Probing Lysine Acetylation in Proteins

STRATEGIES, LIMITATIONS, AND PITFALLS OF *IN VITRO* ACETYLTRANSFERASE ASSAYS*

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The acetylation of proteins at specific lysine residues by acetyltransferase enzymes has emerged as a posttranslational modification of high biological impact. Although lysine acetylation in histone proteins is an integral part of the histone code the acetylation of a multitude of non-histone proteins was recently recognized as a regulatory signal in many cellular processes. New substrates of acetyltransferase enzymes are continuously identified, and the analysis of acetylation sites in proteins is increasingly performed by mass spectrometry. However, the characterization of lysine acetylation in proteins using mass spectrometric techniques has some limitations and pitfalls. The non-enzymatic cysteine acetylation especially can result in false-positive identification of acetylated proteins. Here we demonstrate the application of various mass spectrometric techniques such as matrix-assisted laser desorption/ionization and electrospray ionization mass spectrometry for the analysis of protein acetylation. We describe diverse combinations of biochemical methods useful to map the acetylation sites in proteins and discuss their advantages and limitations. As an example, we present a detailed analysis of the acetylation of the HIV-1 transactivator of transcription (Tat) protein, which is known to be acetylated *in vivo* by the acetyltransferases p300 and p300/CBP-associated factor (PCAF). ***Molecular & Cellular Proteomics 4:1226–1239, 2005.***

The acetylation of proteins by acetyltransferases is increasingly considered a biologically relevant regulatory modification like phosphorylation (1). Acetyltransferases transfer acetyl groups from acetyl-coenzyme A (AcCoA)[1] either to the α-amino group of the amino-terminal residue (*N*-acetyltransferases) or to the ϵ-amino group of specific lysine residues (histone/factor acetyltransferases) of substrate proteins. The reverse reaction is catalyzed by deacetylases that remove acetyl groups from specific acetyllysine residues in their substrates. The reversible lysine acetylation of histones and non-histone proteins plays a vital role in the regulation of many cellular processes including chromatin dynamics and transcription (2–5), gene silencing (6, 7), cell cycle progression (8–11), apoptosis (12–14), differentiation (15–19), DNA replication (20, 21), DNA repair (22–27), nuclear import (28–30), and neuronal repression (31–33). More than 20 acetyltransferases and 18 deacetylases have been identified so far, but the mechanistic details of substrate selection and site specificity of these enzymes remain unclear. Over 40 transcription factors and 30 other nuclear, cytoplasmic, bacterial, and viral proteins have been shown to be acetylated *in vivo* (34, 35), and the investigation of protein acetylation continues.

In recent years, the analysis of protein acetylation by MS has become increasingly popular because MALDI-TOF MS and ESI MS represent fast and sensitive methods for the characterization of co- and posttranslational protein modifications (36–38). In this study, we demonstrate the advantages of various MS techniques for the characterization of protein acetylation. For exemplification we used the human immunodeficiency virus, type 1 (HIV-1) transactivator of transcription (Tat) protein and peptides from the human histone H4 protein that are known substrates of the acetyltransferases p300 and p300/CBP-associated factor (PCAF) (34). Diverse combinations of experiments useful to map the acetylation sites in proteins and peptides are presented, and potential limitations and pitfalls in the interpretation of *in vivo* and *in vitro* acetylation studies are discussed.

EXPERIMENTAL PROCEDURES

Synthesis of Peptides and Proteins—All peptides (Peptide Specialty Laboratories GmbH, Heidelberg, Germany) and proteins used in this study were chemically prepared by solid-phase peptide synthesis in a stepwise fashion using Fmoc chemistry cycles as described before (39). Acetylated lysine residues were incorporated as Fmoc-Lys(Ac)-OH derivatives during the chain assembly. The peptides and proteins were deprotected and cleaved off from the resin with TFA, purified by reversed-phase HPLC, and characterized by MALDI-TOF MS or ESI MS.

MS Analysis of Full-length Tat—For MALDI MS analysis, 0.5 μl of a solution containing 1 nmol/μl full-length Tat (Tat-(1–72)) were co-crystallized with sinapic acid on an AnchorChip™ 800/25 MALDI target plate following the manufacturer's instructions (Bruker-Daltonik, Bremen, Germany). MALDI mass spectra within an *m/z* range of 7200–9200 were recorded in the positive ion linear mode with delayed extraction on a Reflex II™ TOF instrument (Bruker-Daltonik) equipped with a SCOUT-26 inlet and a 337 nm nitrogen laser. The voltage at the ion acceleration plate was set to 20.0 kV, at the first

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Received, February 17, 2005, and in revised form, May 31, 2005
Published, MCP Papers in Press, June 2, 2005, DOI 10.1074/mcp.M500047-MCP200

[1] The abbreviations used are: AcCoA, acetyl-coenzyme A; aa, amino acids; ARM, arginine-rich motif; CRR, cysteine-rich region; HIV, human immunodeficiency virus; PCAF, p300/CBP-associated factor; CBP, cAMP-response element-binding protein (CREB)-binding protein; PTH, phenylthiohydantoin; Tat, transactivator of transcription; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl.

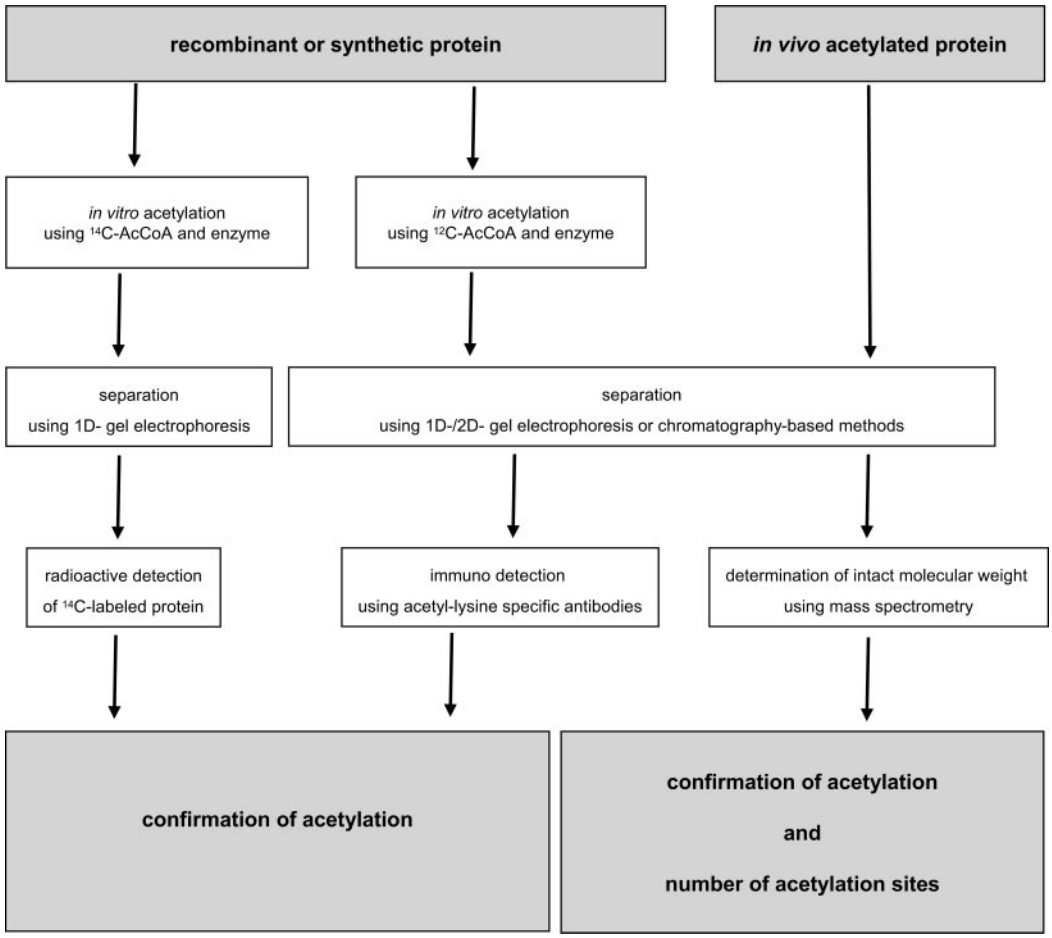

FIG. 1. **Strategies for the detection of acetylated proteins.** Radioactive detection or immunodetection techniques indicate the presence of acetylation in proteins but do not provide information about the degree of acetylation. Mass spectrometry confirms the identity of the protein and reveals the number of acetylation sites. *1D*, one-dimensional; *2D*, two-dimensional.

extraction plate to 16.6 kV, and at the detector to 1.6 kV. Mass spectra were obtained by averaging up to 200 individual laser shots. The spectra were calibrated externally using the signals of singly and doubly charged cytochrome *c* and myoglobin and the singly charged ions of ubiquitin and insulin.

For ESI Q-TOF MS analysis, the proteins were diluted in 0.025% formic acid, 25% acetonitrile to a final concentration of 5 pmol/μl and directly infused into the Ultima^{TM} orthogonal hybrid Q-TOF mass spectrometer (Micromass, Manchester, UK). A constant flow of 200 nl/min was generated by a syringe pump, and PicoTip^{TM} silica tubing emitters with an inner diameter of 10 μm were used for the connection of the syringe with the Z-spray nano-ESI source of the mass spectrometer. To produce the electrospray, a voltage of 1.8 kV was applied to the union, and the cone voltage was set to 50 V. MS spectra were recorded in the V mode under an argon collision gas pressure of 15 p.s.i. and a collision energy of 10 eV.

