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Mass spectrometric and Edman sequencing of lipocortin I isolated by two-dimensional SDS/PAGE of human melanoma lysates

(tandem mass spectrometry/protein microsequencing/acrylamide-modified cysteine/covalent modifications)

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ABSTRACT **We have integrated preparative two-dimensional polyacrylamide gel electrophoresis with high-performance tandem mass spectrometry and Edman degradation. By using this approach, we have isolated and identified, by partial sequencing, a human melanoma protein (34 kDa, pI 6.4) as lipocortin I. To our knowledge, this protein was not previously known to be associated with melanoma cells. The identity of the protein was confirmed by two-dimensional immunoblot analysis. High-energy collision-induced dissociation analysis revealed the sequence and acetylation of the N-terminal tryptic peptide and an acrylamide-modified cysteine in another tryptic peptide. Thus, knowledge concerning both the primary structure and covalent modifications of proteins isolated from two-dimensional gels can be obtained directly by this approach, which is applicable to a broad range of biological problems.**

Two-dimensional polyacrylamide gel electrophoresis (2D PAGE), coupled with microsequencing techniques and the establishment of protein data bases, has proven to be a powerful and sensitive strategy for the detection, purification, and identification of individual proteins from complex mixtures of cellular proteins. In fact, the application of these methods is viewed as both essential and complementary to efforts directed toward mapping and sequencing the entire human genome (1–3). As an analytical technique, 2D PAGE is unequaled in its ability to simultaneously resolve hundreds of proteins from cellular extracts and provide data on their isoelectric points, putative molecular weights, relative abundance, and electrophoretic pattern (4, 5). Utilizing advances in computer-based gel analysis, this laboratory (6–9) and other investigators (10–12) have created protein data bases consisting of catalogues of proteins that characterize a variety of cell and tissue types at the molecular level. Specifically, we have exploited this technology to characterize proteins whose synthesis is regulated by cytokines such as the interferons (IFNs), interleukins, and tumor necrosis factor (TNF) in several cell types, including human fibroblasts (6), ME-180 cervical carcinoma (7), and A375 melanoma cells (9). Extensive data bases exist for human amnion cells (10), rat fibroblasts (11), and *Escherichia coli* (12). The identification and sequencing of these proteins is of major importance because some may regulate cell cycle events and/or represent cytokine or hormonal effects, metastatic potential, tissue-specific functions, or unique structural elements.

Conventional methods of sequencing proteins after purification by 2D PAGE include electroblotting proteins to nitrocellulose or poly(vinylidene difluoride) membranes and N-terminal Edman degradation (1, 3, 13–15). Using this approach, we have sequenced two IFN-γ-induced proteins from human fibroblasts, Mn superoxide dismutase (13) and leucine aminopeptidase (14). Unfortunately, 80–90% of mammalian proteins are acetylated or otherwise modified at their N termini and, consequently, are resistant to Edman degradation (16). Furthermore, proteins can be covalently modified naturally or by reaction with chemical agents in the gel matrix. Internal sequences of proteins have been obtained after enzymatic or chemical digestion after 2D PAGE. The resulting peptides are separated by either reversed-phase HPLC or one-dimensional PAGE and subsequently sequenced by Edman methods (1, 3, 17–19). Although these techniques can be reliable, they do not identify post-translational and chemical modifications. Thus, it was vital to design a protocol to overcome these problems and those that result from the lack of information represented by a blank cycle in Edman degradation or a failure to obtain all peptides generated by cleavage, a problem inherent in immobilized protein digestion procedures. A complete characterization of the structure of proteins isolated by 2D PAGE is essential to an understanding of the biochemistry of a given cell type and how that cell responds to a variety of stimuli.

