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Alkylation of β-Tubulin on Glu 198 by a Microtubule Disrupter

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ABSTRACT

We have shown that β-tubulin was alkylated by a microtubule disrupter, *N*-4-iodophenyl-*N*′-(2-chloroethyl)urea (ICEU), on a glutamic acid residue at position 198 and not on the previously proposed reactive cysteine 239. ICEU belongs to the 4-substituted-phenyl-*N*′-(2-chloroethyl) urea class that alkylates mainly cellular proteins. Previous studies have shown that the *tert*-butyl (tBCEU) and iodo (ICEU) derivatives induce microtubule disruption because of β-tubulin alkylation. tBCEU was supposed to bind covalently to cysteine 239 of β-tubulin, but this binding site was not clearly confirmed (*Cancer Res* **60:**985–992, 2000). We have isolated and analyzed β-tubulin after two-dimensional gel electrophoresis of proteins from B16 cells incubated with ICEU. Alkylated β-tubulin had a lower apparent molecular weight and a more basic isoelectric point than the unmodified protein. Labeled *N*-4-[^{125}I]CEU was effectively bound to the modified β-tubulin but using matrix-assisted laser desorption ionization/time-of-flight mass spectrometry, we demonstrated that none of the cysteine residues of β-tubulin was linked to the alkylating agent. In contrast, peptide masses at *m/z* 4883 and 1792 in trypsin or Asp-N digestions of β-tubulin confirmed binding of iodophenylethylureido moiety to peptides [175–213] or [197–208] respectively. Fragmentation analyses by electrospray mass spectrometry using triply charged ions of peptide [175–213] identified a glutamic acid at position 198 as target for alkylation via an ester bond with ICEU. This amino acid located in the intermediate domain of the β-tubulin should play an essential role in the conformational structure necessary for the interaction between dimers in the protofilament.

Because of their major role in cell proliferation, microtubules constitute a nonspecific but efficient target for antitumor drugs. Among several compounds, two major products, colchicine and paclitaxel (Taxol), impede microtubule polymerization and depolymerization properties, respectively, by binding mainly to β-tubulin (Wilson et al., 1999). Among the class of *N*-aryl-*N*′-(2-chloroethyl)ureas (CEUs) (Mounetou et al., 2001), several molecules mimic the colchicine action and lead to cell death of treated cells by blocking the cell progression cycle at G_2/M transition (Legault et al., 2000; Petitclerc et al., 2004). In vitro studies confirmed that this cytotoxic effect is based on a cytoskeleton modification with microtubules depolymerized as a result of β-tubulin modification (Petitclerc et al., 2004). Two β-tubulin alkylating CEUs [*N*-4-iodophenyl-*N*′-(2-chloroethyl)urea (ICEU) and *N*-4-*tert*-butylphenyl-*N*′-(2-chloroethyl)urea (tBCEU)] induce a specific SDS-PAGE pattern with a faster running additional band, immunoreactive for β-tubulin. This fast-migrating protein is linked covalently to the tBCEU, as shown with the labeled drug (Legault et al., 2000). Binding of CEUs has been attributed to the reactivity of cysteine residues within the target protein, which could form a covalent link by the mean of a nucleophilic substitution with the chloroethyl moiety of the CEU. Indeed, one of the isoforms of β-tubulin (β3), which displays a serine instead of the cysteine at position 239, was not alkylated by tBCEU (Legault et al., 2000). Cysteines 239 and 354 are the best candidates for colchicine-like drug targets (Bai et al., 2000), and crystallography data of β-tubulin-colchicine-stathmin binding protein complex showed that they are buried within the colchicine binding site (Ravelli et al., 2004).

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ABBREVIATIONS: CEU, *N*-aryl-*N*′-(2-chloroethyl)urea; ICEU, *N*-4-iodophenyl-*N*′-(2-chloroethyl)urea; tBCEU, *N*-4-*tert*-butylphenyl-*N*′-(2-chloroethyl)urea; PAGE, polyacrylamide gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption ionization/time of flight; MS, mass spectrometry; MS/MS, MS^2, MS^3, tandem mass spectrometry; ESI, electrospray ionization; 2D, two-dimensional; 1D, one-dimensional; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; IPG, immobilized pH gradient; ACN, acetonitrile; TFA, trifluoroacetic acid.

In the present study, we performed comparative protein and peptide analyses by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF MS) and electrospray ionization tandem mass spectrometry (ESI-MS/MS) on ICEU alkylated β-tubulin isolated on a 2D electrophoresis gel. We first showed that among ^{125}I-CEU-labeled proteins, β-tubulin was recovered in a major spot. MS analyses demonstrated that this ICEU-β-tubulin displays a higher molecular weight although it migrated faster than the native form. In addition, this modified tubulin had a more basic isoelectric point. Analyses of β-tubulin peptides indicated that the CEU molecule was not bound to Cys239 or any other cysteine residue of the protein. ESI-MS/MS demonstrated that ICEU was linked to β-tubulin-5 within peptide [175–213] and was covalently bound to a glutamic acid at position 198.

Materials and Methods

Materials. CEUs (Fig. 1) were synthesized according to the method of Mounetou et al. (2001). ^{125}I-CEU was labeled starting from ^{125}I (GE Healthcare, Orsay, France) with a specific activity of 0.2 mCi/μmol (E. Mounetou, E. Miot-Noirault, R. C. Gaudreault, and J. C. Madelmont, submitted). Cells culture media and additives were from Invitrogen (Cergy-Pontoise, France), and fetal bovine serum was from Sigma-Aldrich (St-Quentin-Fallavier, France). 2D electrophoresis was carried out with Bio-Rad products (Marnes la Coquette, France).