In Vitro Acetylation of Synthetic Peptides—2 nmol of peptide were acetylated *in vitro* with 20 nmol of AcCoA in the presence or absence (negative controls) of 2 nmol of recombinant p300 or PCAF acetyltransferase domain. Recombinant acetyltransferase domains were prepared as described before (39, 40). Incubation was performed at 30 °C for 30 min in a total volume of 20 μl of reaction buffer containing 50 mM HEPES, pH 8.0, 10% glycerol, 1 mM DTT, and 10 mM sodium butyrate. The reaction was stopped by freezing.

MS Analysis of Peptides—For MALDI MS analysis, the *in vitro* acetylated peptides were desalted and concentrated using 10-μl C_{18} ZipTip^{TM} pipette tips (Millipore, Bedford, MA) or HIC_{18} magnetic beads (Bruker-Daltonik) according to the manufacturers' instructions. Co-crystallization with α-cyano-4-hydroxycinnamic acid was carried out directly on ground steel or AnchorChip 800/25 MALDI target plates following the manufacturers' instructions. MALDI mass spectra within an *m/z* range of 700–3500 were recorded in the positive ion reflector mode with delayed extraction on a Reflex II TOF instrument (Bruker-Daltonik) equipped with a SCOUT-26 inlet and a 337 nm nitrogen laser. The voltage at the ion acceleration plate was set to 26.5 kV, at the first extraction plate to 20.6 kV, at the reflector to 30.0 kV, and at the detector to 1.5 kV. Mass spectra were obtained by averaging up to 200 individual laser shots. The spectra were calibrated externally by a two-point linear fit using peptides of angiotensin I and the oxidized B-chain of bovine insulin.

For ESI MS analysis, the *in vitro* acetylated peptides were desalted and concentrated using 10-μl C_{18} ZipTip pipette tips (Millipore) or HIC_{18} magnetic beads (Bruker-Daltonik) according to the manufacturers' instructions. The sample was diluted in 0.05% formic acid, 50% acetonitrile and directly infused into the Ultima orthogonal hybrid Q-TOF mass spectrometer (Micromass). A constant flow of 200 nl/min was generated as described above. Voltages were set to 1.8 kV at the union and to 50 V at the cone. Acquisition of MS spectra was

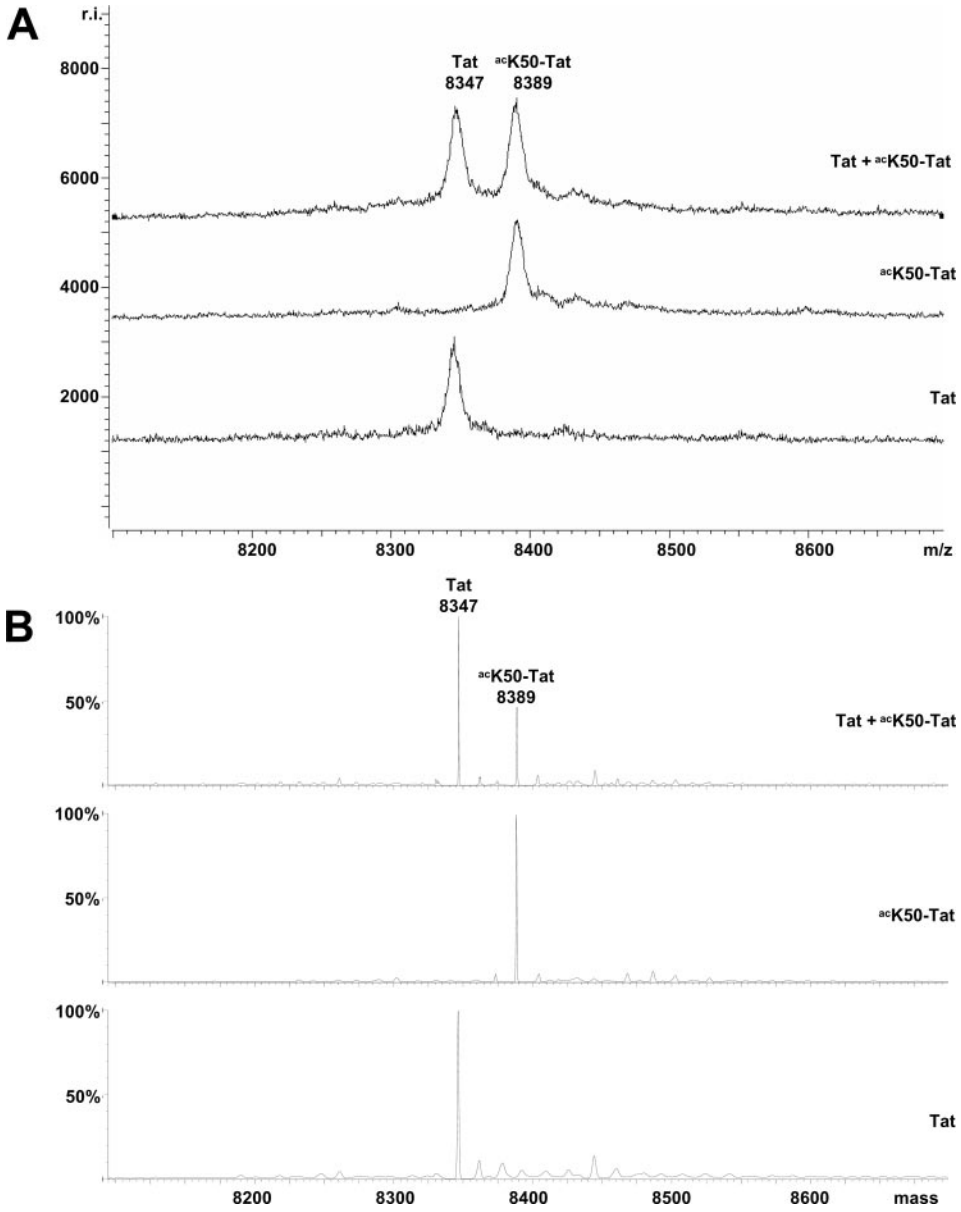

FIG. 2. **Mass spectrometric analysis of non-acetylated and monoacetylated full-length HIV-1 Tat (aa 1–72) and a mixture of both.** *A*, MALDI-TOF mass spectra. *B*, ESI Q-TOF mass spectra. Both techniques yield the correct molecular mass of non-acetylated and monoacetylated Tat with the characteristic mass difference of 42 Da. *r.i.*, relative intensity.

performed in the V mode under a collision energy of 10 eV and an argon collision gas pressure of 15 p.s.i.

Peptide Fragmentation Analysis—MALDI MS/MS analysis was performed in the positive ion mode using the LIFT-TOF/TOF technique on an Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonik). The instrument was controlled by the Flexcontrol software package (Bruker Daltonik).

ESI Q-TOF MS/MS was performed by CID using the instrument parameters described above but with an increased collision energy of 35 eV. If possible, doubly or triply charged peptide ions were selected for fragmentation analysis, and the raw MS/MS spectra were deconvoluted using the MaxEnt3 option of the MassLynx 4.0 software (Micromass). ESI fragmentation analysis of the Tat core peptide was performed by fully automated chip-based nanoelectrospray MS/MS using a NanoMate 100 (Advion Biosciences, Ithaca, NY) mounted to a Q-TOF mass spectrometer as described previously (41).

Digestion with Endoproteinase Lys-C—*In vitro* acetylated Tat peptide 45–58 (20 μg) was precipitated in 80% acetone at −20 °C for 12 h to remove salt from the acetylation assay. After drying the peptide was resuspended in 25 μl of cleavage buffer containing 40 mM ammonium bicarbonate and cleaved at 37 °C for 6 h using 20 ng of endoproteinase Lys-C. The reaction was stopped by freezing.

Edman Sequencing—After *in vitro* acetylation the Tat ARM peptide was desalted and concentrated on a PVDF membrane using the ProSorb sample preparation cartridge technique according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Edman degradation was performed on a Procise 494 cLC protein sequencer using standard programs supplied by the manufacturer

TABLE I
Sequences and masses of the synthetic proteins and peptides used in this study
Proteins and peptides are listed in the order of their appearance in the text. av, average mass, mono, monoisotopic mass.