In recent years, liquid secondary ion mass spectrometry (LSIMS) and tandem mass spectrometry (MS/MS) using both low- and high-energy collision-induced dissociation (CID) have been applied to a variety of biological structural problems (20, 21). These range from peptide mapping to identification of post-translational modifications, such as N-terminal blocking groups (20, 22–24), phosphorylation (25, 26), and glycosylation (24, 27, 28). Recently, 74% of a nurse shark fatty acid binding protein was sequenced by high-performance MS/MS (29). High-energy CID analysis of tryptic peptides from a sialyltransferase led to the cloning of this protein (30). The use of LSIMS and MS/MS is ideally suited to the analysis of proteins isolated by 2D PAGE because of the high sensitivity and unprecedented speed at which an abundance of sequence data can be acquired. Currently, MS/MS is the only method capable of readily sequencing N-terminally blocked or covalently modified peptides and providing relevant information about the structure of such modifications. Previously, we demonstrated the utility of high-energy CID for sequencing known proteins separated by PAGE (31, 32). We now describe an approach to the purification and sequencing of unknown proteins from whole cell lysates that integrates the high resolving power of 2D gels with the sophistication of high-performance MS

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Abbreviations: 2D, two dimensional; CID, collision-induced dissociation; IFN, interferon; TNF, tumor necrosis factor; LSIMS, liquid secondary ion mass spectrometry; MS/MS, tandem mass spectrometry.

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employing electrooptical multichannel array detection. Furthermore, we demonstrate how MS/MS using high-energy CID analysis can be utilized in combination with Edman microsequencing to maximize sequence information from a given protein. Using our approach, we have identified a 34-kDa protein (pI 6.4) from human A375 melanoma cells as human lipocortin I. We not only report the tryptic peptide sequences obtained for this protein isolated by 2D PAGE but also describe its covalent modifications, an acetylated N terminus and an acrylamide-modified cysteine residue.

MATERIALS AND METHODS

Preparation of Melanoma Cell Lysates. Human A375 melanoma cells (CRL 1619, American Type Culture Collection) were grown to confluency in RPMI 1640 medium supplemented with 10% (vol/vol) fetal bovine serum and gentamycin sulfate (50 μg/ml). In indicated experiments, cells were cultured for 48 h with recombinant human TNF (1000 units/ml; specific activity, 4×10^7 units/mg), and recombinant human IFN-γ (100 units/ml; specific activity, $2–4 \times 10^7$ international units/mg) obtained from Genentech. Cells were washed three times with ice-cold phosphate-buffered saline and lysed with 3% (wt/vol) SDS/100 mM dithiothreitol/50 mM Tris·HCl, pH 8.0, and digested with DNase I and RNase A. Proteins were precipitated with acetone, resuspended in 8.5 M urea/100 mM dithiothreitol/4% (vol/vol) *n*-octyl glucoside/2.75% (vol/vol) ampholytes, pH 3–10 (optimized for 2D PAGE, Millipore), and assayed for protein concentration (Bio-Rad protein assay).

Isolation and Purification of Proteins by 2D Preparative Electrophoresis. Electrophoresis of melanoma cell lysates was performed by the method of Garrels (4), as modified by Patton *et al.* (33) and by our laboratory (8), using the Investigator 2D electrophoresis system (Millipore). Preparative gels were used to obtain purified preparations of individual melanoma proteins for sequencing. The methods are similar to those described for analytical gels with the following modifications. Whole cell lysates containing 750–1000 μg of protein were focused for 17.5 h at 1000 V and then for 30 min at 2000 V on prefocused 3-mm tube gels containing 8.5 M urea, 4% (vol/vol) acrylamide/bis solution (Millipore), 5.5% ampholytes, and 2% *n*-octyl glucoside. Tube gels were then equilibrated for 30 min and placed in alignment guides on top of 1-mm-thick 10% or 12.5% polyacrylamide/SDS slab gels (pH 8.8), containing a 3-cm stacking gel (pH 6.8) of 4% or 6% acrylamide/bis solution, and electrophoresed for 5–6 h at 16 W per gel. Sodium thioglycolate (0.1 mM) was included in the running buffer of the second dimension to scavenge free radicals or oxidants trapped in the gel matrix (34). Gels were fixed overnight in 50% (vol/vol) methanol/3% (vol/vol) H_3PO_4, washed twice in H_2O (HPLC grade) for 15 min, and stained for 24 h in 50% methanol/3% H_3PO_4/17% (vol/vol) $(NH_4)_2SO_4$/0.1% Coomassie blue G250. Gels were destained with H_2O and protein gel spots were excised with a scalpel.

