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FAST TRACK

Proteomic Analyses of Arsenic-Induced Cell Transformation With SELDI-TOF ProteinChip® Technology

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Abstract In this study, we demonstrated that low levels (1.5 μM) of arsenite induces B[a]P-treated lung cell transformation. We then used a proteomic approach to identify protein expression by ProteinChips, which could potentially be important for transformation induced by this toxic metal. Most of the protein peaks in cell extracts of all samples, including the control, B[a]P-treated, and B[a]P + As-treated cells are identical. However, surface-enhanced laser desorption/ionization time of flight (SELDI-TOF) analysis with Cu-ProteinChips and WCX-ProteinChips revealed several dramatically different protein peaks that appeared in lung cells after being transformed by a treatment of 1.5 μM arsenite for 12 weeks. SAX2 ProteinChip also identified a prominent protein peak that was preferentially expressed in control cells. Interestingly, by using a SAX2 chip, we were able to detect several protein peaks that increased their expression in lung epithelial cells (LEC) treated with only B[a]P. Identification and characterization of these proteins may reveal the molecular basis of As-induced cell transformation and provide insight into the mechanisms by which arsenic induces carcinogenesis. J. Cell. Biochem. 88: 1–8, 2003.

Key words: arsenic; benzyo[a]pyrene; cell transformation; lung cells; proteinchip; proteomic; SELDI-TOF

Heavy metals represent major environmental hazards to human health. In particular, arsenics are ubiquitously distributed in nature [IARC, 1980; Farmer and Johnson, 1990]. Recent epidemiological studies and clinical observations have associated arsenics with an increased risk of human cancer, including urinary bladder, liver, kidney, skin, lung, nasal cavity, prostate, and other internal sites [Chen et al., 1985; EPA, 1988; Chen and Wang, 1990; Farmer and Johnson, 1990]. Although numerous epidemiological studies have shown that inorganic arsenicals are human lung carcinogens, there is currently no accepted mechanism for arsenic-induced lung carcinogenesis in an established cultured cell and animal model. In this report, we demonstrated sodium arsenite induced rat lung epithelial cell transformation. To gain insight into the biological effects of arsenic and to identify gene activities relevant to its cell transformation, we have analyzed the proteomic response to this toxic metal.

Significant technological advances in protein chemistry in the last decade have established mass spectrometry as an indispensable tool for protein study [Carr et al., 1991; Carr and Annan, 1998; Patterson, 1998]. Although the

Abbreviations: As, arsenic; B[a]P, benzo[a]pyrene; DMSO, dimethyl sulfoxide; LEC, rat lung epithelial cells; SELDI-TOF-MS, surface-enhanced laser desorption/ionization time of flight mass spectrometry.

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resolving power of 2D gels remains unchallenged, the high sensitivity, speed, and reproducibility of mass spectrometry have boosted its application in all aspects of protein analysis, including discovery, identification (i.e., peptide mapping, sequencing), and structural characterization. Analogous to the DNA chip technologies that allow the study of gene expression profiles, Ciphergen Biosystems, Inc. (Fremont, CA) has recently developed the ProteinChip technology coupled with SELDI-TOF-MS (surface-enhanced laser desorption/ionization time of flight mass spectrometry) to facilitate protein profiling of complex biological mixtures [Hutchens and Yip, 1993; Fung et al., 2001]. This technology utilizes patented ProteinChip arrays to capture individual proteins from complex mixtures that are subsequently resolved by mass spectrometry. The efficacy of the SELDI technology for discovery of prostate cancer protein markers in serum, seminal plasma, and cell extracts has early been demonstrated [Wright et al., 1999; Paweletz et al., 2000]. This report describes our initial evaluation using the ProteinChip SELDI-TOF-MS system to detect potential alteration of protein expression in rat lung epithelial cells (LEC) during As-induced transformation.