Sequence	MH^+	Description
MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCF-HCQVCFITKALGISYGRKKRRQRRRPPQGSQT-HQVSLSKQ	8346.9 (av)	aa 1–72 of HIV-1 Tat
MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCF-HCQVCFITKALGISYGR ^{Ac}KKRRQRRRPPQGS-QTHQVSLSKQ	8388.9 (av)	aa 1–72 of HIV-1 Tat, acetylated at Lys^{50}
GGLGISYGRKKRRQRRRP	2141.3 (mono)	aa 43–58 of HIV-1 Tat (ARM), amino-terminal GG
GGLGISYGR^{Ac}KKRRQRRRP	2183.3 (mono)	aa 43–58 of HIV-1 Tat (ARM), amino-terminal GG, acetylated at Lys^{50}
Biotin-SGRGKGG^{Ac}KGLGK	1368.7 (mono)	aa 1–12 of histone H4, biotinylated, acetylated at Lys^{8}
ISYGRKKRRQRRRP	1857.1 (mono)	aa 45–58 of HIV-1 Tat (ARM)
ALGISYGRKKRRQRRRP	2098.3 (mono)	aa 42–58 of HIV-1 Tat (ARM)
ISYGRAKRRQRRRP	1801.1 (mono)	aa 45–58 of HIV-1 Tat (ARM), K50A exchange
ISYGRKARRQRRRP	1801.1 (mono)	aa 45–58 of HIV-1 Tat (ARM), K51A exchange
CTNCYCKKCCFHCQ	1683.6 (mono)	aa 22–35 of HIV-1 Tat (CRR)
LHKSMGRTWQFDYNPEACVIK	2523.2 (mono)	The 20 proteinogenic aa
LHKSMGRTWQFDYNPEAVIK	2420.2 (mono)	The 20 proteinogenic aa without cysteine
VCFITKALGISYGRK	1655.9 (mono)	aa 36–50 of HIV-1 Tat (core)

(Applied Biosystems), and the HPLC column of the sequencing system was preprocessed by highly concentrated TFA washes using the Fast Column Break IN version 2.0 program (Applied Biosystems).

RESULTS AND DISCUSSION

Strategies for the Detection of Acetylated Proteins—Different strategies can be used for the detection of intact proteins that are acetylated at internal lysine residues (Fig. 1). Most of the known acetylated proteins have been identified by radioactive detection or immunodetection. Radioactive detection is only applicable after complex *in vivo* labeling or after enzymatic *in vitro* acetylation of recombinant or synthetic proteins using [^{14}C]AcCoA. Detection occurs via autoradiographic imaging or scintillation counting after separation of the acetylated proteins from radioactive AcCoA and other by-products that is predominantly achieved by one-dimensional gel electrophoresis. Immunodetection using acetylation-specific antibodies can be performed with *in vivo* and *in vitro* acetylated proteins using [^{12}C]AcCoA. By avoiding radioactive labeling immunodetection is more convenient than radioactive detection with respect to subsequent separation methods such as one-dimensional/two-dimensional gel electrophoresis or chromatography-based methods. In addition, a growing number of site-specific antibodies for the analysis of protein acetylation is commercially available. However, both radioactive detection and immunodetection provide evidence for acetylation but do not give information about the number of acetylation sites in a protein. In contrast, mass spectrometric measurement of the molecular weight of *in vivo* or *in vitro* acetylated proteins not only allows their characterization but also reveals the number of acetyl groups attached to the protein. Because the signal of an acetylated protein is shifted by +42 Da per covalently bound acetyl group as compared with the signal of the unmodified protein the number of acetylation sites in a protein can easily be determined using MS.

Both MALDI-TOF MS and ESI MS have proven to be suitable techniques for the determination of the molecular weight of intact proteins. Fig. 2 shows exemplarily the analysis of chemically synthesized full-length HIV-1 Tat protein by MALDI-TOF MS and ESI Q-TOF MS. First unmodified Tat protein, Tat chemically acetylated at Lys^{50}, and a mixture of both were analyzed by MALDI-TOF MS (Fig. 2*A*). The signal of non-acetylated Tat was observed at *m/z* 8347 (Fig. 2*A*, *bottom*), which is in accordance with the average molecular mass calculated for Tat-(1–72) (Table I). The monoacetylated Tat was also detected correctly at *m/z* 8389 (Fig. 2*A*, *middle*). The ion signal for the acetylated Tat was shifted by +42 Da as compared with the signal of the unmodified Tat (Fig. 2*A*, *top*) indicating the presence of a single acetyl group. The same results were obtained by ESI MS analysis on a Q-TOF mass spectrometer (Fig. 2*B*). Here the acetyl characteristic mass shift of +42 Da was clearly detectable after deconvolution of the corresponding mass spectra of unmodified and monoacetylated Tat. Hence both MALDI and ESI MS serve well for the detection of acetylated proteins.

Strategies for the Identification of Acetylation Sites in Proteins—The identification of acetylation sites in proteins can be performed using different approaches (Fig. 3). Mapping the acetylation sites within a substrate is preferentially performed using peptides rather than full-length proteins because peptides serve as well defined and versatile substrates of acetyltransferases and reduce the number of potential acetylation sites in the analyte molecule (42). Therefore, *in vivo* acetylated proteins and *in vitro* acetylated recombinant or synthetic proteins should be cleaved into peptides by a suitable endoproteinase like trypsin, Arg-C, or Lys-C prior to MS analysis (Fig. 3, *top part*). Alternatively chemically synthesized peptides

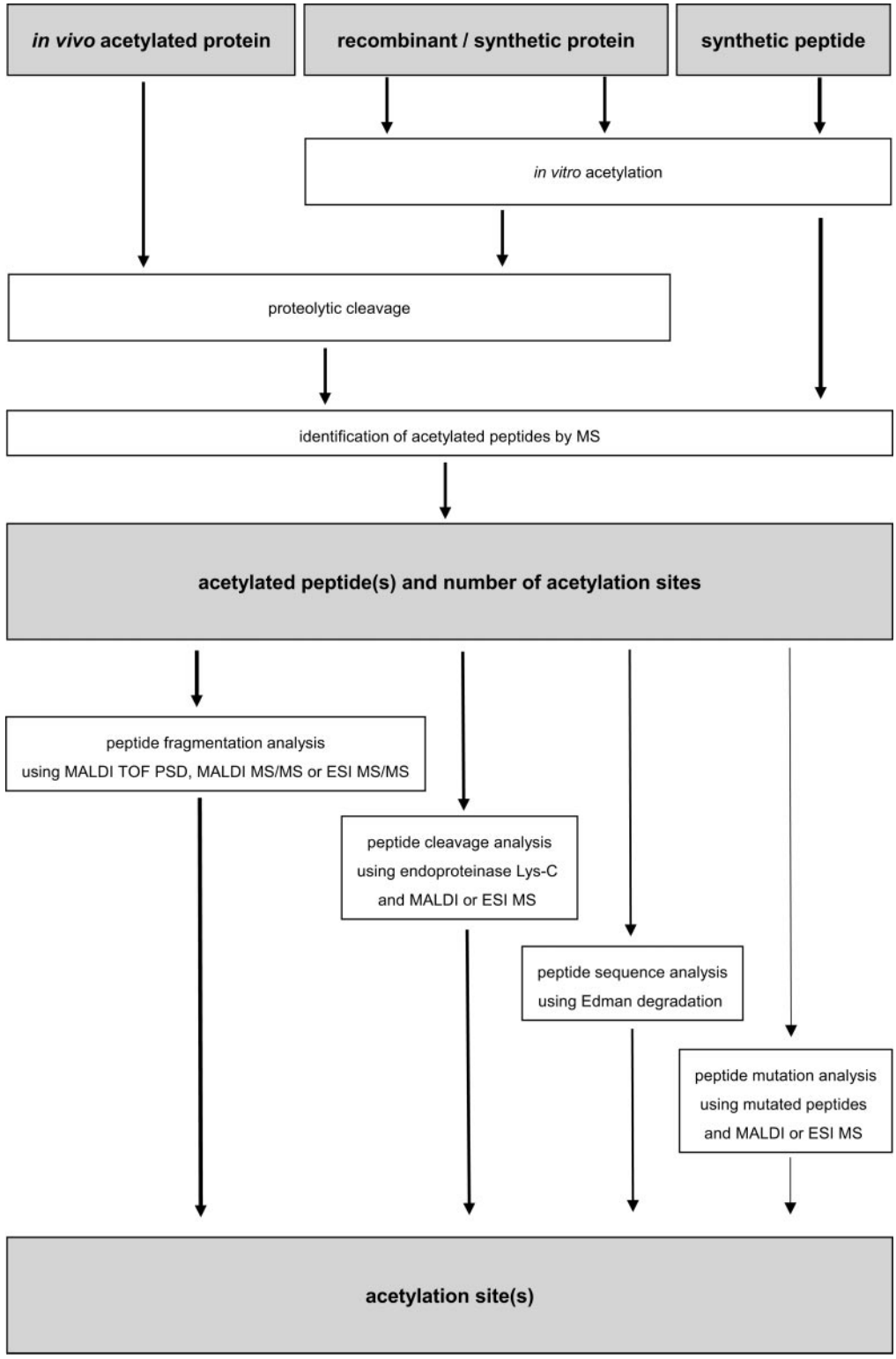

FIG. 3. **Strategies for the identification of acetylation sites in proteins.** *In vivo* or *in vitro* acetylated full-length proteins are cleaved into peptides prior to MS analysis. Alternatively synthetic peptides are *in vitro* acetylated and analyzed directly. MS identifies the acetylated peptides and defines the number of covalently bound acetyl groups. Subsequent mapping of the acetylation site(s) within the peptide can be performed using fragmentation, cleavage, sequence, or mutation analysis. *Arrow width* in the flow chart correlates with straightforwardness of the approach.