Cell Culture Treatment and Protein Extracts. Mouse melanoma B16 cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum, 1 mM nonessential amino acids, 1 mM sodium pyruvate, and 4 μg/ml gentamicin. Cells were subcultured every 2 days and plated at 3 to 5 × 10^5 cells/ml 24 h before treatment. The CEUs solubilized in dimethyl sulfoxide were added to the medium at 100 μM for 24 h; corresponding controls were performed with 0.5% dimethyl sulfoxide.

Protein extracts for electrophoresis were prepared essentially as described previously (Rabilloud, 1999). The cells were harvested by scraping and were subsequently pelleted and washed two times with phosphate-buffered saline. The resulting pellet was resuspended in 1 volume of 10 mM Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose and 10 mM EDTA, and 4 volumes of solubilization buffer (8.4 M urea, 2.4 M thiourea, 50 mM dithiothreitol, and 5% CHAPS) were added with a cocktail of protease inhibitors (Roche Diagnostics, Meylan, France). Extraction was carried out for 30 min at room temperature by vigorous shaking and was followed by ultra centrifugation at 100,000 g for 30 min. Supernatants were recovered and protein concentration was measured by Coomassie blue using bovine serum albumin as standard.

One- or Two-Dimensional Electrophoresis, Immunoblotting. SDS-PAGE was performed on 10% polyacrylamide gel by loading samples of protein extracts without heat denaturation. Proteins were transferred to nitrocellulose membrane (Immobilon NC; Millipore, St-Quentin-en-Yvelines, France) and were subjected to an anti β-tubulin antibody (Anti TUB2.1; Sigma). Further incubation with a horseradish peroxidase-labeled anti mouse antibody (DakoCytomation, Trappes, France) allowed the localization of the detected proteins by chemiluminescence (GE Healthcare).

Analytical 2D gels (100 μg of protein on a 7-cm IPG strip, focused at 8000 Vhours) were prepared to determine modification of β-tubulin migration by Western blot analysis.

Preparative 2D gels were performed with 350 to 500 μg of total cellular proteins loaded on a 17-cm IPG strip nonlinear pH 3 to 10 or pH 4 to 7 in solubilization buffer containing 0.8% ampholytes, pH 3 to 10, and focused at 40,000 Vhours. IPG strips were further treated in a buffer at pH 8.8 containing 6 M Urea, 2% SDS, 30% glycerol, 50 mM Tris-HCl, with 1% dithiothreitol for the first 15 min and 4% iodoacetamide for the next 15 min. The IPG strips were then loaded onto 12.5% SDS polyacrylamide gels. After migration, gels were stained with colloidal blue. For autoradiography, gels were dried and exposed to films. Protein spots encompassing β-tubulin area were excised and stored at −20°C for mass spectrometry analysis.

MALDI-TOF MS Analysis of Intact Protein. Passive elution from polyacrylamide gels was performed according to Claverol et al. (2003). In brief, protein spots were incubated overnight at 37°C in 30 μl of 0.1 M sodium acetate and 0.1% SDS, pH 8.2. The samples were then sonicated for 15 min to facilitate elution. The eluted proteins were further cleaned by removing contaminants with a $ZipTip_{HPL}$ (Millipore). For this, the protein was loaded onto the $ZipTip_{HPL}$ in 90% acetonitrile (ACN) containing 0.1% aqueous acetic acid, and eluted in 1 μl of 50% ACN containing 0.1% aqueous acetic acid. The eluted protein was deposited directly onto the target, and the matrix solution (sinapinic acid) was further added onto it.

MALDI-TOF MS analysis was performed on a Voyager DE-PRO mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a delayed extraction MALDI source and a pulsed nitrogen laser (337 nm). Analysis of the intact molecules was performed in a positive linear mode. Calibration was performed in a close external mode using bovine serum albumin (singly charged M^+ and doubly charged M^{2+}) ions.

MALDI-TOF MS Analysis of Protein Digests. Proteins were digested in-gel with proteases. For this, the gel pieces were extensively washed with 50 mM ammonium bicarbonate in 50% aqueous ACN, and the enzyme was added to the dried gel pieces. Trypsin digestion was performed with 120 ng of trypsin (Promega, Madison, WI) per gel piece. After 18 h at 36°C, the resulting peptides were extracted with 70% ACN in 0.5% aqueous TFA and the peptide mixture was analyzed by MALDI-TOF MS using cyano-4-hydroxycinnamic acid as a matrix in a positive reflector mode. Internal calibration of samples was done using tryptic autolytic peptides (*m/z* at 842.510 and 2211.104). Identification of the protein using these mass fingerprinting data were carried out using the MS-FIT software (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm). Analyses in a positive linear mode were performed for higher sensitivity in larger peptide analysis using trypsin autolytic peptides at *m/z* 3339.85 and 5561.32 (average masses) for calibration.

For Asp-N endoprotease treatment (Roche Diagnostics), 15 ng of enzyme was added to each protein spot and incubated in ammonium bicarbonate buffer containing 10% ACN. The reaction was stopped after 3 h, and the resulting peptides were extracted with 70% ACN in 0.5% aqueous TFA. The peptide mixture was analyzed by MALDI-TOF MS using cyano-4-hydroxycinnamic acid as a matrix in positive and negative reflector modes.

