQUEST results.

The hypothetical m/z values of peptides used for generation of ion tracing chromatograms were derived using the PEPSTAT algorithm in the SEQUEST package. The EXPLORE program (Finnigan) was used to plot the ion tracings.
RESULTS

Determination of p300-mediated in vitro acetylation sites on p53 by LC/MS/MS

In our previous report, we established a selected ion tracing (SIT) method to identify phosphorylated peptides (32). Here, we set out to test whether the SIT method could be adapted to identify acetylated peptides. Previous studies have demonstrated that p53 could be acetylated by p300 in vitro (10). Therefore, we analyzed acetylated peptides derived from FLAG-tagged recombinant p53 protein that was acetylated in vitro by p300. Baculovirus-expressed recombinant FLAG-tagged p53 was immobilized on M2-agrose beads and served as the substrate for the in vitro acetylation reactions. Various amounts of HAT domain from the p300 acetyltransferase (HAT-p300) were incubated with a fixed amount of FLAG-p53 in the presence of [14C] acetyl-CoA (Fig. 1A). After in vitro acetylation, the reaction mixtures were analyzed by SDS-PAGE followed by autoradiographic analysis (Fig. 1B). The FLAG-tagged p53 was acetylated by the enzyme in a dose-dependent fashion. Quantitative determination of the autoradiogram showed that the acetylation was not saturated.

The acetylated p53 resolved in the gel was excised and divided into two portions. One was treated with Arg-C and the other was digested with Asp-N. An aliquot of each digested peptide mixture was analyzed by LC/MS/MS. The raw data were then interpreted by the SEQUEST software with optional addition of 44 daltons to the Lys residues. This analysis yielded fifteen Arg-C peptides and six Asp-N peptides (Table 1). These two peptide groups were estimated to account for 87% (350/401) of amino acid
residues of FLAG-p53 and 95% (21/22) of lysine residues (Fig. 2A). Two acetylated residues were found directly by SEQUEST software, Lys-373 and Lys-381/-382. Subsequently, we used the SIT approach for examining all of these peptides and their putative acetylated analogues were chromatographed. We found that three Arg-C peptides and one Asp-N peptide were probably acetylated (Table 1).

In particular, we found that the triply charged, unmodified P291-306 with an \( m/z \) of 600.4, had a retention time of 35.66 min in our analysis (Fig. 2B). The +3 ion tracing for singly acetylated peptide has multiple peaks migrating closely with the unmodified peaks (Fig. 2B, middle trace). In order to distinguish whether these contained the acetylated peptide ions, the +4 ion tracing was also chromatographed (Fig. 2B, lower trace). The result revealed that there were two major peaks, at 35.6-min and 39.8-min, which were commonly seen in two tracings. On the other hand, many signals found between 50~60 min in the +3 ion tracing were not observed in the +4 ion tracing. This is consistent with the notion that these peaks contain ions that may have different molecular masses but have similar \( m/z \) values as the triply charged P291-306. Also, based on their relative abundances, these lysine residues appeared to be modified at a lower efficiency (Fig. 2B).

The collision-induced dissociation (CID) spectra of the 615-\( m/z \) ion in the 35.6-min peak were acquired for identification of the acetylation sites (Fig. 2C). The presence of \( y_4 \) to \( y_5 \) ions argues against the possibility that Lys-305 is acetylated, while the ion masses of \( b_9 \) and \( b_{10} \) indicates that the acetyl group was on the lysine residue at the N-terminal end. The observation of \( y_{15} \) ion indicated that Lys-292 is the acetylated residue.

The CID spectra of the 615-\( m/z \) ion in the 39.8-min peak are shown in Fig. 2D. Observation of \( b_7 \) to \( b_{12} \) ions indicated that the acetyl group was on the C-terminal three amino acids. Lys-305 is presumably the acetylation target base on the molecular sizes of \( y_1 \) and \( y_2 \) ion. This assumption is further supported by the fact that it is the only acetylatable residue in the region. Therefore, we identified two distinct acetylation sites,
Lys-292 and Lys-305, in this particular peptide.