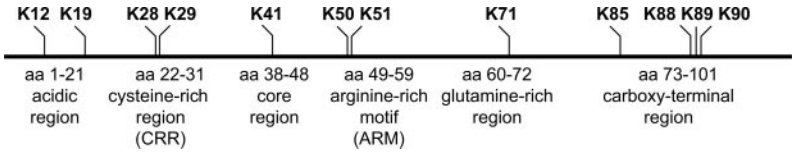

FIG. 4. **Schematic presentation of full-length Tat protein.** The location of the 12 lysine residues within the six functional domains is indicated.

covering putative acetylation sites of a protein are also suitable for *in vitro* acetylation and subsequent MS analysis. Initial mass spectrometric analyses identify the proteolytic or synthetic peptide(s) that are acetylated and define the number of acetylation sites within the respective peptide via the number of characteristic mass shifts. For the subsequent mapping of the acetylation sites in the peptides MALDI and ESI MS techniques can be applied directly or in combination with a variety of accessory experiments (Fig. 3, *bottom part*). Peptide fragmentation analysis such as MALDI MS/MS or PSD MS and ESI MS/MS experiments identify acetylated residues in the most straightforward way. If fragmentation analysis does not give a conclusive result peptide cleavage analysis using endoproteinase Lys-C in combination with MALDI or ESI MS is successful in most cases. Peptide sequence analysis by Edman degradation is a complementary biochemical approach to determine acetylated lysine residues in a peptide. In case that none of these techniques provides clear evidence peptide mutation analysis can be performed using synthetic peptides mutated at specific amino acid positions in combination with MALDI or ESI MS.

In the following, detailed analyses of the acetylation of the HIV-1 Tat protein will be presented to exemplify the advantages, limitations, and pitfalls of the individual steps of the above named strategies. HIV-1 Tat is a unique viral transactivator of HIV transcription that comprises 101 amino acids forming six different regions (Fig. 4). Tat harbors 12 lysine residues and is known to be acetylated *in vivo* by the acetyltransferases p300 and PCAF (43–45). To identify those regions within the Tat sequence that serve as substrate for p300 and PCAF various synthetic peptides of suitable lengths were designed spanning different regions of Tat (Table I).

Identification of Acetylated Peptides by Mass Spectrometry—The presence of acetyl groups in proteolytic or synthetic peptides can easily be detected by MALDI-TOF MS and ESI MS techniques. As in proteins, the number of mass shifts characteristic for an acetylation indicates the number of acetylation sites in the peptide. Fig. 5 shows exemplarily the mass spectrometric analysis of the synthetic peptide GGLGISYGRKKRRQRRR representing the ARM region of Tat. The non-acetylated peptide and its singly acetylated counterpart that was acetylated during the course of the chemical synthesis were analyzed by MALDI-TOF MS and ESI MS. In MALDI MS and deconvoluted ESI MS spectra, the acetylated peptide at *m/z* 2183 directly displays the characteristic mass difference of 42 Da in comparison with the unmodified peptide at *m/z* 2141 because these spectra display singly charged ions (Fig. 5, *A* and *B*). In unprocessed ESI MS spectra, the value of the mass shift characteristic for a single acetylation depends on the charge state of the peptide ion, *e.g.* +21 Da for doubly charged ions, +14 Da for triply charged ions, and +10.5 Da for quadruply charged ions. Accordingly the raw ESI Q-TOF MS spectrum of the Tat ARM peptide displays the signals of the unmodified and the monoacetylated quadruply charged peptide at *m/z* 536.1 and 546.6, the signals of the corresponding triply charged peptides at *m/z* 714.4 and 728.4, and the signals of the doubly charged peptides at *m/z* 1071.1 and 1092.1 (Fig. 5*C*).

Peptide Fragmentation Analysis—Fragmentation analysis by MALDI-TOF PSD MS, MALDI MS/MS, or ESI MS/MS experiments represents the most straightforward and thus most favored approach for the characterization of acetylation sites in a peptide. Generally fragmentation induced by MALDI and ESI mainly takes place at the peptide bond, the softest bond in a peptide, yielding the so called b- and y-ions (46). The position of the acetylated residues can be directly identified by the acetyl characteristic mass shift of +42 Da of the b- and y-ion signals. Lysine-acetylated peptides are stable during the MALDI and ESI MS ionization process and show no decay indicative for the modification in contrast to serine- and threonine-phosphorylated peptides, which sustain substantial losses of H_3PO_4 and HPO_3 (47). However, a specific acetyllysine marker ion at *m/z* 126.1 is detected in MALDI PSD MS, MALDI MS/MS, and ESI MS/MS experiments in addition to the acetyllysine immonium ion at *m/z* 143.1 (48) and can be utilized as proof that an acetylated lysine residue is indeed present in the peptide of interest. These acetyl-specific ions are absent when other than lysine residues or the amino terminus of the peptide are acetylated. Furthermore they can serve to differentiate between acetyllysine and the isobaric trimethyllysine because the latter amino acid does not exhibit these ion signals in the corresponding fragmentation spectrum (data not shown).

Fig. 6 shows the peptide fragmentation analysis of a synthetic histone H4 peptide (biotin-^{1}SGRGKGG^{Ac}KGLGK^{12} where ^{Ac}K is acetylated lysine). Continuous series of b- and y-ion signals were detected in both MALDI MS/MS (Fig. 6*A*) and ESI Q-TOF MS/MS (Fig. 6*B*). The position of the acetyl group at Lys^{8} was confirmed because the observed y-ion series y_1– y_9 starts to shift by +42 Da at the y_5-position. Accordingly the +42-Da shift in the detected b-ion series b_2–b_8 starts at the position b_8 (Fig. 6, *A* and *B*, not labeled). The nature of the acetylated amino acid was identified via the mass difference between the y_4- and the y_5-ion and between the b_7- and the b_8-ion, respectively, which was 170.11 Da, characteristic for an acetylated lysine, instead of 128.17 Da, characteristic for an unmodified lysine.

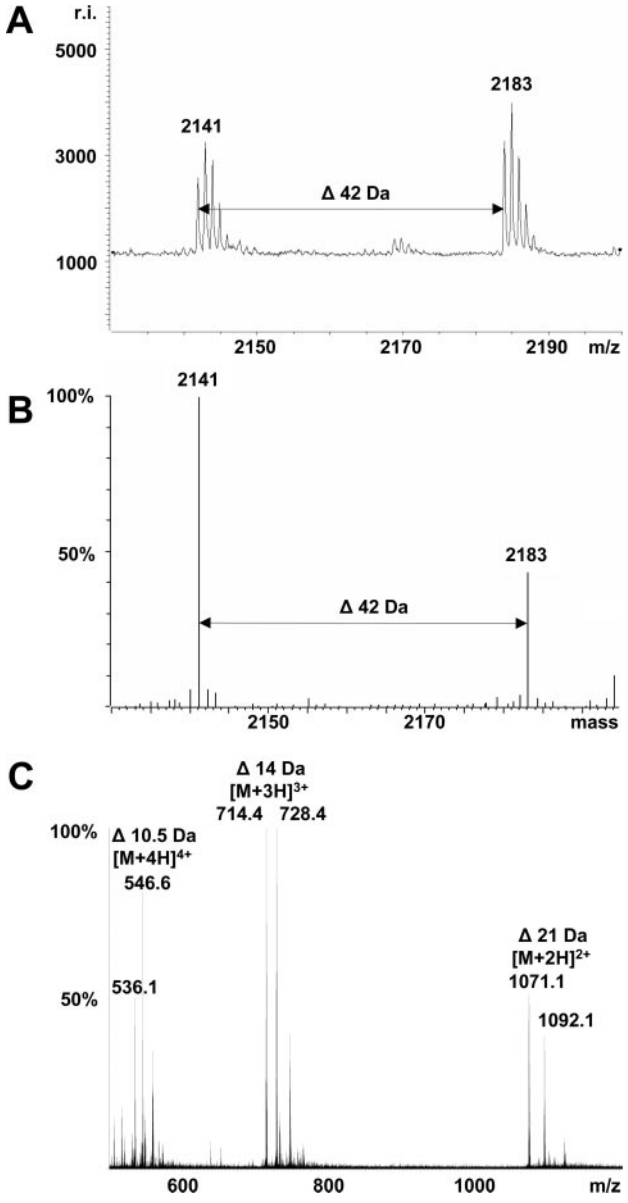

FIG. 5. **Mass spectrometric analysis of the non-acetylated and monoacetylated synthetic peptide GGLGISYGRKKRRQRRR representing the ARM region of HIV-1 Tat.** *A*, MALDI-TOF MS spectrum displaying the singly charged non-acetylated peptide at *m/z* 2141 and the singly charged acetylated peptide at *m/z* 2183. *B*, deconvoluted ESI Q-TOF MS spectrum displaying the singly charged unmodified peptide at *m/z* 2141 and the singly charged acetylated peptide at *m/z* 2183. *C*, unprocessed ESI Q-TOF MS spectrum displaying the quadruply charged non-acetylated peptide at *m/z* 536.1, the triply charged non-acetylated peptide at *m/z* 714.4, and the doubly charged non-acetylated peptide at *m/z* 1071.1. The corresponding monoacetylated ion signals are observed at *m/z* 546.6, 728.4, and 1092.1. *r.i.*, relative intensity.