Nano-ESI MS/MS Analyses of Protein Digests. Nano-ESI-MS/MS analyses of trypsin digests were carried out on an LCQ ion trap mass spectrometer equipped with a nanoelectrospray source (Thermo Electron Corporation, Waltham, MA). The nanoelectrospray capillaries (Protana, Odense, Denmark) were loaded with 3 μl of peptide mixture in 50% ACN in water containing 0.1% TFA. The

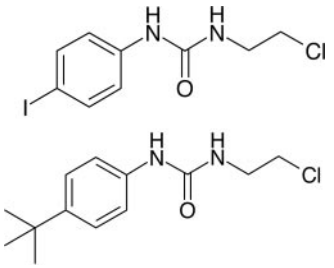

Fig. 1. Chemical structure of ICEU and tBCEU. The calculated monoisotopic masses for ICEU and tBCEU are 323.95 and 254.12, respectively for the complete molecules, and 288.98 and 219.15 without the chlorine atom.

peptides were directly analyzed by infusion, and the ionization was performed with liquid junction with a noncoated capillary probe (New Objective, Woburn, MA). Data acquisition was performed in a manual mode, and the collision-induced dissociation of selected precursor ions was performed using 30% relative collision energy. The MS/MS data from the nonalkylated peptide were searched against the mammalian database subset from the NCBI database (with the SEQUEST search engine, LCQ Deca software package; Thermo Electron). MS^2 and MS^3 data from alkylated peptide were interpreted manually, assuming the modified masses (see Fig. 7A). Fragments are assigned according to the nomenclature of Roepstorff and Fohlman (1984).

Results

Modification of β-Tubulin Migration Properties by ICEU. The modification of β-tubulin migration in SDS-PAGE by tBCEU or ICEU (Fig. 1) was previously demonstrated in several cell lines (Legault et al., 2000; Mounetou et al., 2003; Petitclerc et al., 2004). To assess whether this phenomenon was also present in B16 melanoma cells, we performed Western blot analysis using a monoclonal antibody against rat purified β-tubulin (Fig. 2A). We observed an additional lower band in proteins extracted from cells incubated for 24 h with 100 μM ICEU (Fig. 2A, lane 2) that was absent in control cells (lane 1). After 2D gel electrophoresis, controls and ICEU B16 proteins were Coomassie-stained (Fig. 2B, top images) or immunodetected using the same antibody (Fig. 2B, bottom images). An additional spot characterized by a lower apparent molecular weight and a more basic isoelectric point in ICEU treated cells was observed (right) compared with the control extracts (left). 2D electrophoresis analysis allowed discrimination of two β-tubulin spots after ICEU treatment.

To ascertain that this additional β-tubulin was linked to ICEU, B16 cells were incubated with [^{125}I]CEU in the same conditions as with the nonradioactive molecule. On 1D electrophoresis gel, we observed a major radioactive band at 50 kDa (Fig. 3A, top). This labeled band could be superimposed onto the lower band of β-tubulin as revealed by Western blotting (Fig. 3A, bottom).

The labeled proteins were analyzed in 2D electrophoresis on a 17-cm IPG strip pH 4 to 7 and detected by autoradiography (Fig. 3B, top); the spot in the β-tubulin area (Fig. 3B, bottom) was excised and further identified as β-tubulin by MALDI-TOF MS after at least two periods of radioactivity decay. This protein was identified as mouse tubulin β-5 chain (Swiss-Prot number P99024). These data clearly demonstrated that β-tubulin was alkylated by ICEU and that ICEU was still fixed to the protein after 2D electrophoresis.

β-Tubulin Alkylation Demonstrated by MALDI-TOF-MS Analysis of Intact Proteins. To verify that modified β-tubulin was not truncated as a result of alkylation by ICEU, an analysis by MALDI-TOF-MS of the intact proteins was performed after passive elution of the two corresponding spots from the 2D gel. A mass of 49990 was obtained for the upper spot, whereas a mass of 50320 was measured for the lower one (Fig. 4A), indicating that the protein was complete in both cases and that the alkylating agent was effectively bound to the protein. The difference between the two masses was in agreement with ICEU structure (Fig. 1) despite a poor resolution caused by the low recovery of a 50-kDa protein from the gel. In a tryptic digest, N- and C-terminal peptides could be clearly identified, thus confirming that β-tubulin is not truncated (data not shown). It should be noticed that the fast migrating spot (lower spot) actually had a higher mass than the slow migrating one (upper spot).

β-Tubulin Alkylation Does Not Result from Cys Modification. Upper and lower β-tubulin 2D spots were further analyzed by MALDI-TOF MS to characterize CEU binding to the protein. For this, a comparative study was performed on the tryptic digest of the two spots (Fig. 4B). In the *m/z* range from 1000 to 3000, 60% of β-5 tubulin sequence was covered, including four cysteine residues of the eight present along the molecule. This was the case especially for Cys239, which has been proposed as the main target for tBCEU (Legault et al., 2000). The corresponding peptide [217–241] was measured at *m/z* 2708.30, which corresponded to the mass of the peptide carbamidomethylated by iodoacetamide during the 2D process but not alkylated by ICEU. The same result was obtained for the peptides containing the three other cysteine

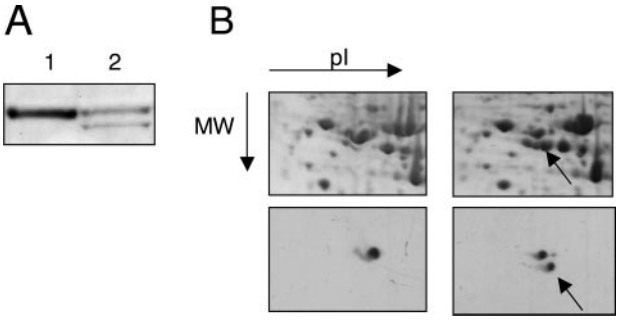

Fig. 2. ICEU treatment modifies 1D and 2D migration pattern of B16 β-tubulin. After incubation for 24 h with 100 μM ICEU, B16 proteins were extracted and loaded on a 10% SDS PAGE or submitted to 2D electrophoresis using pH 4 to 7 IPG strip and 12.5% SDS PAGE for the first and second dimension, respectively. After protein transfer on membrane and hybridization with an anti-rat tubulin antibody, 1D analysis (A) revealed two bands in ICEU treated B16 cells (lane 2), whereas one band was present in control cells (lane 1). These extracts were studied by 2D electrophoresis in a 4 to 7 pI range and gels stained with colloidal Coomassie blue (B, top images) were subsequently studied by Western blot (B, bottom images). Two spots appeared in the treated samples (right), corresponding to the upper and lower band observed in the 1D approach. The lower spot displayed a more basic pI than the upper spot. Arrows indicate ICEU β-tubulin spots.