METHODS AND EXPERIMENTAL PROCEDURES

Cell Culture and Arsenic Exposure

An adult rat LEC, that morphologically resembles type II pneumocytes was derived originally from the lungs of an adult Fisher 344 rat [Li et al., 1983] as described [Shukla et al., 2000]. LEC cells exhibit contact inhibition during growth, are unable to grow in soft agar, and do not form tumors when injected subcutaneously into syngeneic hosts. LEC cells were routinely grown in culture at 37°C in an atmosphere of 5% CO_2/95% air in F-12 medium, containing 10% Newborn bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin.

Cell Transformation in Vitro

LEC cells were seeded at a density of 3×10^5 cells/100 mm plate. Twenty four hours after seeding, cells were exposed to 100 nM of benzo[a]pyrene (B[a]P, Sigma) in the presence or absence of 1.5 μM arsenite for another 24 h; cells incubated with 0.25% dimethyl sulfoxide (DMSO) were served as a negative control. After exposure to B[a]P, cultures were continuously treated with or without arsenic for 12–16 weeks. At the end of experiments, cell cultures were harvested. The tumorigenecity of these cells was determined by soft agar method.

Anchorage-Independent Transformation

Arsenite or/and B[a]P-treated LEC cells were harvested by trypsinization. Cells (4×10^5) were suspended in 2.0 ml of 0.3% agar containing medium plus serum and placed over 10 ml of 0.6% agar with medium and serum. The cultures were maintained in a 37°C incubator for 2–3 weeks, and the anchorage-independent colonies greater than eight cells were scored by a computerized image analyzer.

ProteinChip SELDI Analyses of Cellular Extracts

Protein extracts were prepared from LEC cells of control (treated with 0.25% DMSO), B[a]P-treated, and B[a]P-treated plus arsenic respectively, by resuspending the cells in 20 mmol/L Hepes containing 0.1% Nonidet P-40, vortexing for 5 min, and then centrifugation at 14,000 rpm for 1 min. One μl of cell extract diluted into 4 μl of starting buffer was spotted onto each of following ProteinChip arrays (Ciphergen Biosystems) with variety of chemical surfaces: IMAC-Cu (chelated copper ion), WCX2 (weak cation exchanger: Carboxylate) or SAX2 (strong anion exchanger: Quaternary ammonium). The chips were then incubated at room temperature for 20 min on a shaker. Based on different chemicals coated on the chip, various proteins were captured and retained. Nonbound proteins and other contaminants were washed from the WCX and SAX2 ProteinChip arrays with 20 mmol/L Tris, pH 7.5, 0.1% Triton X-100, for three times, and from the IMAC-Cu ProteinChip arrays with 20 mmol/L Tris, pH 7.5, 0.1% Triton X-100, 0.5 mol/L NaCl for three times. Finally all chips were washed with HPLC-deionized water for three times to remove interfering substances such as salts and detergents. After drying, 0.5 μl of saturated energy absorbing molecule solution [α-cyano-4-hydroxycinnamic acid in 50% acetonitrile (v/v), 0.5% trifluoroacetic acid (v/v)] was added two

times and allowed to air-dry. Mass spectrometry analysis was performed by time-of-flight mass spectrometry in a PBS-II mass reader (Ciphergen Biosystems). Spectra were collected using an average 80 nitrogen laser shots. Spectrum analysis was performed using the ProteinChip software version 2.1b (Ciphergen Biosystems).

RESULTS AND DISCUSSION

We analyzed the proteomic response of arsenic action to identify gene activities, which could be potentially important for transformation induced by this toxic metal. Since arsenic is not a mutagen and has been implicated as a tumor promoter, we used B[a]P as a tumor initiator to mutate cellular DNA. B[a]P, one of the most potent pro-carcinogens, may cause tumor formation in several organs in a multistep process [Doll and Peto, 1978]. The viability of the LEC cells after 24 h exposure to the various concentrations (25, 50, 100, 200, and 500 nm) of B[a]P, as determined by the tryptan blue dye-exclusion method, showed only a slight decrease (3%) and the mitochondrial activity of the cells as determined in the MTT test was not significantly changed in the cells treated with 100 nm B[a]P (data not shown).