Isolation and Purification of Proteins from 2D Gels. Electroelution was performed at 23°C in an apparatus designed by Hunkapiller *et al.* (34) and fitted with a Spectra-Por dialysis membrane (8-kDa cut off) washed with H_2O prior to use. Gel slices were diced in H_2O without exposure to any SDS buffer to prevent protein loss. *N*-Ethylmorpholine acetate (NEMOAc, pH 8.2) was used as the buffer component, since fewer bubbles were formed with NEMOAc and less residue was left after lyophilization than with ammonium bicarbonate buffer.

Before enzymatic digestion, an acetone precipitation using solvent system A (35) was performed to remove SDS and Coomassie blue from the protein. The blue protein solution (≈250 μl) and the membrane were removed from the recovery side of the elution cell and transferred to a microcentrifuge tube. The solution/membrane mixture was agitated periodically for 15 min at 23°C to aid removal of protein from the membrane. The membrane was removed and the entire solution was lyophilized in a SpeedVac. The protein pellet was resuspended in 400 μl of the extraction solvent with sonication, held at 4°C for 30 min, and pelleted again by centrifugation for 15 min. The blue supernatant was carefully removed with a fine-tipped Eppendorf pipette under very close scrutiny. This procedure was repeated once and followed by two rinses of the protein pellet with ice-cold acetone. The final pellet was allowed to air dry to avoid complete dehydration in a SpeedVac. Amino acid analysis was performed on 10% of the protein pellet.

Tryptic Digestion and Microbore HPLC Separation of Peptides. The protein pellet was dissolved in 10 μl of 0.4 M Tris·HCl (pH 8.0) containing 8 M urea and 0.4 mM $CaCl_2$, followed by sonication, vortex mixing, and heating in a 37°C water bath for 30 min. The solution was diluted to 40 μl with H_2O. L-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (United States Biochemical) was added at an enzyme/substrate ratio of 10:1 (wt/wt), followed by incubation at 37°C for 16 h. The peptides were chromatographed immediately or stored at 4°C for no more than 24 h.

The peptides were separated on a microbore C_{18} column (1.0 mm i.d. × 100 mm; 7 μm, particle size; 300 Å, pore size) equilibrated with 0.1% trifluoroacetic acid in H_2O. The column was eluted isocratically for 10 min followed by a linear gradient (0.5%/min) to a final mobile-phase composition of 70% (vol/vol) acetonitrile/30% H_2O/0.1% trifluoroacetic acid at a flow rate of 50 μl/min by using an Applied Biosystems 140A dual syringe pump equipped with a Rheodyne model 8125 injector. Peptide elution was monitored at 215 nm using a Kratos 783A variable wavelength detector fitted with an LC Packings U-Z View flow cell. Fractions were manually collected in 0.5-ml polypropylene tubes and concentrated to ≈5 μl on a SpeedVac.

Mass Spectrometric Sequence Determination. The molecular weight of all components of each HPLC fraction was determined by LSIMS using a VG Analytical 70SE double focusing mass spectrometer, operating at a mass resolution of 2500 in the positive ion mode, equipped with a high field magnet and a cesium ion gun. A Cs^+ primary ion beam of 8 keV was used with an 8 keV secondary ion accelerating voltage. A scan rate of 10 sec per scan was utilized and data were analyzed using a VAX station 3100 with an OPUS data system. One-fifth to one-third of each HPLC fraction was dissolved in a matrix of glycerol/thioglycerol, 1:1 (vol/vol), with 1% trifluoroacetic acid before spectra were acquired. High-energy CID mass spectra were obtained for tryptic peptides with a Kratos Analytical Instruments Concept IIHH four-sector high-performance tandem mass spectrometer equipped with a 4% electrooptical multichannel array detection system (36).