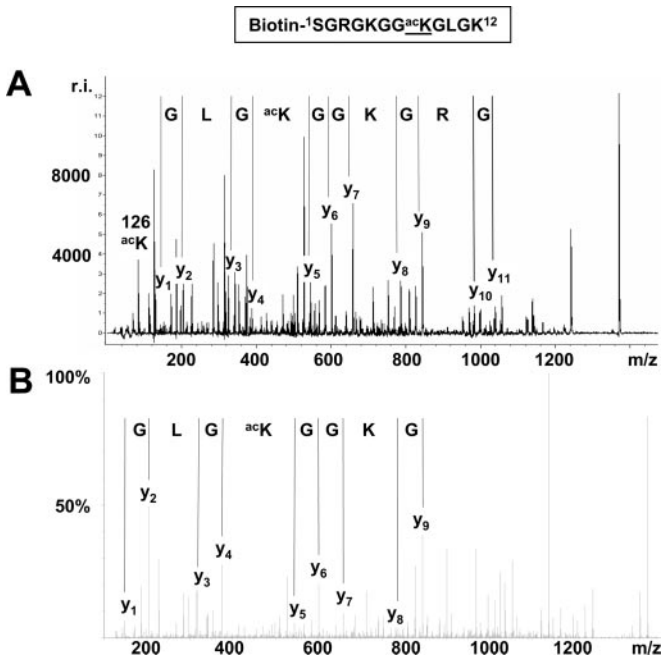

FIG. 6. **Peptide fragmentation analysis of monoacetylated histone H4 peptide (biotin-^{1}SGRGKGGP^{Ac}KGLGK^{12}).** *A*, MALDI MS/MS spectrum. *B*, deconvoluted ESI Q-TOF MS/MS spectrum. Both spectra display a continuous y_1–y_9 series mapping the acetylation site to Lys^{8}. Note the acetyllysine marker ion at *m/z* 126 in the MALDI MS/MS spectrum. *r.i.*, relative intensity.

In the same way the synthetic Tat peptide 45–58 corresponding to the Tat ARM (^{45}ISYGRKKRRQRRRP^{58}) was examined. Prior to analysis the ARM peptide was acetylated *in vitro* with AcCoA in the presence and absence of the acetyltransferase p300 (Fig. 7). The MALDI-TOF MS spectra of the ARM peptide in the absence of enzyme showed no mass shift of the peptide signal at *m/z* 1857 (Fig. 7, *lower trace*), whereas an additional signal at *m/z* 1899 was detected after *in vitro* acetylation of the peptide in the presence of p300 (Fig. 7, *upper trace*). This indicated an enzymatic transfer of a single acetyl group to the Tat ARM peptide by p300. In addition, the signal of the singly charged unmodified peptide at *m/z* 1857 was detected due to the non-quantitative acetylation of the peptide during the short *in vitro* incubation. Because no signal characteristic for the diacetylated peptide was found at *m/z* 1941 it must be concluded that only one of the lysine residues embedded in the Tat ARM, Lys^{50} or Lys^{51}, was specifically acetylated by p300. The same results were obtained when the Tat ARM peptide was acetylated in the presence of PCAF (not shown).

To determine which of the two lysine residues was acetylated by p300 and PCAF the peptide was subjected to fragmentation analysis. However, neither by MALDI MS/MS nor by ESI MS/MS could a consecutive ion series be obtained (spectra not shown). Because of its sequence and amino acid composition the peptide sustained strong internal fragmentation leading to a complete loss of b- and y-ion series. Therefore, the acetylation site in the Tat ARM peptide could not be identified by peptide fragmentation analysis, and therefore peptide cleavage analysis was applied (see below).

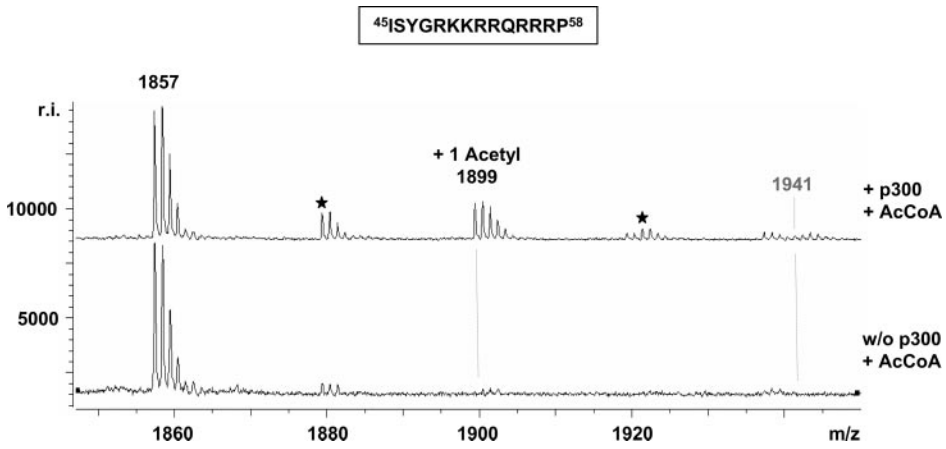

FIG. 7. **Mass spectrometric analysis of HIV-1 Tat ARM peptide (^{45}ISYGRKKRRQRRRP^{58}).** After *in vitro* acetylation in the presence of AcCoA and p300 an ion signal at *m/z* 1899 was observed in the MALDI MS spectrum in addition to the signal of the unmodified peptide at *m/z* 1857 (*upper trace*). This indicates an enzymatic transfer of one acetyl group to one of the two lysine residues. No such signal could be detected in the absence of enzyme (*lower trace*). ⋆, sodium adduct; *w/o*, without; *r.i.*, relative intensity.

Peptide Cleavage Analysis—Peptide cleavage analysis is advisable when peptide fragmentation analysis does not result in an unambiguous localization of the acetylated residue within the sequence. The cleavage specificity of the endoproteinase Lys-C is especially useful for the analysis of peptides harboring more than one lysine because Lys-C hydrolyzes peptide bonds at the carboxyl terminus of unmodified lysines, whereas peptide bonds carboxyl-terminal of modified lysines are resistant to cleavage (43, 49, 50). This is exemplified for the characterization of the acetylation site in the Tat ARM where peptide fragmentation analysis failed completely. To determine whether Lys^{50} or Lys^{51} was the target of acetylation by p300 and PCAF an extended ARM peptide was first digested *in silico*. In theory, Lys-C cleavage of Tat peptide 42–58 acetylated at Lys^{50} (^{42}ALGISYGR^{Ac}KKRRQRRRP^{58}) takes place exclusively carboxyl-terminally of the non-acetylated Lys^{51} yielding the acetylated amino-terminal cleavage product ^{42}ALGISYGR^{Ac}KK^{51} at *m/z* 1135 and the corresponding non-acetylated carboxyl-terminal fragment ^{52}RRQRRRP^{58} at *m/z* 1025. In contrast, cleavage of the peptide acetylated at Lys^{51} (^{42}ALGISYGRK^{Ac}KRRQRRRP^{58}) occurs carboxyl-terminally of the non-acetylated Lys^{50} yielding the non-acetylated amino-terminal fragment ^{42}ALGISYGRK^{50} at *m/z* 965 and the monoacetylated carboxyl-terminal product ^{51Ac}KRRQRRRP^{58} at *m/z* 1195. In the following assignment of the cleavage pattern that was carried out by MALDI-TOF MS only one additional peptide signal at *m/z* 1135 was detected after incubation of the peptide with AcCoA and PCAF and subsequent Lys-C digestion in comparison to the negative control reaction without acetyltransferase (Fig. 8*B*). The same result was obtained with the Tat ARM peptide that was acetylated in the presence of p300 (not shown). The additional signal corresponded to the monoacetylated form of cleavage product ^{42}ALGISYGRKK^{51} with *m/z* (1093 + 42) originating from proteolytic cleavage carboxyl-terminally of Lys^{51}. Because Lys-C cleaves exclusively carboxyl-terminally of unmodified lysine residues, the only possible acetyl acceptor site in this peptide is Lys^{50} (^{42}ALGISYGR^{Ac}KK^{51}). Again the acetylated peptide signal was accompanied by the signals of the cleavage products of the non-acetylated peptides ^{42}ALGISYGRK^{50} at *m/z* 965 and ^{42}ALGISYGRKK^{51} at *m/z* 1093 due to the non-quantitative acetylation reaction. No signal was observed for the peptide ^{51Ac}KRRQRRRP^{58} at *m/z* 1195 excluding acetylation of Lys^{51}. Hence Lys^{50} was identified as the exclusive acetylation site of PCAF and p300 in the Tat ARM by peptide cleavage analysis.