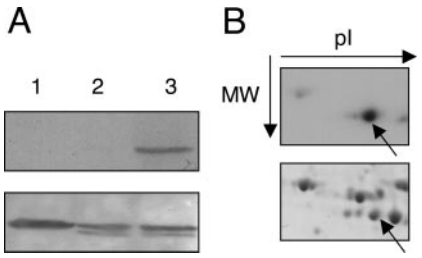

Fig. 3. [^{125}I]CEU binds to β-tubulin. B16 cells were cultured for 24 h with 100 μM [^{125}I]CEU or 100 μM unlabeled ICEU. Protein extracts (A) were analyzed by autoradiography of the gel (top) and immunoblotting (bottom). Lane 1, control cells; lane 2, 100 μM ICEU-treated cells, lane 3, 100 μM [^{125}I]CEU-treated cells. The lower additional band observed on the Western blot of treated ICEU cells (lane 3) overlaps the radioactive band in the 50 kDa area in the [^{125}I]CEU extracts (lane 3). 2D electrophoresis performed on [^{125}I]CEU extracts (B) allowed detecting one major spot in the 50 kDa range, which was identified as mouse β-5 tubulin (Swiss-Prot P99024) by MALDI-TOF MS analysis (autoradiogram and Coomassie stained gel, top and bottom, respectively). Arrows indicate ICEU β-tubulin spots.

residues (Cys354 on peptide [351–359] at *m/z* 1028.5, Cys303 on peptide [298–306] at *m/z* 1065.4, and Cys12 on peptide [3–19] at *m/z* 1822.9). As demonstrated ahead, ICEU was not removed by the different treatments of the gel; thus, it was clear that neither Cys239 nor any of these three other cysteine residues were the binding site of ICEU.

Because in this analysis only four cysteine residues of β-5 tubulin could be detected, peptide analysis was extended to *m/z* 6000 in a linear mode to determine whether a different cysteine could be the target for ICEU. The four other Cys residues were detected in two peptides in upper and lower β-tubulin spots, and each of these Cys residues was carbamidomethylated [*m/z* 3313.7 compared with 3199.6 for unmodified cysteine residues in peptide [123–154] (data not shown), and *m/z* 4596.0 compared with 4481.9 for unmodified cysteine residues in peptide [175–213]] (Fig. 5A). It was thus evident that ICEU did not bind to a cysteine residue of β-tubulin. One extra unidentified peptide at *m/z* 4883.8 was detected in the lower spot (Fig. 5A). Although the peptide at *m/z* 4596 was still present in this sample, the mass difference between these two peptides (Δm = 288) corresponded to the expected mass difference because of alkylation by ICEU (Fig. 1) and was thus a good candidate for further analysis.

***β*-Tubulin Alkylation Generates an Additional Tryptic Peptide.** Using ICEU, an extra tryptic peptide was observed exhibiting a Δm = 288 with the peptide [175–213] at *m/z* 4596 (Fig. 5A). A comparable experiment was performed with tBCEU, which had been shown to alkylate β tubulin in a similar way (Legault et al., 2000). An extra tryptic peptide at *m/z* 4814.6 was also observed, and the mass difference to peptide [175–213] was 218 (Fig. 5B), which corresponded to the expected mass difference for this tBCEU structure (Fig. 1). These data indicated that ICEU as well as tBCEU were bound to peptide [175–213].

To confirm that this extra peptide was effectively related to peptide at *m/z* 4596 and to locate the alkylation site along the peptide [175–213] more precisely, a digestion was performed using an enzyme with a different specificity. For this, unmodified or ICEU-modified β-tubulin from two 2D gel spots were digested with endoprotease Asp-N. Because of the poor ionization of peptides having no basic residue, MALDI-TOF MS analyses had to be performed in a negative reflector mode. A peptide at *m/z* 1791.53 was present in the lower spot, whereas *m/z* 1503.58, corresponding to peptide [197–208] in the upper spot, was absent from the lower one (Fig. 5C). The mass difference between these two peptides was consistent with the presence of ICEU. Thus, these data delimitated CEU alkylation site between Asp197 and Tyr208.

***β*-Tubulin Alkylation Localization.** To further localize the CEU molecule along the peptidic chain and to identify the modified amino acid, the two tryptic peptides at *m/z* 4596 and 4884 (measured at 4593 and 4881 in a monoisotopic resolution) obtained in the lower band were fragmented using a nano-ESI source. The triply charged precursor ion at *m/z* 1532 ($[M+3H]^{3+}$) from peptide 4593 ($[M+H]^{+}$) was isolated and fragmented, and b (amino-terminal) as well as y (carboxyl-terminal) ions were obtained (Fig. 6A). y_3 to y_{14}, b_5, and b_7 singly charged fragments, as well as y_{16}, y_{26} to y_{33}, b_{23}, and b_{25} doubly charged fragments obtained from peptide 4593 allowed the identification of peptide [175–213] from β-tubulin.