Next we investigated whether arsenic promotes cell transformation in cultures. LEC cells were treated with 100 nm B[a]P for 24 h and then divided into two groups. Each treatment contains three plates. The first group of culture continued to grow under a regular culture medium. The second group of cultures grew in a regular medium, plus 1.5 μM sodium arsenic for 12 weeks. Cells were then plated in soft agar to see the effect of arsenite on cell transformation by determining anchorage independent growth. Figure 1A shows the colonies on soft agar formed by cells treated with 100 nm B[a]P for 24 h and 1.5 μM arsenite for 12 weeks. Figure 1B is a summary of data on the number of colonies formed by LEC cells treated with B[a]P or/and arsenic.

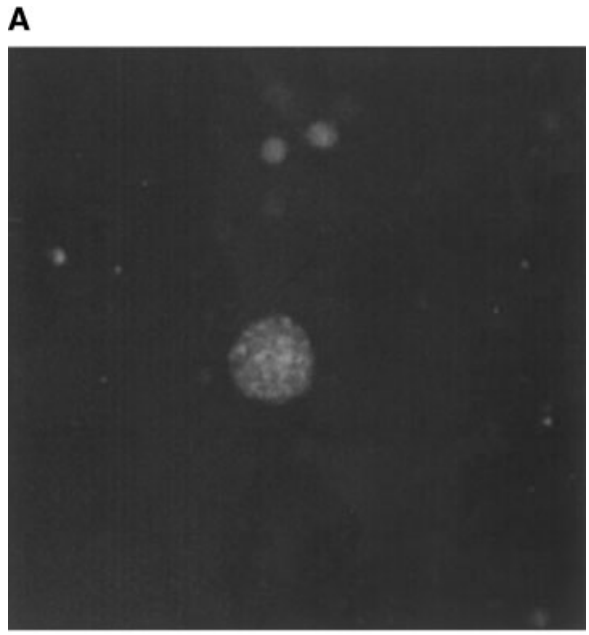

Fig. 1. Arsenite induces LEC cell transformation (**A**) Microscopic view of cell colonies on soft agar. Original magnification: × 4. **B**: Colony formation of LEC cells after treatment of B[a]P for 24 h or/and 1.5 μM of sodium arsenite for 12 weeks. Cells were then grown on soft agar for 20 days.

One μl of protein extracts from LEC cells of control (treated with 0.25% DMSO), B[a]P-treated, and B[a]P-treated plus arsenic (As) respectively, was added directly onto a spot of a chemically derivatized coated aluminum ProteinChip array (Ciphergen Biosystems) and processed as described in the Methods. After insertion of the ProteinChip array into the ProteinChip reader, a laser beam was applied to ionize and desorb proteins from ProteinChip array surface. The ionized proteins were detected and their molecular masses were

determined by TOF analysis using ProteinChip Reader. Figure 2 is a representative protein spectra of LEC cell extracts that were assayed in Cu-affinity ProteinChip and analyzed by SELDI mass spectrometry. Most of protein peaks in cell extracts of control, B[a]P-treated, and B[a]P + As-treated cells are identical. However, SELDI-TOF analysis revealed two dramatic different protein peaks appeared in LEC cells after transformed by long-term treatment with low level of arsenic (B[a]P + As). One of these transformation-associated protein peaks has molecular mass of 4,099.3 Da (Fig. 2A), and the other has molecular mass of 8,175.5 Da (Fig. 2C). In addition to the dramatic changes in these two prominent protein peaks, we also identified three protein peaks around at 4,424.9; 4,590; and 8,990 Da that increased in LEC cells treated with both B[a]P or B[a]P plus arsenic (Fig. 2B and C).