Subsequently, we used the SIT approach to analyze peptides P354-379 and P380-401. Both peptides could take up as much as two acetyl groups. The three sets of Arg-C peptides were subjected to LC/MS/MS analysis that was carried out in a mass-specific mode such that the necessary MS/MS spectra were acquired. The MS/MS spectra of P380-401 peptide ion indicated that the acetylation group could locate on either Lys-381 or Lys-382. Additionally, double acetylation on Lys-381 and Lys-382 was also observed (data not shown). These results are in agreement with the previous finding that both Lys-381 and Lys-382 are acetylated. Although the SEQUEST analysis revealed that the P354-379 peptide could be singly acetylated, this region could incorporate as many as two acetyl groups based on SIT analysis. Nevertheless, our current data could not pinpoint the exact modified residues in the four-amino acid stretch from Lys-370 to Lys-373. Thus, p53 could be in vitro acetylated by p300 on lysine residues 370/372/373 as well as 382 and 383. Moreover, novel p53 acetylation sites by p300 were also found on lysine residues 292 and 305.

Detection of in vitro acetylated p53 by antibodies specific to acetyl-Lys-305 and acetyl-Lys-382 of p53

The rabbit antibodies raised against Lys-305 (Ab-165) and Lys-382 (Ab-166) were purified by peptide affinity chromatography. The specificity of the purified antibodies was verified by a dot-blot analysis (Fig. 3A). We found that Ab-165 specifically recognized acetylated Ac-P300-310 (Ac-K305), and it did not recognize acetylated Ac-P376-387 (Ac-K382) or an acetylated peptide from HMG-14 peptide (Ac-HMG) (Fig. 3A, upper panel). Likewise, Ab-166 only recognized Ac-K382 but neither Ac-K305 nor
Ac-HMG (Fig. 3A, lower panel).

We next tested if these antibodies can recognize full-length p53 acetylated by p300 in vitro (Fig. 3B). After in vitro acetylation, recombinant FLAG-p53 was analyzed by immunoblotting using Ab-165 and Ab-166 antibodies. The amount of p53 used for each reaction was about the same (Fig. 3B lower panel). Ab-165 recognized p53 that was acetylated in the presence of p300 and acetyl-CoA. The acetylated signals increased proportionately to the p300 amounts in the reaction (Fig. 3B upper panel). Similarly, Ab-166 specifically recognized acetylated, but not the non-acetylated p53 (Fig. 3B middle panel). Ab-165 and Ab-166 did not recognize p53 in the absence of acetyl CoA or p300 in the acetylation reactions (Fig. 3B). These results indicated that Ab-165 and Ab-166 could specifically recognize acetylated p53. Together with the fact that Ab-165 could specifically recognize acetylated Lys-305, these data further corroborated the conclusion that Lys-305 was indeed acetylated in vitro by p300 in a dose-dependent fashion.

Lys-305 is acetylated by p300 in vivo

To address whether Lys-305 of p53 might be acetylated in vivo, we performed an in vivo acetylation assay that previously identified Lys-382 as an acetylated target. The H1299 cells were transfected with the pcDNA-p53 plasmid expressing wild-type p53 (Fig. 4). Without further treatment, no acetylated signal was detected on the Western blot using either antibody. Upon addition of sodium butyrate, an inhibitor of HDACs, acetylation at Lys-305 as well as at Lys-382 were detected (Fig. 4). Overexpression of HA-tagged p300 also enhanced acetylation at Lys-305, like Lys-382. This enhancement is correlated with the HA-p300 levels (Fig. 4). Synergism between sodium butyrate and p300 overexpression was also observed for the Lys-305 acetylation (Fig. 4). These data
indicated that Lys-305, like Lys-382, was an acetylation target of p300.

Acetylation of p53 at Lys-305 is induced by various stress stimuli.

Previous studies have shown that various stress stimuli are capable of inducing p53 acetylation (3, 15). To determine whether Lys-305 acetylation was induced in response to these stimuli we used Ab-165 and Ab-166 antibodies to monitor the acetylation levels of p53 in normal and stressed cells. First, we tested whether Lys-305 acetylation was induced by UV or ionization irradiation. MCF-7 cells were exposed to 50 J/m² UV-C or 20 Gy γ-rays (IR) and the cell lysates were prepared at various time points after treatment. Before initiation of DNA damage, these cells were treated with sodium butyrate to reduce the rapid deacetylation by endogenous histone deacetylase activity (27).