A comparable analysis was performed from the triply charged precursor ion at *m/z* 1628 ($[M+3H]^{3+}$) from peptide 4881 ($[M+H]^{+}$) (Fig. 6B). The fragments obtained were com-

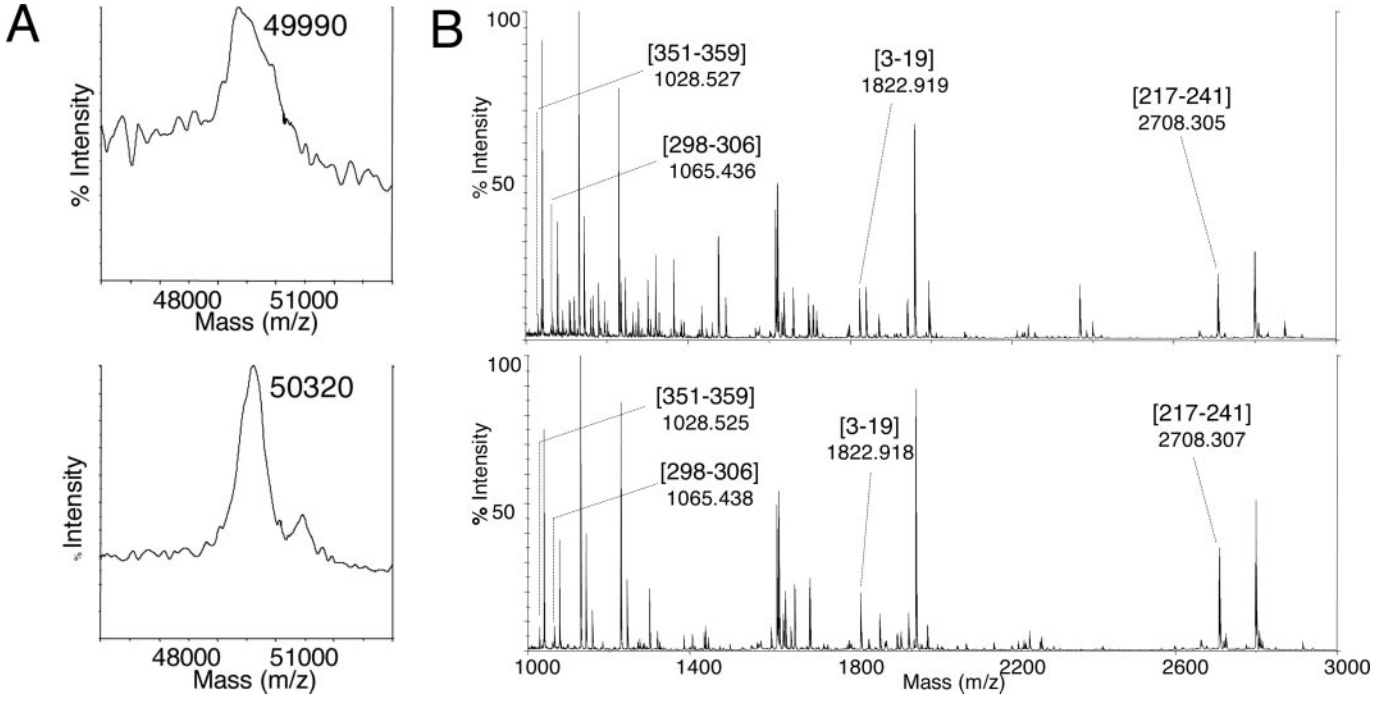

Fig. 4. ICEU modifies β-tubulin mass but is not linked to Cys 239. Proteins from the upper and lower β-tubulin spots were eluted from the 2D gel and analyzed by MALDI-TOF MS (A). The upper spot (top) had a mass of 49990 and the lower one (bottom) a mass of 50320. These masses are in agreement with the fixation of ICEU to β-tubulin. Tryptic peptides extracted from upper and lower β-tubulin spots (B, top and bottom, respectively) were analyzed by MALDI-TOF MS. Four cysteine-containing peptides were identified and are localized on the spectra. They corresponded to Cys354 from peptide [351–359], Cys303 from peptide [298–306], Cys12 from peptide [3–19], and Cys239 from peptide [217–241]. All of them were detected as carbamidomethylated, and thus could not be the binding sites for ICEU.