Edman Degradation. In some instances, where particular HPLC fractions did not yield molecular ion data, sequence information was obtained using an Applied Biosystems model 470A gas-phase protein sequencer with an Applied Biosystems 120A phenylthiohydantoin analyzer (15).

Immunoblot Analysis. The identity of lipocortin I was confirmed on immunoblots of 2D gels as follows: Protein lysates (200 μg) containing 5×10^5 cpm of ^{14}C-labeled lysate (8) were electrophoresed as above and gels were blotted to poly(vinylidene difluoride) (Immobilon-P, Millipore) at 400 $\mu A/cm^2$ for 60 min in a modified Towbin (37) transfer buffer. Immunoblots were prepared by the method of Lopez *et al.* (38) using a monoclonal antibody to lipocortin I (39) obtained from B. Pepinsky (Biogen) and AuroProbe BLplus (Amersham). Positive spots on immunoblots were matched to autoradiograms and stained gels and quantitated using a

Computing Densitometer and IMAGEQUANT software (Molecular Dynamics, Mountain View, CA).

RESULTS AND DISCUSSION

A typical 2D preparative gel separation of human A375 melanoma proteins is illustrated in Fig. 1. A protein having the molecular mass and isoelectric point of 34 kDa and 6.4, respectively, was isolated from 6 to 18 gels and partially sequenced using both mass spectrometric and Edman protocols on three occasions. We identified it as lipocortin I by matching experimentally determined tryptic peptide sequences to those of proteins catalogued in the Protein Identification Resource data base (40). The sequence corresponds to that of Wallner *et al.* (41). Four of our sequences were determined by MS/MS and five sequences were determined by Edman degradation. Fig. 2 depicts the amino acid sequence of human lipocortin I derived by cDNA sequence analysis (41). The peptides that we sequenced by high-energy CID are underlined with a bold bar and the peptides sequenced by Edman degradation are underlined twice. Four additional HPLC fractions contained components whose molecular ion masses matched the predicted monoisotopic molecular ion (MH^+) masses corresponding to other protonated lipocortin I tryptic peptides (underlined once). These latter peptides were recovered at levels too low to obtain sequence data by MS/MS or by Edman chemistry. Several HPLC fractions contained more than one peptide. MS/MS enabled us to select a single peptide from the entire fraction for sequencing without further purification, thus providing a distinct advantage over Edman degradation. The totality of all our LSIMS data and sequences obtained experimentally by either MS/MS or Edman methods account for 43% of the lipocortin I sequence.

Sufficient amino acid sequence to correctly identify the protein as lipocortin I was obtained from as little as 62 pmol (2 μg) of protein, as determined by amino acid analysis. Protein yields did not always correlate with the number of gel plugs excised, indicating that losses may have occurred during purification. However, enough lipocortin I for sequencing was isolated from as few as six preparative gels.

MS/MS was crucial in sequencing and determining the nature of the covalent modification of two tryptic peptides. The experimentally determined CID spectrum of the lipocortin I N-terminal tryptic peptide, amino acid residues 1–8, MH^+ = 966.6 Da is shown in Fig. 3. The sequence of this peptide was established by a complete C-terminal *y*-ion series[‖] as well as a nearly complete N-terminal *b*-ion series. This allowed us to take advantage of another unique feature of mass spectrometric sequencing. Once the C-terminal sequence had been established, it was verified by identifying additional masses representative of peptide backbone cleavages originating from the N terminus. Also, all of the N-terminal fragment ions found for this peptide are 42 Da greater than that predicted. This mass difference corresponds to the incremental mass of an acetyl moiety and thus establishes its presence on the N-terminal alanine of lipocortin I. Currently, MS/MS is the only method available capable of readily sequencing peptides that are acetylated at their N terminus (20, 22–24, 48). In fact, human placental and *E. coli*-expressed human lipocortin I have been shown (48) to be N-terminally acetylated by this technique.