Peptide Sequence Analysis—An alternative technique to map the site of lysine acetylation in a peptide is sequence analysis by Edman degradation. Here the amino-terminal amino acids of a peptide are sequentially modified with phenylisothiocyanate, cleaved off from the peptide chain, converted into the stable phenylthiohydantoin (PTH) derivatives, and identified by their retention time in reversed-phase C_{18} HPLC (51, 52). Among the 20 proteinogenic amino acids, the derivative of an unmodified lysine residue shows the highest hydrophobicity and consequently the longest retention time on a C_{18} HPLC column because it carries two chromophores: a PTH moiety originating from the modification of the amino terminus of the peptide and an additional phenylthiocarbamoyl moiety originating from the modification of the ϵ-amino group in the lysine side chain. In contrast, the derivative of an acetylated lysine residue shows a significantly reduced retention time because the acetyl group attached to the lysine side chain prevents the additional phenylthiocarbamoyl modification and thereby decreases the hydrophobicity of the derivative (50, 53). The PTH derivative of the isobaric amino acid trimethyllysine elutes significantly later than the acetyllysine derivative and yields a much broader peak (data not shown). Because no other amino acid derivative shows an identical elution profile the acetylated lysine in a peptide can be identified by its characteristic retention time. Fig. 9 exemplarily shows the identification of Lys^{50} as the acetylation site of PCAF in the Tat ARM peptide (^{45}ISYGRKKRRQRRRP^{58}). In the negative control reaction performed in the absence of PCAF both Lys^{50} and Lys^{51} showed a retention time identical to the lysine standard in the PTH amino acid control mixture (Fig. 9, *left column*). However, after addition of PCAF to the *in vitro* acetylation assay a peak appeared for Lys^{50} with a

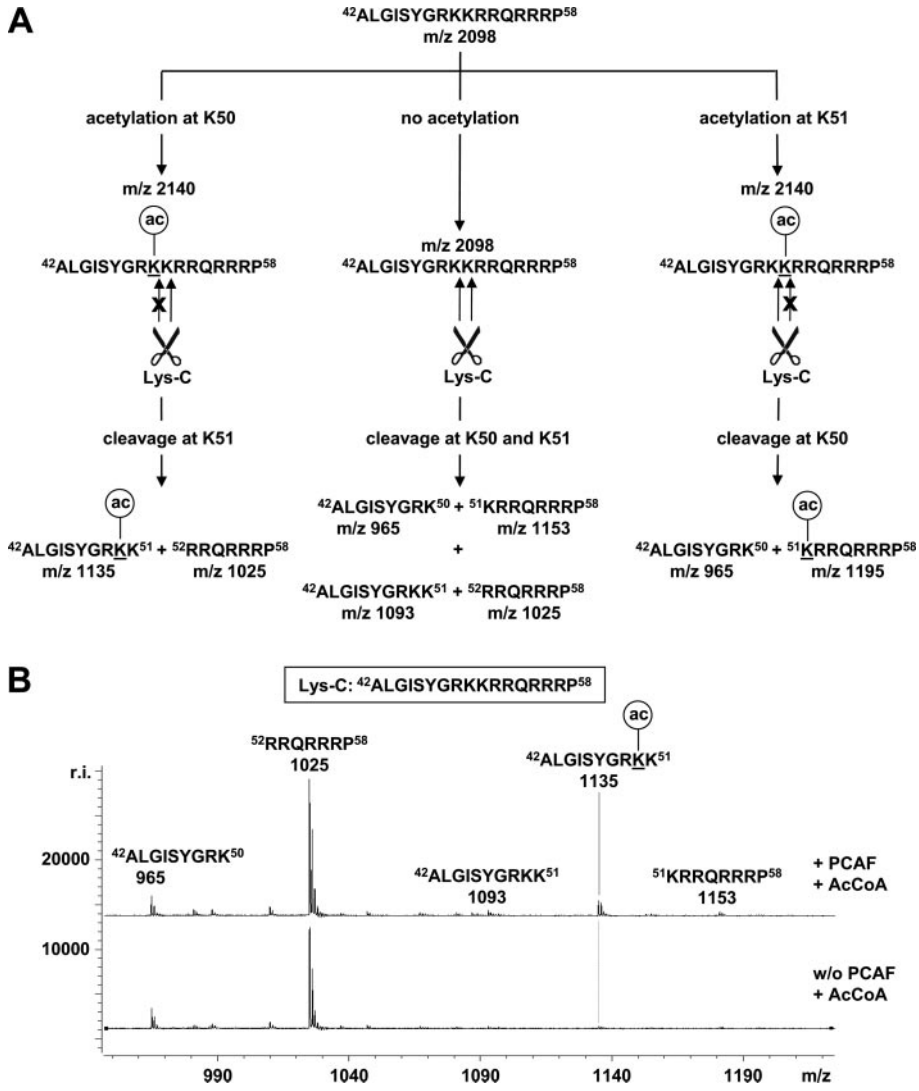

FIG. 8. **Peptide cleavage analysis of HIV-1 Tat ARM peptide (^{42}ALGISYGRKKRRQRRRP^{58}).** *A*, *in silico* endoproteinase Lys-C cleavage. Because endoproteinase Lys-C hydrolyzes peptide bonds only at the carboxyl-terminal side of unmodified but not of modified lysines acetylation at Lys^{50} would yield an acetylated peptide fragment at *m/z* 1135, whereas acetylation at Lys^{51} would yield an acetylated fragment at *m/z* 1195. *B*, MALDI-TOF MS analysis of Tat ARM peptide after *in vitro* acetylation in the presence (*upper trace*) or absence (*lower trace*) of PCAF and subsequent endoproteinase Lys-C cleavage. The presence of an ion signal at *m/z* 1135 unambiguously mapped the site of acetylation to Lys^{50}. *w/o*, without; *r.i.*, relative intensity.

retention time of ~10 min (Fig. 9, *right column*). This retention time is characteristic for the derivative of an acetylated lysine residue as was confirmed with a synthetic model peptide containing an acetyllysine residue. For Lys^{51} the peak specific for the unmodified lysine residues was dominant, whereas the peak specific for acetylated lysines was present at a very low intensity. Because incomplete degradation steps and slightly fluctuating repetitive yields of the protein sequencer are common phenomena in Edman sequencing (51) this was no indication for an additional acetylation of Lys^{51} by PCAF. The same result was obtained after acetylation of the Tat ARM peptide with p300 (50). Conclusively Edman sequencing confirmed that Lys^{50} in the Tat ARM is acetylated by PCAF and p300.

Peptide Mutation Analysis—The three so far described strategies for the identification of acetylation sites in peptides can fail for several reasons. In peptide fragmentation analysis by MALDI MS/MS, MALDI PSD MS, or ESI MS/MS, certain peptides may be poorly ionized or may lack the potential to generate conclusive ion series due to their amino acid composition and sequence. In peptide cleavage analysis, unspecific remodeling and fragmentation events can result in spectra too complex for an unambiguous interpretation. In peptide sequence analysis, some amino acids are not stable, form undetectable derivatives, or generate disturbing by-products leading to the abortion of the Edman reaction chain. Basic peptides and cysteine-containing peptides especially are difficult to analyze by MS and Edman sequencing due to their labile side chains and highly reactive sulfhydryl moieties. In these cases, the method of choice is peptide mutation analysis although this approach is time- and cost-consuming and requires well considered controls.

Generally it is well accepted to mutate the potential acetylation sites in a peptide to alanine, a non-reactive small amino acid that cannot bind acetyl groups. In the example of Tat two peptides corresponding to the ARM region were designed in which Lys^{50} and Lys^{51} were replaced by alanine, respectively (Table I). These peptides were chemically synthesized and *in*

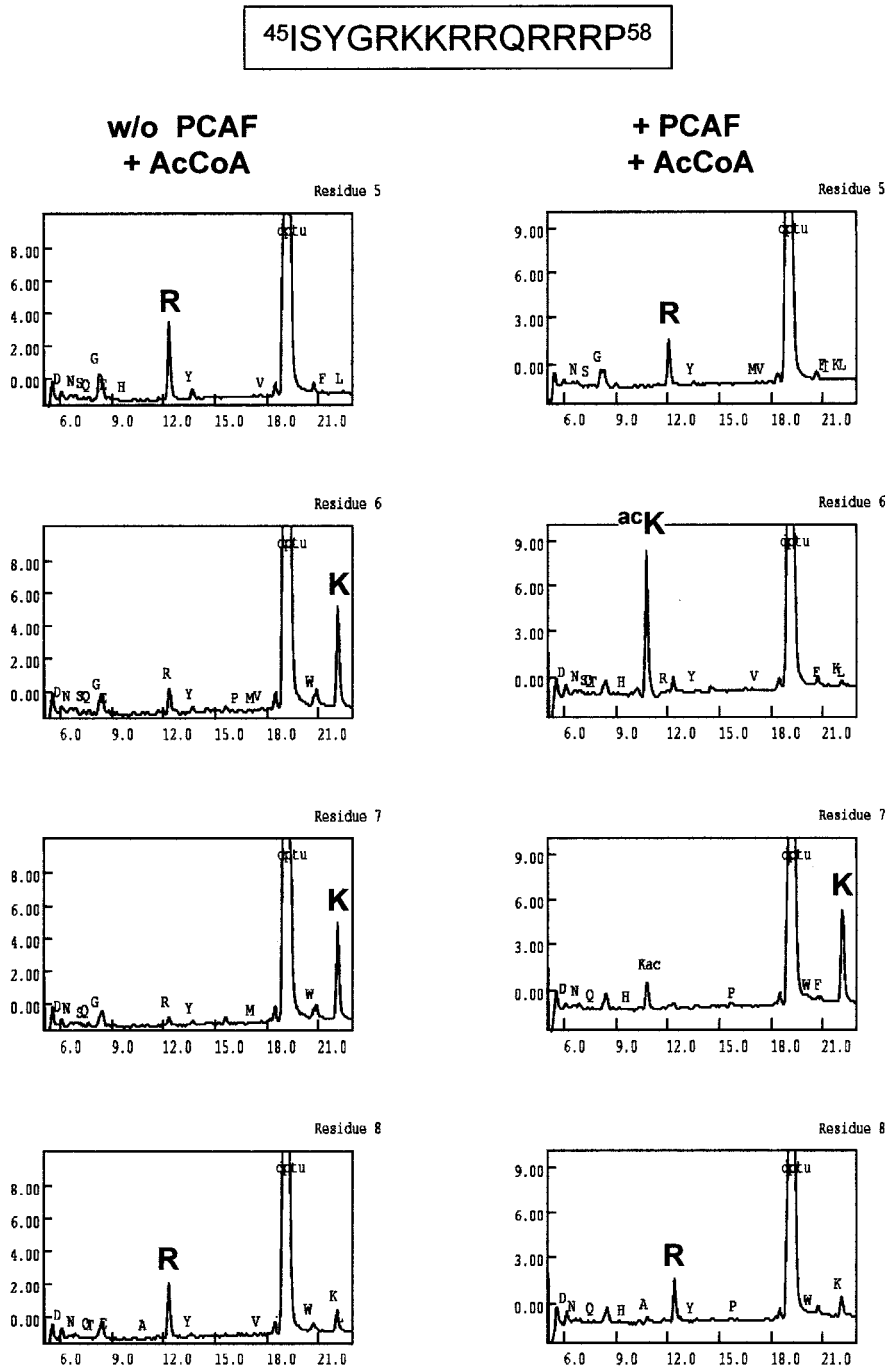

FIG. 9. **Peptide sequence analysis by Edman degradation of the HIV-1 Tat ARM peptide (^{45}ISYGRKKRRQRRRP^{58}) after *in vitro* acetylation in the presence (*right column*) and absence (*left column*) of PCAF.** Degradation cycles of residues 5–8 are shown, and identified amino acids are indicated. Enzymatic acetylation by PCAF was observed for Lys^{50}. *w/o*, without.