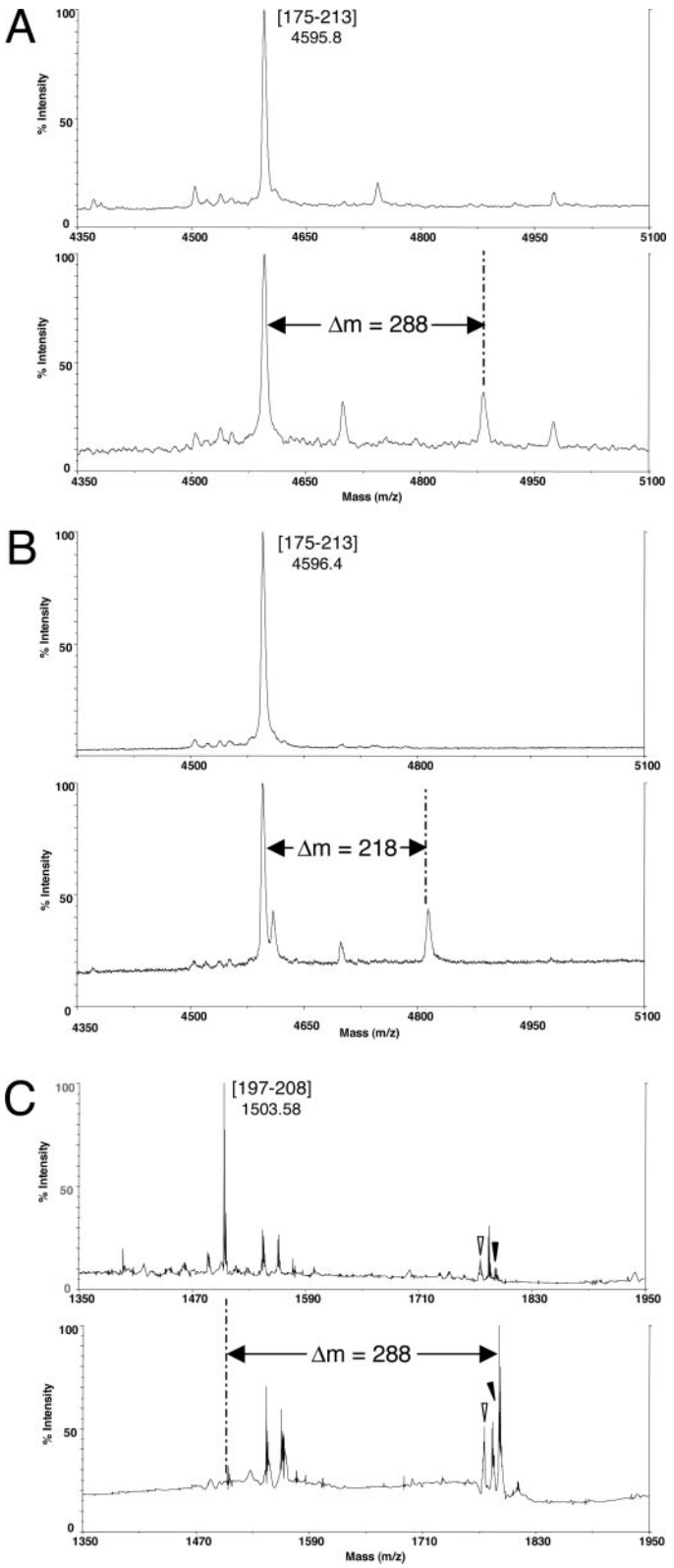

Fig. 5. β-Tubulin is alkylated within peptide [197–208]. Tryptic peptides were obtained after digestion of ICEU-treated (A) or tBCEU-treated (B) B16 cell tubulin. Peptides extracted from slow migrating (top) and fast migrating (bottom) tubulin spots were analyzed by MALDI-TOF in a positive linear mode. The part of the spectra showing differences between the two proteins are presented. Peptide at *m/z* 4595.8 to 4596.4 was identified as peptide [175–213]. An additional peptide was observed in both cases with a mass difference of 288 and 218, respectively, from the peptide [175–213]. These mass differences corresponded to expected mass differences for ICEU and tBCEU, respectively, and indicated the binding of CEU molecules on peptide [175–213]. Peptides extracted from upper and lower β-tubulin spots were digested using endoprotease AspN (C, top and bottom, respectively) and analyzed by MALDI-TOF MS in a negative reflector mode. A peptide at *m/z* 1503.58 present in the upper spot was absent in the lower one, whereas a peptide at *m/z* 1791.53 appeared in the lower spot. This mass shift corresponded to the binding of ICEU on peptide [197–208]. A minor peptide at *m/z* 1790.92 (▼) corresponding to the oxidized form of peptide [161–176] at *m/z* 1774.87 (▽) was present in the untreated protein and thus partially overlapped the modified peptide at *m/z* 1791.53.

pared with fragments from peptide 4593, and fragments y_4 to y_{14} indicated that peptide 4881 derived from peptide 4593. On the contrary, fragments with higher masses (doubly and triply charged fragments, boxed in Fig. 6B) could be assigned assuming an alkylation. Specific *m/z* observed in the two series of fragments allowed the localization of this alkylation: on the one hand the mass difference between *m/z* 1852.4 (y_{14}) and *m/z* 1186.0, attributed to ${y_{16}}^{2+}$, corresponded to the mass of Glu plus Thr plus 288; on the other hand, *m/z* 1256.6, 1465.4, and 1515.6 were assigned to the fragmentations framing an alkylated Glu (+288) and a Thr, respectively, along the peptidic chain, and were thus identified as ${b_{23}}^{2+}$, ${b_{24}}^{2+}$, and ${b_{25}}^{2+}$. Alkylation occurred thus on Glu24 of this peptide.

As important fragments were present at *m/z* 1546.2, an MS^3 experiment was performed on this triply charged precursor ion (Fig. 6C). It corresponded to a fragmentation within the ICEU molecule, at its "peptidic bond" (Fig. 7A), and contained modified fragments (Δm 42) starting from y_{16} on the one hand and from b_{23} on the other hand (Fig. 7B). It was thus confirmed from this MS^3 experiment that the iodophenylethylureido moiety of ICEU was localized on Glu24 residue of this peptide, which corresponds to Glu198 of β-tubulin.

Discussion

We have demonstrated in B16 melanoma cells that ICEU alkylates β-tubulin 5 isoform by binding covalently to a glutamic acid residue at position 198. β-tubulin 5 represents the major isoform in B16 cells (present study), whereas we identified β3 and β4 isoforms (which display Glu at position 198) in too low amounts to detect alkylated counterparts. Mouse β5-tubulin isoform is homologous to human β2 tubulin; the two of them are expressed ubiquitously.

The alkylation of Glu198 is in agreement with the more basic isoelectric point observed for tubulin after ICEU treatment (Figs. 1 and 2) because of the loss of an acidic charge; in addition, this amino acid is characterized by a pK_a value of 4, which is compatible with an esterification reaction at physiological pH. In alkali conditions, trypsin digestion at pH 8.2 for 16 h leads to partial loss of the ester linkage, giving rise to the nonmodified peptide [175–213], which was detected by MS in addition to the modified peptide (Fig. 5A). Identical alkylation of Glu198 probably occurs with tBCEU, in that the same data were observed in tryptic digests from modified tBCEU β-tubulin (Fig. 5B). This artifactual loss of alkylation, which permitted the analysis of both peptides in the same sample, was not observed in the case of a 2- to 3-h incubation with endoprotease Asp-N (Fig. 5C) and resulted from the lability of this link. The involvement of an acidic amino acid in an alkylation reaction via an ester bond has been estab-