The importance of employing high-performance MS/MS in this investigation is clearly illustrated by analysis of one of the HPLC fractions that contained a component that gave a MH^+ = 818.5 Da that did not correspond to the mass of any

[‖]Throughout the text the nomenclature first proposed by Roepstroff and Fohlman (42) and modified by Biemann (43) is used for peptide fragment ions.

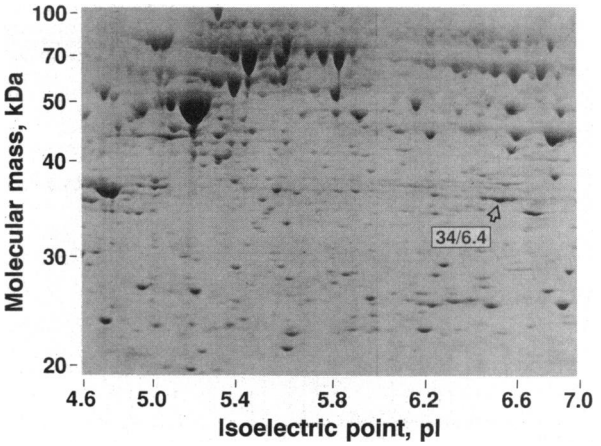

FIG. 1. Coomassie blue G250-stained 2D preparative gel of human A375 melanoma proteins. The 34-kDa protein (pI 6.4) that was identified as lipocortin I by mass spectrometric and Edman sequencing is designated by the arrow (34/6.4).

predicted lipocortin I tryptic peptide. However, interpretation of the CID spectrum of this component (Fig. 4) revealed that it was the tryptic peptide CLTAIVK, residues 262–268 of lipocortin I, in which the cysteine had been covalently modified by an acrylamide moiety of incremental mass 72 Da. The sequence of this peptide was clearly established by the presence of a complete C-terminal *y*-ion series as well as an almost complete N-terminal *b*-ion series. Isoleucine rather than leucine is established as the fifth residue by the appropriate *v* and *w* ions present at masses 485.2 and 498.3 Da, respectively. Ions produced by side-chain fragmentation present at masses 774.4, 746.5, 713.4, and 700.5 Da proved that the modification is an acrylamide moiety bound to the cysteine sulfur atom rather than an N-terminal alanine residue. Acrylamide and an N-terminal alanine have identical atomic compositions and molecular weights. To our knowledge, this acrylamide-modified amino acid residue provides the first direct proof that proteins can be covalently modified during electrophoresis. It is essential that this type of covalent modification be considered when interpreting CID data of proteins isolated from polyacrylamide gels.

LSIMS analysis of several HPLC fractions exhibiting substantial absorbance peaks did not produce molecular ions, possibly because of the presence of unanticipated impurities. During our initial attempts at sequencing lipocortin I, LSIMS analysis showed a nonpeptide component that gave a mass spectrum similar to a polyethylene glycol mixture. Subsequent analysis showed that the most probable source was residual Nonidet P-40, a detergent mixture originally used in the preparation of melanoma cell lysates. Once Nonidet P-40 was replaced with β-octyl glucoside, subsequent preparations were free of this contaminant. Moreover, HPLC fractions containing the five tryptic peptides of lipocortin I that

AMVSEFLKQAWFIENEEQEYVQTVKSSKGGPGSAVSPYPTFNPSSDVAALHKAIMVK
GVDEATIIDILTKRNNAQRQQIKAAYLGETGKPLDETLKKALTGHLEEVVLALLKTPAQFDADELR
AAMKGLGTDEDTLIEILASRTNKEIRDINRVYREELKRDLAKDITSDTSGDFRNALLSLAKGDR
SEDFGVNEDLADSDARALYEAGERRKGTDVNVFNTILTTRSYPQLRRVFQKYTKYSKHDMNKVL
DLELKGDIEKCLTAIVKCATSKPAFFAEKLHQAMKGVGTRHK ALIRIMVSRSEIDMNDIKAFYQKM
YGISLCQAILDETKGDYEKILVALCGGN

FIG. 2. Lipocortin I amino acid sequence. Heavy underlines, sequences identified by high-energy CID; single underlines, peptides whose theoretical MH^+ values correspond to experimentally determined masses of tryptic digest components for which no sequence information was obtained; double underlines, sequences identified by Edman degradation.