vitro acetylated with AcCoA in the presence and absence of the acetyltransferase PCAF. The MALDI-TOF mass spectrum of the peptide bearing the Lys^{50} to Ala^{50} exchange showed only the ion signal of the non-acetylated peptide at *m/z* 1801, whereas no signal could be detected at *m/z* 1843 indicating that acetylation was abrogated in this mutant peptide (Fig. 10*A*). In contrast, the characteristic mass shift by +42 Da from *m/z* 1801 to 1843 could be observed in the spectrum of the peptide with the Lys^{51} to Ala^{51} mutation (Fig. 10*B*). The same spectra were obtained after acetylation of the Tat ARM peptide with p300. These results confirm the previous findings by cleavage analysis and Edman sequencing that Lys^{50} is the only residue in the ARM that is enzymatically acetylated by PCAF and p300 and exemplify the use of peptide mutation analysis for the mapping of acetylation sites in peptides.

The Non-enzymatic Cysteine Acetylation—For mapping acetylation sites of p300 and PCAF in HIV-1 Tat we analyzed various peptides spanning the different regions of Tat includ-

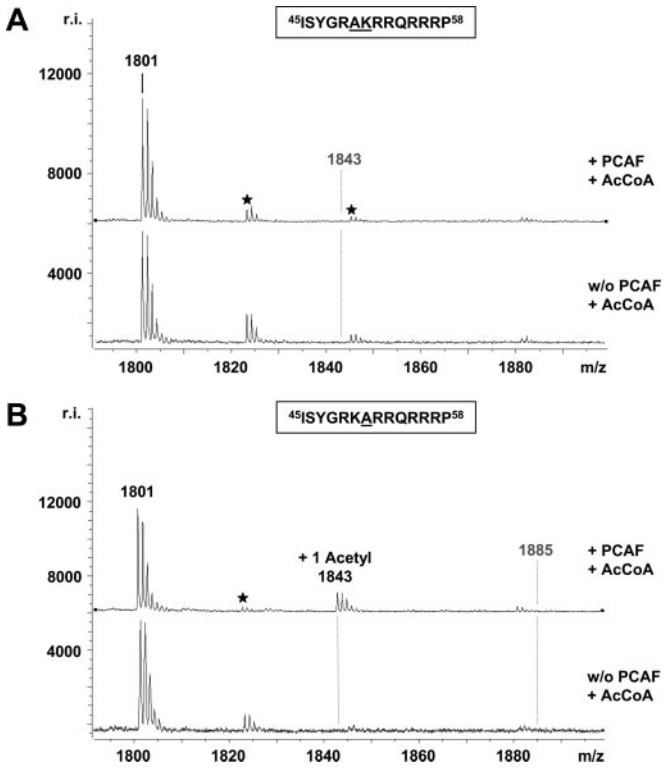

FIG. 10. **Peptide mutation analysis of HIV-1 Tat ARM peptide 45–58 after *in vitro* acetylation in the presence (*upper traces*) and absence (*lower traces*) of PCAF.** *A*, MALDI-TOF MS spectrum of peptide ^{45}ISYGRAKRRQRRRP^{58} where Lys^{50} was replaced by alanine. No ion signal at *m/z* 1843 with the acetyl characteristic mass shift of +42 Da compared with the signal of the non-acetylated peptide at *m/z* 1801 could be observed indicating that enzymatic acetylation was completely abrogated. *B*, the corresponding mass spectrum of mutant peptide ^{45}ISYGRKARRQRRRP^{58} where Lys^{51} was replaced by alanine showed an ion signal at *m/z* 1843 confirming an enzymatic acetylation of Lys^{50} by PCAF. ⋆, sodium adduct; *w/o*, without; *r.i.*, relative intensity.

ing a peptide representing the Tat CRR (^{22}CTNCYCKKCCFHCQ^{35}) (Fig. 4 and Table I). After *in vitro* acetylation with p300 the peptide showed intensive ion signals at *m/z* 1726, 1768, 1810, and 1852 in the MALDI-TOF MS analysis (Fig. 11, *upper trace*). This indicated that up to four acetyl groups were attached to the peptide, although the Tat CRR contains only two lysines at positions 28 and 29. Consequently the multiple acetylation of the peptide must involve at least two other amino acid residues than lysines. The signals of the acetylated peptides were significantly higher than the signal of the unmodified peptide at *m/z* 1684, but this does not allow conclusions about the degree of the non-enzymatic acetylation because peptides containing unmodified cysteines are poorly ionized and are barely detected in MALDI MS (54). Surprisingly the four acetylation signals were also present in the spectrum of the negative control reaction that was performed in the absence of p300 signifying that the transfer of acetyl groups from AcCoA to the peptide had occurred in a non-enzymatic manner (Fig. 11, *lower trace*). The pattern of peptide signals detected after incubation with AcCoA in the presence and absence of p300 was identical with respect to number of acetyl groups attached to the peptide as well as signal intensity. This suggests that no enzymatic acetylation of the peptide by p300 had occurred in addition to the non-enzymatic acetylation. Detailed studies of a series of synthetic Tat CRR mutant peptides confirmed that the multiple acetylation of the CRR peptide is based on the non-enzymatic acetylation of cysteine residues (50).

The knowledge of the number of potential acetyl acceptor residues in a peptide sequence is mandatory for the interpretation of acetylation experiments. To address the question of which amino acids other than cysteine can undergo non-enzymatic acetylation a synthetic peptide containing all 20 proteinogenic amino acids in an arbitrary order (^{1}LHKSMGRTWQFDYNPEACVIK^{21}) was subjected to *in vitro* acetylation in the absence of enzyme. MALDI-TOF MS analysis revealed the addition of only one acetyl group to the peptide identified by a mass shift of +42 Da from *m/z* 2523 to 2565, whereas a control peptide lacking the cysteine residue at position 18 showed no acetylation signal at all (Fig. 12). Thus, no other amino acid than cysteine is able to act as an acetyl acceptor in the absence of acetyltransferase in *in vitro* acetylation reactions.

During the analysis of p300 and PCAF acetylation sites in Tat a non-enzymatic acetylation was also observed for the peptide corresponding to the Tat core region (^{36}VCFITKALGISYGRK^{50}) (Fig. 4 and Table I). After incubation of the peptide with AcCoA alone in the absence of acetyltransferase the signal of the monoacetylated peptide at *m/z* 1698 was detected in the MALDI-TOF MS analysis together with the signal of the unmodified peptide at *m/z* 1656 (data not shown). Again the ion signal pattern of the Tat core peptide was the same after incubation with AcCoA in the presence and absence of p300 or PCAF. No further mass shift of +42 Da and no significant change of signal intensities were found after addition of acetyltransferase to the *in vitro* acetylation assay excluding an additional enzymatic acetylation of the Tat core.

For the Tat core peptide, confirmation for the non-enzymatic acetylation of cysteine was achieved by ESI MS/MS analysis. In the ESI Q-TOF MS spectrum the signals of the triply charged unmodified and of the triply charged monoacetylated peptide were observed (not shown) and subsequently subjected to peptide fragmentation analysis by ESI MS/MS. In both cases, a continuous y-ion series was detected (Fig. 13). An acetyl characteristic mass shift of +42 Da was observed for the y_{14}-ion of the monoacetylated peptide (Fig. 13*B*). The mass difference between the y_{14}- and the y_{13}-ion is 145.02 Da, characteristic for an acetylcysteine, instead of 103.01 Da, characteristic for an unmodified cysteine. Hence the non-enzymatic acetylation of a cysteine residue was directly proven by peptide fragmentation analysis.