Without any stimuli, the level of p53 in MCF-7 cells is low while the acetylation cannot be detected by either Ab-165 or Ab-166 (Figure 5A, lane 1). Sodium butyrate treatment slightly increased the p53 level, which could be sustained for as long as 8 hr post treatment (Figure 5A, lanes 2-4). After IR, acetylation at Lys-382 was detected as early as one hour and become prominent at 4 hr post irradiation. A similar course was also seen upon IR treatment for Lys-305 (Figure 5A, lanes 5-7). Meanwhile, UV irradiation dramatically increased the acetylation for Lys-305 acetylation at 8 hr post treatment (Figure 5A, lanes 8-10). The kinetics of irradiation-induced p53 acetylation for Lys-305 and Lys-382 were very similar. This suggests that Lys-305 and Lys-382 acetylation might be activated through a similar mechanism under these conditions.

The effects of H₂O₂ and actinomycin D (ActD) on the p53 acetylation in MCF-7 cells were also investigated. Like irradiation-induced responses, actinomycin D
induced the accumulation of p53 as well as the acetylation of Lys-305 (Fig. 5B). The 
H$_2$O$_2$-mediated oxidative stress, however, resulted in a slower accumulation of p53 than 
that elicited by actinomycine D. Intriguingly, it appeared that the kinetics of Lys-305 
acetylation was quite different from that of Lys-382 (Fig. 5B). Lys-305 acetylation 
reached the highest level within 1 hr post treatment, while Lys-382 acetylation appeared 
to be higher at 4-8 hr post treatment (Fig. 5B). Hence, the H$_2$O$_2$-induced p53 acetylation 
at Lys-305 and Lys-382 showed different kinetic patterns, implicating that different 
mechanisms are responsible for acetylation at these two sites under this particular 
condition.

Together, these results confirmed that acetylation of Lys-305, like that of Lys-382, is 
responsive to all p53-activating agents, which further suggested that Lys-305 acetylation 
may play important roles in p53 activation.

Lys-305 is important for p53 transcriptional activity

To further determine the function of Lys-305, we tested whether it might affect 
p53-mediated transcriptional activation. We analyzed the effects of site-specific mutants 
of Lys-305 on transactivation function. Plasmids expressing various Lys-305 and 
Lys-382 mutants as well as wild-type proteins were introduced into the p53-null human 
lung cancer cell line H1299. These proteins were examined for their ability to activate a 
p53 responsive CAT reporter plasmid, $p50$-2, that contains two copies of p53 binding 
 motif in its promoter region (12).

Overexpression of wild-type p53 generated a ~50-fold stimulation of the CAT 
activity (Fig. 6). It is similar to the previous reports that p53 is sufficient to stimulate
this promoter activity of the reporter gene, p50-2 (29, 35). When cells expressed p53 mutant K305R, the p50-2 promoter activity was about 2 folds higher than that induced by wild-type p53. In contrast, overexpression of the K305A mutant resulted in a 25-30% decrease of p50-2 promoter activity. These mutations have similar effects as seen with the Lys-382 mutants (Fig. 6A), further suggesting that Lys-305 may influence the transcriptional activity of p53 through a similar mechanism mediated by Lys-382 acetylation. Unexpectedly, overexpression of glutamine substituted p53 mutants, K305Q and K382Q, could not activate p50-2 promoter and the CAT activities were similar to mock control (Fig. 6A). These glutamine mutants seem to completely lose the intrinsic p53 transcriptional activity.

To further study the functional significance of Lys-305 acetylation, we tested whether it might affect p53-mediated transcriptional activation under stress stimuli (e.g. UV irradiation). After UV-irradiation, the wild-type p53-mediated transcriptional activity was elevated up to ~2-fold stimulation of the CAT activity (Fig. 6B). However, none of p53 mutants could respond to UV-irradiation to induce higher CAT activities. The transcriptional activity of K305Q or K382Q was completely abolished whether the transfectants were treated with UV irradiation or not.
DISCUSSION

In this report, we first illustrated how partially acetylated peptides can be identified using a LC/MS/MS-based SIT approach. Using this approach, we successfully verified the previously reported in vitro p53 acetylation sites by acetyltransferase CBP/p300. We further demonstrated the prowess of this method by discovering two additional acetylation targets, Lys-292 and Lys-305. Several lines of evidence indicated that Lys-305 was acetylated both in vivo and in vitro by p300. The acetylation of Lys-305, just like Lys-382, may be induced by various stress stimuli that are known to activate p53. Transfection assay also established the important roles of Lys-305 acetylation.