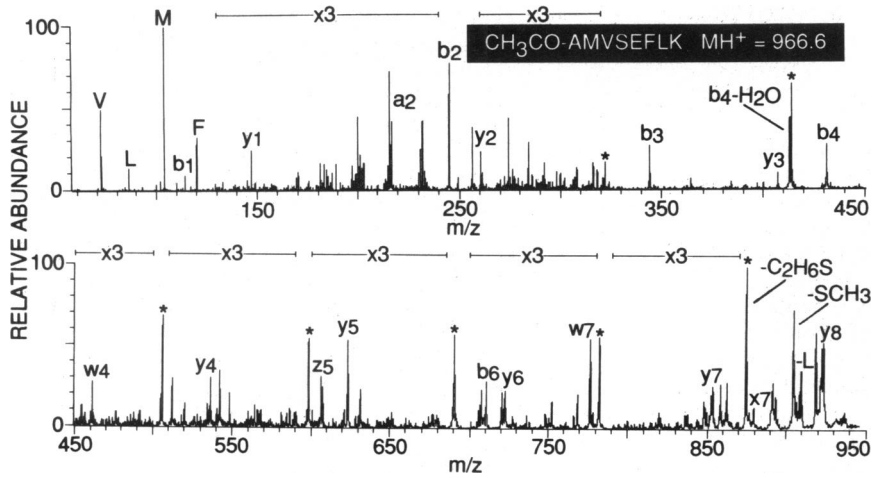

FIG. 3. High-energy CID spectrum of lipocortin I N-terminal tryptic peptide, MH^+ = 966.6 Da. Immonium ions corresponding to individual amino acid residues are denoted by the single-letter code. Peptide backbone cleavage ions formed from fragmentation indicative of charge retention at the C terminus are denoted by x, y, and z and at the N terminus are denoted by a and b. Ions formed by side-chain fragmentation are denoted as v and w. *, Glycerol cluster ions. CID spectrum was obtained from ≈64 pmol of peptide.

were sequenced by the Edman degradation gave no molecular weight information by LSIMS. It is possible that more hydrophobic impurities present in the fractions resulted in a suppression of peptide ionization due to their greater surface activity (44). Thus, data obtained by Edman degradation complemented, but did not duplicate, sequence data obtained by mass spectrometry.

One potential problem in microsequencing peptides of a protein containing several cysteines is that they usually have formed disulfide bridges and subsequently limit enzymatic digestion or result in two peptides that are disulfide-linked. Although lipocortin I contains four cysteine residues that presumably can form disulfide bridges (41), if present, they did not interfere with our ability to digest the protein with trypsin nor with our ability to identify the protein using MS/MS or Edman degradation.

The identity of the protein we sequenced as lipocortin I was also confirmed by 2D immunoblot analysis. Fig. 5*A* shows the autoradiogram of the 2D A375 melanoma protein blot that contains lipocortin I. The arrow points to the larger spot that has the molecular mass and pI coordinates that match those of the protein that we excised from preparative gels for sequencing. The same area is depicted in Fig. 5*B* after the blot was probed with a monoclonal antibody specific to human lipocortin I. The result clearly demonstrates that the protein we selected for sequencing is lipocortin I. Another spot of lesser intensity also reacted with the monoclonal antibody and probably represents another form of lipocortin I. Lipocortin I contains potential phosphorylation sites (41) and the molecule becomes dephosphorylated prior to binding calcium and phospholipids and blocking phospholipase A_2 activity (for review, see ref. 45).

Lipocortin I is an important regulator of inflammation (45). Therefore, we determined whether various cytokines that mediate inflammation could affect lipocortin I production. Quantitative densitometric analysis of autoradiograms and immunoblot analysis revealed that TNF and IFN-γ have no apparent effect on lipocortin I synthesis in A375 melanoma cells *in vitro* (data not shown). Similar results were found by Beyaert *et al.* (46) in several other cell types treated with TNF. The constitutive presence of lipocortin I in melanoma cells is of particular importance, as it is known to block phospholipase A_2 activity (45), a key molecule in the signal pathway of TNF (47).