Pitfalls in the Interpretation of in Vitro Acetylation Experiments—The interpretation of *in vitro* acetylation experiments holds some pitfalls, namely the non-enzymatic cysteine acety-

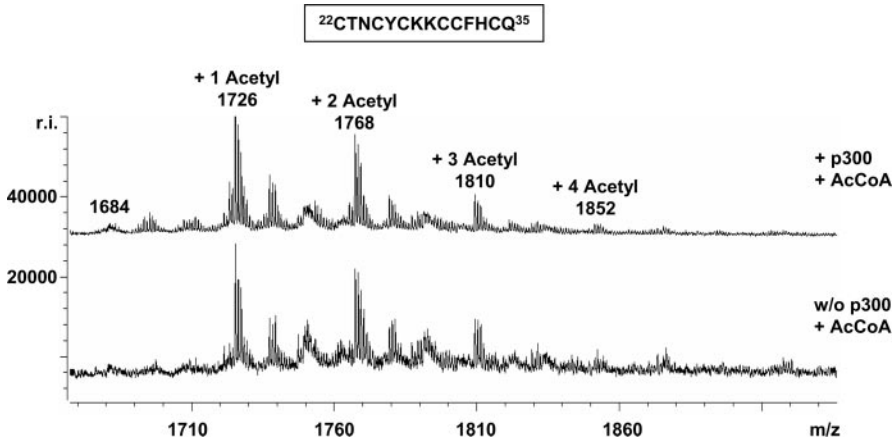

FIG. 11. **MALDI-TOF MS analysis of peptide ^{22}CTNCYCKKCCFHCQ^{35} representing the CRR of HIV-1 Tat.** In addition to the non-acetylated peptide at *m/z* 1684 ion signals are observed at *m/z* 1726, 1768, 1810, and 1852 indicating that up to four acetyl groups are attached to the peptide. Acetylation occurred independently of the presence (*upper trace*) or absence (*lower trace*) of p300. *w/o*, without; *r.i.*, relative intensity.

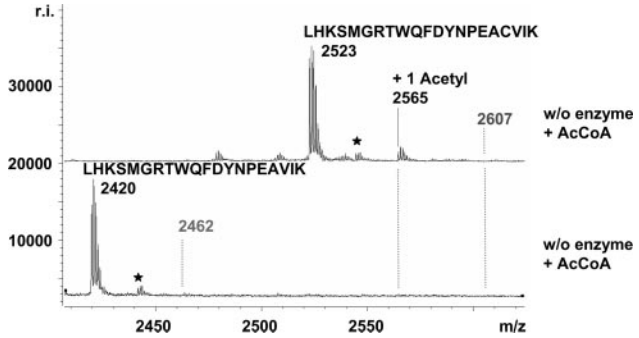

FIG. 12. **MALDI-TOF MS analysis of peptide ^{1}LHKSMGRTWQFDYNPEACVIK^{21} containing all 20 proteinogenic amino acids (*upper trace*) and the same peptide sequence but without cysteine (*lower trace*).** *In vitro* acetylation was carried out with AcCoA in the absence of enzyme. Only the cysteine-containing peptide showed the characteristic mass shift of +42 Da from *m/z* 2523 to 2565. No mass shift was observed for the peptide lacking the cysteine residue confirming cysteine as the only amino acid that can undergo non-enzymatic acetylation. ⋆, sodium adduct; *w/o*, without; *r.i.*, relative intensity.

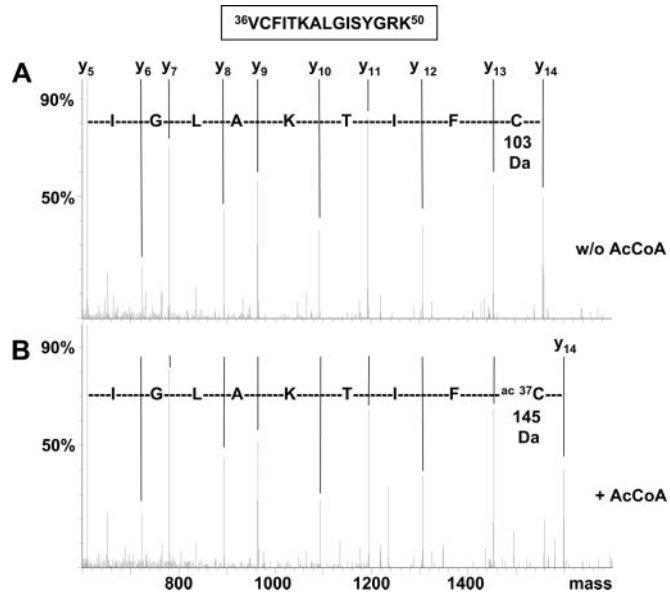

FIG. 13. **Peptide fragmentation analysis of non-acetylated and monoacetylated HIV-1 Tat core peptide (^{36}VCFITKALGISYGRK^{50}).** *A*, deconvoluted ESI Q-TOF MS/MS spectrum of the unmodified peptide. *B*, deconvoluted ESI Q-TOF MS/MS spectrum of the peptide after incubation with AcCoA in the absence of acetyltransferase. Both spectra display a continuous y_6–y_{14} series but show a mass difference of 42 Da for the y_{14}-ion indicating the non-enzymatic acetylation of Cys^{37}. *w/o*, without.

lation, the chain length of chemically synthesized peptide substrates, and the generation of artificial acetylation sites by peptide mutations. Regarding the identification of enzymatically acetylated residues in peptides or proteins the disregard of the non-enzymatic cysteine acetylation can lead to false-positive results. Often acetylation sites are mapped on the assumption that lysine is the only amino acid with acetyl acceptor function in the peptide or protein so that negative control reactions excluding a possible non-enzymatic acetylation are omitted. It is now clear that the non-enzymatic acetylation of cysteine residues generally takes place in any arbitrary peptide independent from its amino acid composition and sequence (50). The non-enzymatic cysteine acetylation must therefore be considered in every interpretation of *in vitro* acetylations of cysteine-containing peptides or proteins to prevent false-positive results. In the example of Tat, the reactivity of cysteines with AcCoA is probably the real cause for the reported enzymatic acetylation of the Tat CRR and the so called autoacetylation of the Tat core region. Disregarding the non-enzymatic cysteine acetylation it was concluded from radioactive acetylation assays that Lys^{28} in the Tat CRR is enzymatically acetylated by PCAF (44) and that Lys^{41} is autoacetylated (55). By resolving the number of attached acetyl groups to the wild-type and to various mutant peptides by MS it became evident that the acetylation of these regions was exclusively due to the non-enzymatic acetylation of the cysteine residues present in the sequence (50). Special attention must be paid when mapping an enzymatic acetylation site in a peptide containing multiple cysteines like the Tat CRR because the number of cysteine residues complicates the signal patterns in the mass spectra and consequently the interpretation of *in vitro* acetylation assays.

When model peptides are designed to study acetylation in

proteins the chain length of these synthetic substrates is another important parameter. Synthetic peptides consisting of at least 5 amino acids are suitable for MS analysis, but the minimum peptide length required for the *in vitro* acetylation reaction must be determined for every individual acetyltransferase enzyme. In the example of Tat, Lys^{50} acetylation by PCAF was only observed when an ARM peptide consisting of 14 amino acids was used in the *in vitro* acetylation assay, whereas p300 but not PCAF was able to acetylate Lys^{50} in an ARM peptide consisting of only 9 amino acids (data not shown). The fact that the acetylation of Lys^{50} by PCAF takes place exclusively in long Tat ARM peptides demonstrated that acetylation of a lysine residue by more than one acetyltransferase depends on specific but yet unknown substrate prerequisites of the peptide including its chain length. Furthermore it is known that the proteolytic removal of a protein domain may lead to the creation of new acetylation sites (56). This mechanism is taking place in the cell but may unintentionally be mimicked by the choice of short peptides for *in vitro* acetylation reactions.

In peptide mutation analyses, the design of the synthetic peptides is critical with regard to the position and the character of the introduced amino acid. Depending on the sequence of the analyzed peptide new acetylation sites can unintentionally be generated and can lead to incorrect results. For example, the acetyltransferase activity of p300 could be directed to Lys^{51} in the Tat ARM when Arg^{49} was exchanged by glycine (^{45}ISYGGKKRR^{53}) (data not shown). It is therefore important to confirm the acetylation site in mutant peptides after each amino acid exchange using one of the techniques described above.

Conclusively for the identification of acetylation sites in peptides or proteins it is recommended to choose an approach as straightforward as possible, to consider potential non-enzymatic acetylation of the peptide or protein, to vary the length of the analyzed peptides, and to reconfirm the acetylation site when amino acid exchanges are performed within the analyzed peptide. This can be achieved by peptide fragmentation analysis such as MALDI PSD MS, MALDI MS/MS, or ESI MS/MS using suitable mutation and cysteine protection in control peptides; by cleavage analysis using endoproteinase Lys-C in combination with MALDI or ESI MS; or by sequence analysis using Edman degradation.

Acknowledgments—We thank the following people for giving us a helping hand: Hans-Richard Rackwitz (Peptide Specialty Laboratories GmbH, Heidelberg, Germany) for peptide synthesis, Peter Henklein (Humboldt Universität Berlin, Berlin, Germany) for the chemical synthesis of Tat and acetylated Tat, Alexander Dörr for acetyltransferase purification, Sabine Fiedler and Bettina Helfert for MALDI MS, Tore Kempf and Hans Heid for Edman sequencing, Uwe Warnken for Q-TOF MS, Jörg Niebel (Advion Biosciences, Ithaca, NY) and Jasna Peter-Katalinic (Westfälische Wilhelms-Universität Münster, Münster, Germany) for fragmentation analysis of the Tat core peptide, Arndt Asperger (Bruker Daltonik GmbH, Leipzig, Germany) for the LIFT spectrum of the biotinylated H4 peptide, and Claudia Hetzer-Egger for helpful discussions.

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§ Recipient of a Ph.D. scholarship from the German Cancer Research Center.

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