LC/MS/MS analysis is very useful in identification of residues bearing modifications such as phosphorylation (32). The SIT method is based on the hypothesis that the small modification groups do not change much the overall physiochemical properties of peptides and thus the modified peptides have similar retentive behaviors in chromatographic analyses as the unmodified peptides. Based on the principles we previously discovered, modestly phosphorylated peptides could be identified by close examination of specific ion chromatograms (32). Here, our present data also indicate it can be applied to studying protein acetylation. Base on these observations, we surmise that this method should be applied to studies of other protein modifications, like methylation. Recently, we have indeed located the dimethylarginine residues on several nuclear proteins using similar approach (Tsay, unpublished observation).

The most intriguing finding in the present report is the identification of additional novel p53 acetylation sites, Lys-292 and Lys-305. Considering that they have not been
reported in the literature, it is quite a surprise why acetylation at Lys-292 and Lys-305 was only detected in our system. There are several plausible explanations for our unique observation. The first is that the efficiency for acetylation at Lys-305 is probably much lower than that occurring at other sites (Fig 2B). The ion counts of singly acetylated peptide for acetylation at Lys-381 and Lys-382 is about half of that for unmodified isoform (data not shown). In contrast, the amounts of the acetylated peptides at Lys-292 and Lys-305 are only about one thirtieth of the corresponding unmodified peptides. Therefore, acetylation at Lys-292 and Lys-305 residues may have been neglected in previous experiments due to their suboptimal labeling under these conditions.

Another possibility is that we used a full-length p53 as the substrate, rather than the deletion mutants used by other investigators (10). The reductionistic approach has been frequently used in experiments involving identification of modified residues. However, an important pitfall is that construction of these deletion mutants, either randomly or deliberately, is associated with distortion of important local protein conformation. This can lead to introduction of false targets or obliteration of true ones. As these two sites are positioned over the region between DNA-binding and tetramerization domains, the context surrounding them may have been disrupted in mutants only consisting of individual domains.

Specific protein cleavage by endoproteinases is very important for SIT method. One basic premise is that protein digestion is minimally or not affected by protein modification per se. If the moiety prevents the modified residue from being cleaved, it is conceivable that unmodified peptides derived from this region should have lengths different from those of modified peptides. Therefore, it becomes unlikely to identify the modified peptides using the unmodified counterparts as the references. Considering acetyl-lysine residues are very poor substrates for both trypsin and Lys-C, either enzyme
is not suitable for our experiment. Therefore, we digested substrate proteins with enzymes whose cleavage is minimally affected by acetylation, for example, Asp-N and Arg-C.

Previous studies have demonstrated that different post-translational modifications of p53 occurred under different stress-induced conditions. It may be activated through different signal transduction pathways (5, 18). Our data demonstrated that the acetylation of p53 at Lys-305, like Lys-382, was responsive to all p53-activating agents. Intriguingly, the acetylation profiles elicited by H$_2$O$_2$ at Lys-305 and Lys-382 are quite different. The induction of Lys-382 acetylation is slower than that of Lys-305 (Fig. 5B). It is likely that Lys-305 could be acetylated by a mechanism different from that of Lys-382. This interesting observation remains to be studied.

We have attempted to investigate the mechanism via which Lys-305 acetylation may modulate its transcriptional activity. Alteration of p53 subcellular distribution has been an attractive possibility since Lys-305 has been documented as part of bipartite nuclear localization signal (21). Nevertheless, we found that both K305R and K305A mutants and wild-type p53 have very similar immunofluorescence staining pattern (data not shown). The results are the same as those previously documented by other investigators (17). Therefore, it seems unlikely that alteration of subcellular/subnuclear distribution may fully account for the stimulatory/inhibitory effects of these mutants. In accordance with these findings, we also found that subcellular/subnuclear localization pattern of the acetyl-Lys-305, as well as acetyl-Lys-382, is identical to the overall population of p53 molecules (data not shown). This also supports the idea that Lys-305 acetylation is not directly associated with subcellular/subnuclear targeting of p53 protein.
To further examine whether glutamine/arginine substitutions may mimic constitutively acetylated/nonacetylated lysine residues, cells were cotransfected with K305Q or K382Q mutants, the p50-2 promoter activity was markedly decreased in both cases (Fig. 6). The glutamine substitution mutants do not mimic constitutively acetylated lysine. Contrary, the arginine-substituted mutants, K305R and K382R, led to an increased transcriptional activity. Normally, arginine substitution would mimic a non-acetylatable lysine and thus could not alter the p53 transcriptional activity while the glutamine substitution would mimic the acetylated lysine and thus could increase the p53 transcriptional activity. Previous studies (25, 26) demonstrated that single and multiple K-to-R p53 mutations resulted in higher transcriptional activities. The amino acid substitutions may not properly reflect the acetylated lysine and non-acetylated lysine. It is possible that p53 C-terminal domain is a multifunctional domain, responsible for oligomerization, nuclear translocation, protein interaction and negative regulation domain to the specific DNA binding activity of p53. In addition, the C-terminal domain of p53 may be modified by other posttranslational modifications, such as phosphorylation and ubiquitination. Even substitution for only one amino acid may still affect several functions of p53. Alternatively, while it may be possible that acetylation is indeed involved in downregulating p53 function and the increased acetylation that occurs under stress conditions reflects a negative autoregulatory loop that serves to limit the extent of p53 activation.