In summary, by integrating preparative 2D PAGE with Edman degradation and MS/MS, we have isolated and identified lipocortin I, to our knowledge, a protein previously not known to be associated with melanoma cells. Specific covalent modifications, an acetylated N terminus and an acrylamide-modified cysteine residue, were determined by MS/MS. The combination of these technologies maximizes the amount of information pertaining to the sequence and primary structural modifications of a given protein. This combined approach could be applied to the creation of a multitude of protein data bases and should be of considerable utility in complementing data obtained from the Human Genome Project, as partial sequences of unknown proteins can be used to generate specific oligonucleotides that can then be used to isolate and clone unidentified genes from cDNA libraries. Also, it could be applied to a broad range of

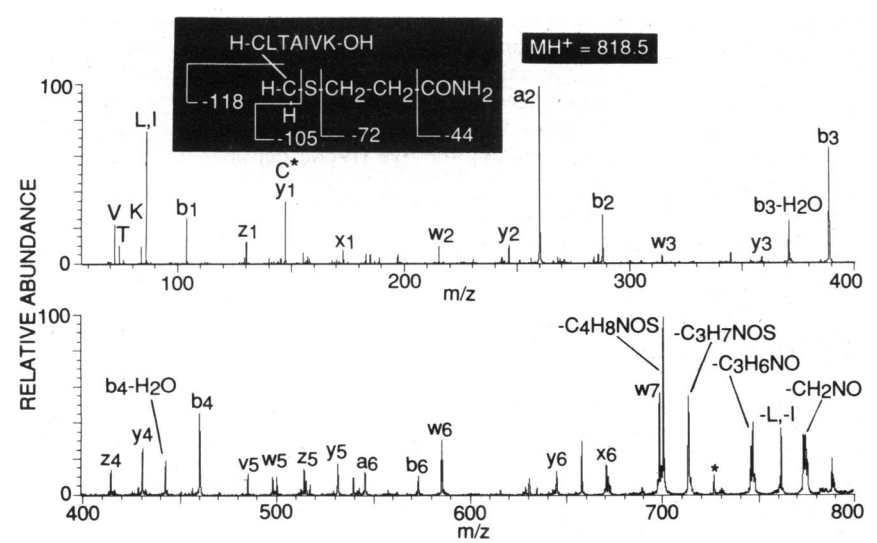

FIG. 4. High-energy CID spectrum of lipocortin I tryptic peptide, containing an acrylamide-modified cysteine, MH^+ = 818.5 Da. Immonium, C- and N-terminal, and glycerol cluster ions are labeled as in Fig. 3. Peaks observed at masses 774.4, 746.5, 713.4, and 700.5 Da correspond to ions formed by side-chain fragmentation from the acrylamide-modified cysteine (C*). CID spectrum was obtained from ≈64 pmol of peptide.

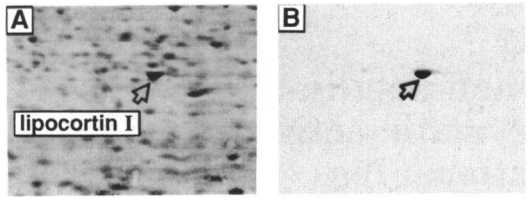

FIG. 5. Identification of A375 melanoma proteins as lipocortin I by 2D immunoblot analysis. (*A*) Autoradiogram of a 2D gel blotted to a poly(vinylidene difluoride) membrane showing the position of the sequenced protein. (*B*) Immunoblot of the same 2D gel probed with a monoclonal antibody to lipocortin I and stained with IgG conjugated to immunogold.

biological problems to determine how various agents (i.e., cytokines, hormones, or chemotherapeutic agents) affect a wide variety of either normal or malignant cells.

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