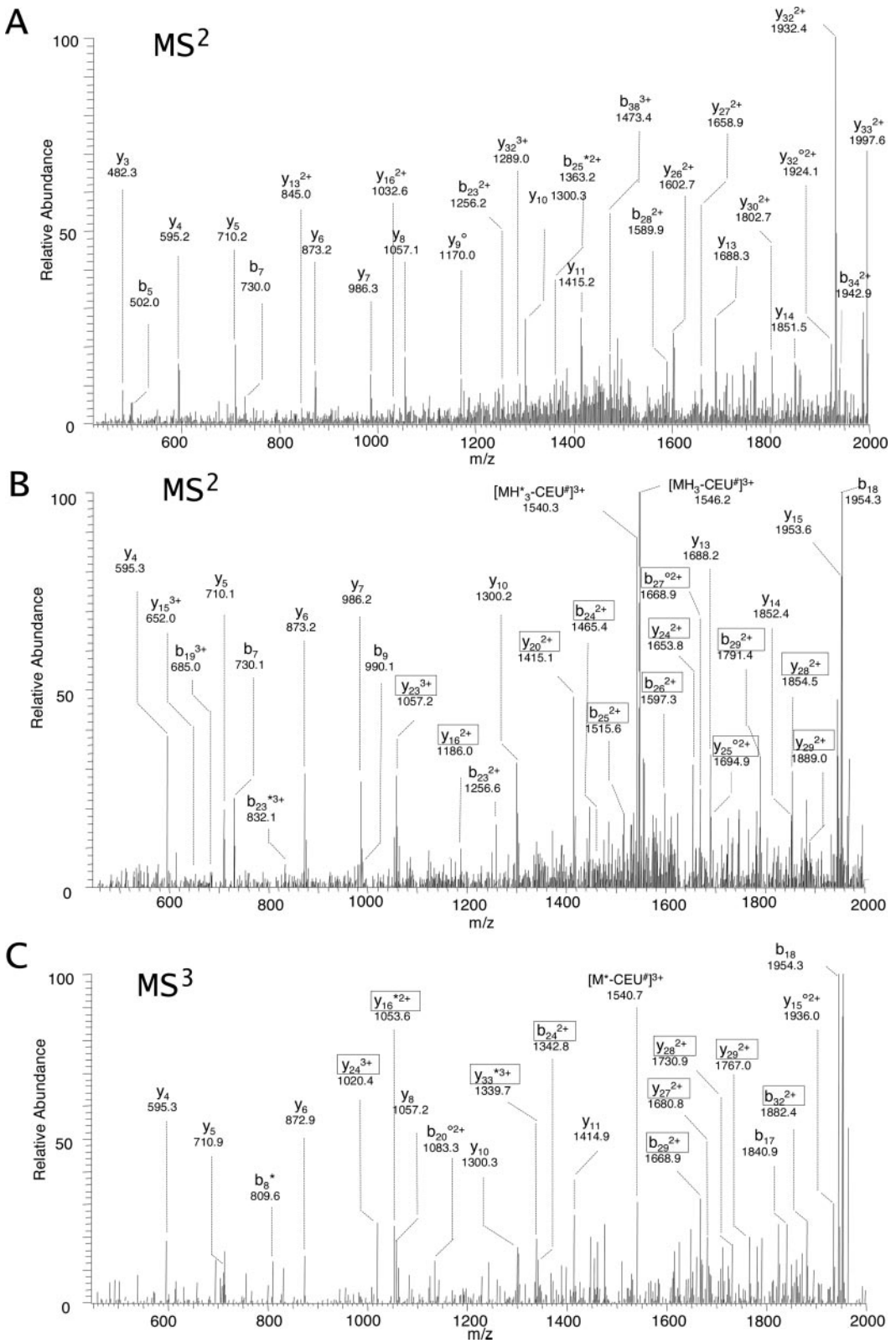

Fig. 6. ICEU binds to Glu198 β-tubulin. β-Tubulin was digested from a fast migrating spot (lower spot). Peptides at *m/z* 4593 and *m/z* 4881 were analyzed by nanoESI MS/MS. The MS^2 spectrum of the precursor ions at *m/z* 1532^{3+} (A) allowed to identify peptide [175–213] of mouse β-tubulin-5. In the MS^2 spectrum of the precursor ion at *m/z* 1628^{3+} (B), singly charged fragments allowed the identification of peptide [175–213] of β-tubulin, whereas doubly charged fragments with higher masses than b_{23} and y_{15} corresponded to modified fragments compared with the original spectrum in A. These modified fragments were assigned assuming an alkylation at Glu24 (Δm 288). They are presented in boxes. The MS^3 spectrum of a precursor ion at *m/z* 1546.2^{3+} (C) from the MS^2 spectrum (precursor ion at *m/z* 1628^{3+}) confirmed peptide [175–213] identity as well as the alkylation position. The alkylating agent had been fragmented at its amide bond (see Fig. 7), and several b and y fragments, mostly doubly charged (in boxes) exhibited a Δm value of 42 compared with the unalkylated peptide fragments in 6A. y, carboxyl-terminal ions; b, amino-terminal ions; M, nonfragmented peptides; *, loss of one water molecule, °, loss of one NH_3 molecule; CEU#, fragmented alkylated molecule (see Fig. 7).