Although the glutamine/arginine substitutions may not mimic constitutively acetylated/nonacetylated lysine residue as we initially presumed, there is paralleled change of p53 transcriptional activities by different amino acid substitutions at Lys-305 and Lys-382. Meanwhile, these p53 mutants could not respond to UV-irradiation as efficiently and failed to produce optimal transcriptional activity that is intrinsic to
wild-type p53. These results suggest that Lys-305, like Lys-382, played an important role in modulating the precise transcriptional activity of p53.

The Lys-292 of p53 was also identified as an *in vitro* p300 acetylated target by SIT approach (Fig. 2D). Unfortunately, we have not been able to generate the specific anti-acetyl-Lys-292 antibody to test the possibility that Lys-292 can also be acetylated in vivo. However, we have constructed K292 mutants and found that these mutants also had altered the p53 transcriptional activity. Interestingly, it was reported that some cancer and cell lines have mutations at Lys-292 (see http://www.lf2.cuni.cz/projects/germline_mut_p53.htm). The function of Lys292 is in the subject of future investigations.

We have also tested the possibility that Lys-305 acetylation might actually modulate the transcriptional activity of p53 through influencing the modification of other p53 residues. It has been documented that post-translational modification at certain sites can alter the properties of p53 such as protein stability (9, 33), tetramerization (30), and DNA-binding capacity (4, 16). In order to test this possibility, we investigated how modification states of other residues are changed in K305A and K305R mutants as compared to the wild-type protein. Our preliminary data appeared to show that K382 acetylation is suppressed in K305A mutant but enhanced in K305R mutant. It remains to be examined whether this is the primary mechanism responsible for the altered transcriptional activity of these mutants. It is also intriguing to examine whether the modification of other sites may also be affected by K305 acetylation.
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FOOTNOTES

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ABBREVIATIONS

The abbreviations used are: CREB, cAMP-response element-binding protein; SIT, selected ion tracing; CBP, CREB binding protein; PCAF, p300/CBP associated factor; CAT assay, Chloramphenicol acetyltransferase assay; CID, collision-induced dissociation; HDAC, histone deacetylase; ActD, actinomycin D; HMG, high mobility group;
Figure legends

Fig. 1. *In vitro* acetylation of recombinant FLAG-p53 by HAT domain of p300 acetyltransferase.  

A, 4 µg of FLAG-p53 were incubated with various amounts of HAT-p300 in the presence of [¹⁴C] acetyl-CoA for 1 hr at 37℃. The reaction mixtures were subjected to SDS-PAGE followed by staining with Coomassie blue. The amount of HAT-p300 used was 0 ng (lane 1), 3 ng (lane 2), 10 ng (lane 3), 30 ng (lane 4), and 100 ng (lane 5), respectively. The dried gel was then subjected to autoradiography. The numbers by the bars on the left side at left indicate the migration positions of molecular weight markers (in kDa).  

B, quantitative analysis of the autoradiogram from (A) for acetylated p53 polypeptides.

Fig. 2. Identification of p300-mediated acetylation sites of FLAG-p53 *in vitro*.  