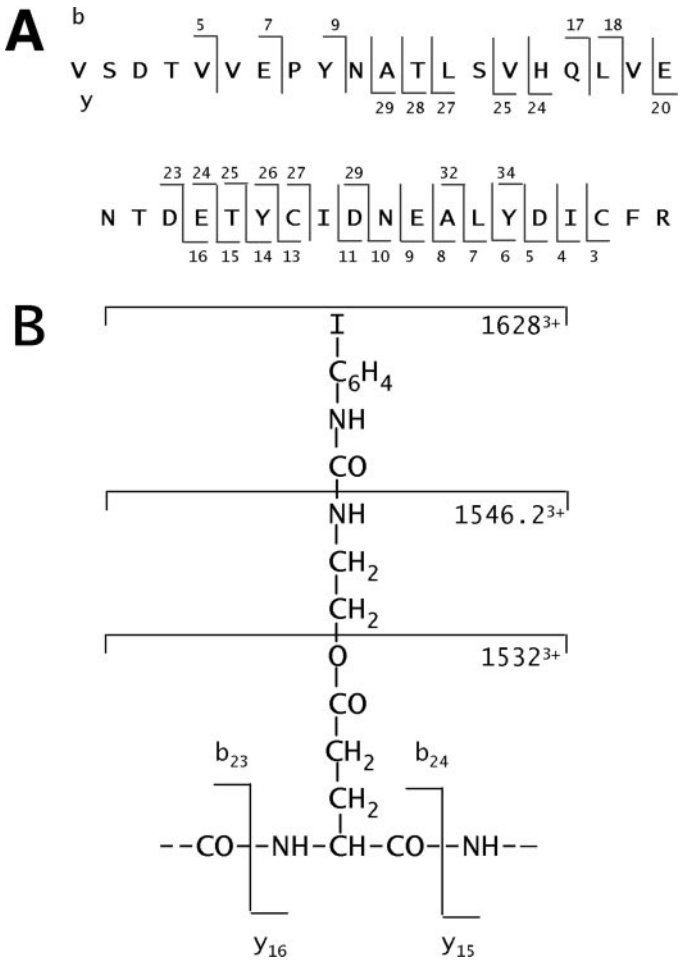

Fig. 7. Peptide [175–213] is alkylated by ICEU through an ester linkage. The sequence of peptide [175–213] possessing two cysteine residues and eight putative alkylation sites at glutamic and aspartic acid residues is presented, including most fragmentations observed in MS/MS analyses (A). The acidic moiety of Glu198 reacted with ICEU to create an ester bond (B). ICEU could be fragmented at its peptidic linkage (1546.2^{3+}) giving rise to a fragmented CEU. Because of the lability of the ester bond in alkaline conditions, this linkage could be lost during tryptic incubation, and the unmodified peptide could thus be analyzed (1532^{3+}) in the same tryptic digest.

lished for chlorambucil (a CEU parent drug) and angiotensin I-converting enzyme by an enzymatic approach (Harris and Wilson, 1982). In our study, despite the large molecular mass of the interesting peptides (*m/z* 4596 and 4884, average masses), we performed comparative nano-ESI MS/MS experiments with the triply charged ions of the native and modified peptides to determine which of the eight acidic amino acids within peptide [175–213] was alkylated. This allowed to localize the alkylated amino acid between fragments b_{23} and b_{24} of the peptide (Fig. 7A) and thus identified Glu198 as a candidate for alkylation in MS^2 experiments (precursor ion at 1628). ICEU contains an amide bond that is fragmented as easily as any peptidic bond, giving rise to a truncated ion at 1546. MS^3 fragmentation of this ion clearly confirmed that Glu198 is the target for the fixation of the ICEU molecule. Mutation of yeast β-tubulin at position 198 conferred a resistance phenotype toward a microtubule disrupter, showing that this residue is important in the interaction between this protein and antimicrotubule drugs (Richards et al., 2000). Glu198 is located in the T6-loop fragment of β-tubulin, essential to the interface between α and β subunits (Nogales et al., 1998; Lowe et al., 2001), near the buried colchicine site. This location is consistent with the competition experiment performed earlier (Legault et al., 2000). In this latter study, tBCEU could not alkylate tubulin in cells pretreated with colchicine, suggesting that the targets for both drugs were identical or located near each other within the protein scaffold. In the same experiments, β-tubulin 3 (which has a serine at position 239 instead of a cysteine residue) was not alkylated, and it was thus hypothesized that cysteine 239 was the binding site. This is consistent with the stronger reactivity of cysteine 239 and cysteine 354, compared with the other β-tubulin cysteine residues, to colchicine and its derivatives (Bai et al., 2000) and to reducing compounds (Britto et al., 2002). In fact, changing cysteine 239 to serine should modify the structural folding of the protein so strongly that an interaction with exogenous products or with protein ligands can no longer occur. Our MALDI-TOF MS analyses have shown that all cysteine residues were free in the control β-tubulin, as it had been shown previously in the rat brain α/β tubulin dimer (Britto et al., 2002) but also in ICEU β-tubulin. This clearly eliminates cysteine 239 or any other cysteine residue as a target in the β-tubulin 5 alkylation process. We presume that prebinding of colchicines or the conformational changes induced by replacing cysteine 239 with serine prohibit alkylation of glutamate 198 within the T6 loop.

The intermediate fragment containing T6 loop should be implicated in the rotation movement from straight to curved structures in protofilaments (Ravelli et al., 2004). The observed effects of microtubule depolymerization in cells treated with ICEU or tBCEU (Petitclerc et al., 2004) are in agreement with a potential role of Glu198 in lateral contacts in the microtubules. Alkylation of β-tubulin with ICEU and tBCEU lead to a faster migrating protein (Fig. 2 and 3) although the protein was complete and the alkylkation agent still present (Fig. 4). We suggest that in treated B16 cells, β-tubulin adopts a curved structure, inducing depolymerization of microtubules, but also keeps this conformation after protein extraction and in SDS-PAGE under denaturing conditions, thus allowing faster migration. The basis of this conformational change must be further elucidated.

β-Tubulin is one of the main target proteins for ICEU alkylation. Identification of the Glu198 as an alkylation site refutes previous hypothesis that reacting amino acids in tubulins would be mainly cysteine residues. Current work will be helpful to evaluate the interactions of other β-tubulin-alkylating drugs and for modeling studies based on crystallography data. Identification of ICEU binding sites in other alkylated proteins should help to elucidate whether the nature of the reacting amino acid participates more in the CEU alkylation mechanism than in the folding of the protein. This is of particular interest because ICEU is a potential drug for the treatment of colon cancer (Miot-Noirault et al., 2004).

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