A, the peptide coverage of p53 protein by automated LC-MS/MS analysis. Boldface letters represent the peptides identified by LC-MS/MS analysis and gray ones are the residues that were not covered. The dashed underlines denote the sequence coverage of Arg-C peptides, and the solid underlines indicate the residues corresponding to Asp-N peptides. The identified acetylated lysine residues are indicated by closed triangles, while these acetylated residues are marked by open triangles.  

B, the chromatographic tracing (SIT) of the 600.4- (upper), 615.0- (middle) and 462.5- (lower) m/z ions. The retention times are denoted above the major peaks.  

C, CID spectrum of the 615-m/z ion at 35.6 min.  

D, CID spectrum of the 615-m/z ion at 39.8. The aK indicates the acetylated lysine residue.

Fig. 3. The specificities of Ab-165 and Ab-166.  

A, Dot blot analysis of purified
Ab-165 and Ab-166 against synthetic acetylated peptides. 20µg of acetylated peptides were spotted onto Hybond-C membrane: Ac-K305 peptide (lane 1), Ac-K382 peptide (lane 2) and Ac-HMG-14 peptide (lane 3) were used. The membrane was then subjected to immunoblotting with the indicated antibodies. B, Ab-165 and Ab-166 recognized in vitro acetylated recombinant FLAG-p53. Recombinant FLAG-p53 (0.2 µg) was acetylated by HAT-p300 0.1 µg (lanes 3 and 4) or 0.4 µg (lane 5) in the presence of acetyl CoA (100 pmol, lanes 2, 4 and 5) or in the absence of acetyl CoA (lanes 1 and 3). The acetylated p53 was detected by immunoblotting with the indicated antibodies. The levels of p53 protein in these reactions were revealed by immunoblotting with anti-p53 monoclonal antibody, DO-1.

Fig. 4 Sodium butyrate and p300 enchaned acetylation at Lys-305 in vivo. H1299 cells were transiently transfected with plasmid encoded with FLAG-p53, together with or without p300, and subsequently treated or untreated with sodium butyrate (50 µM, for 6 hr). 48 hours after transfection, whole cell lysate from each transient transfection group was prepared and then analyzed by SDS-polyacrylamide gel electrophoresis. The acetylation level at each residue was determined by immunoblotting with the indicated antibodies. The amounts of p53 protein were revealed by immunoblotting with DO-1.

Fig. 5. Lys-305 acetylation was elevated in response to various stress stimuli. MCF-7 cells were treated with a variety of p53-activating agents. A, the cells were treated with sodium butyrate (50 µM, lanes 2-4), IR irradiation (20 Gy, lanes 5-7), and UV (50 J/m², lanes 8-10) and then the cell lysates were analyzed by Western blots using the indicated antibodies. B, the cells were treated with H₂O₂ (1 mM, lanes 2-5), and actinomycin D (5 nM, lanes 6-9) and then lysates were analyzed by Western blots using the indicated antibodies. The cells were harvested at various time points after these
treatments.

**Fig. 6. Effects of K305 mutations on the transcriptional activity of p53.** A. H1299 cells were transfected with p50-2 CAT reporter gene together with a plasmid expressing wild-type p53 or p53 mutants (K305A, K305R, K305Q, K382A, K382R, and K382Q). B. The transcriptional activities of wild-type p53 and p53 mutants were determined after the transfectants were treated with UV irradiation. After 48 hr, β-gal and CAT activities were measured. Relative CAT activities were calculated from two independent experiments, normalized by the β-gal expression, and the standard deviations were derived from the activity means. The Western blots showed that the protein expression levels of wild-type mutant variants of p53 were done using anti-FLAG (*bottom panel*).
| Group | Peptide sequence | Presence of acetyl | Peptide position |
|-------|------------------|--------------------|------------------|
|       |                  |                    |                  |

Table 1. Characteristics of acetylated residues on p53 protein.
**Autoradiography**

**Coomassie blue staining**

**p300**

*Fig. 1A*
Figure 4

Table: Sodium butyrate

|        | HA-p300 | Flag-p53 |
|--------|---------|----------|
| +      | -       |          |
| 4      | 4       | 2        |
| +      | +       | +        |

Articles:

- DO-1
- Ab-166
- Ab-165
Figure 5A
Identification and characterization of a novel p300-mediated p53 acetylation site, lysine 305
Yan-Hsiung Wang, Yeou-Guang Tsay, Bertrand Chin-Ming Tan, Wen-Yi Lo and Sheng-Chung Lee

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