A WCX chip that consists of a weak cationic exchanger was used for second sets of SELDI analysis of cellular proteins in LEC cells (Fig. 3). Most of proteins bound to this chip are also

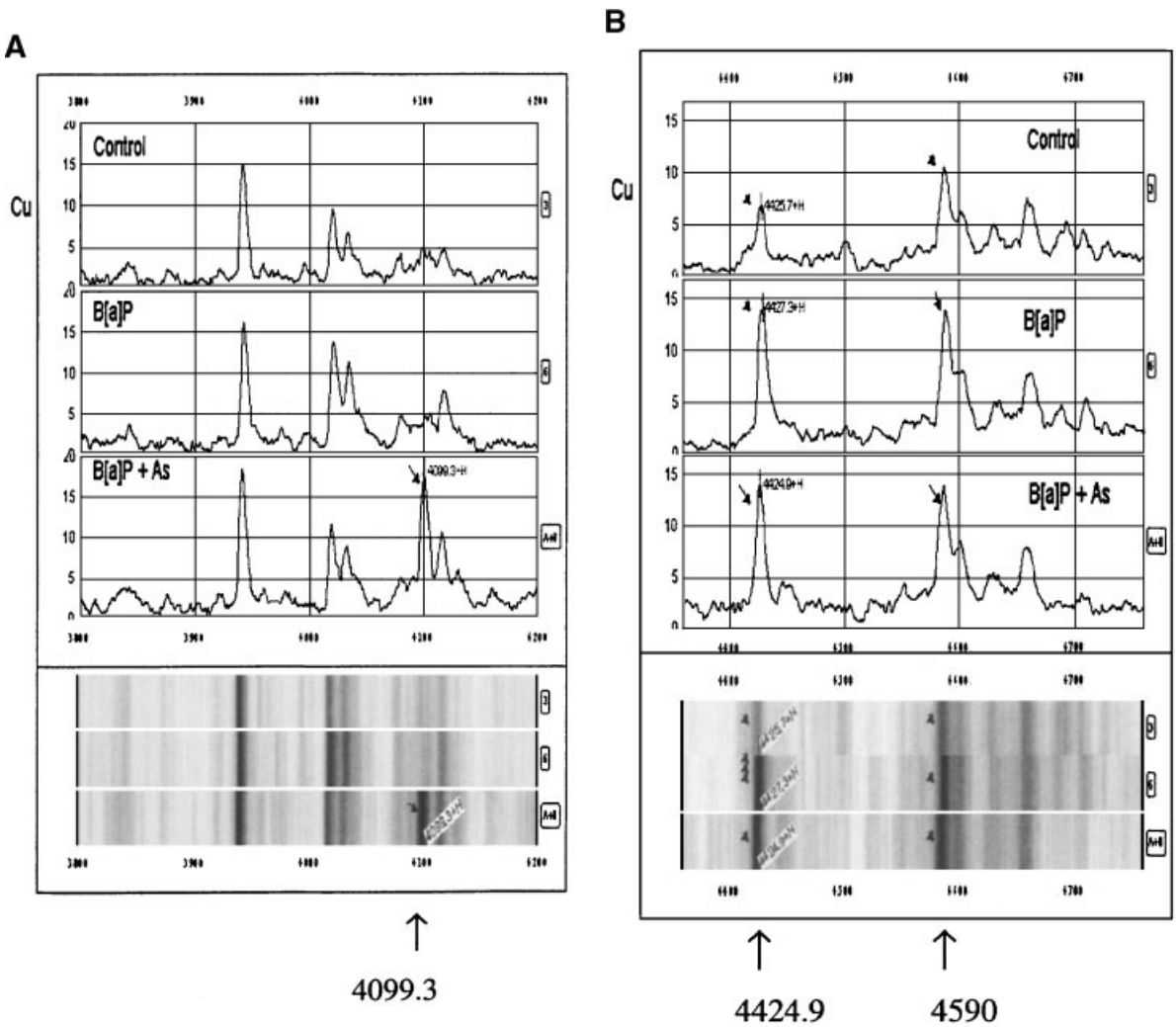

Fig. 2. Representative protein spectra (**upper panel**) and gel views (**lower panel**) of SELDI analysis of cellular proteins bound to copper ProteinChip array. Cellular extract of control LEC cells, cells treated with B[a]P, or treated with B[a] + arsenite were spotted onto Cu-ProteinChip array and processed as described in the Methods. Two protein peaks with MW of 4,099.3 and 8,175.5 were present in the As-transformed LEC cells, but absent in the control and B[a]P-treated LEC cells.

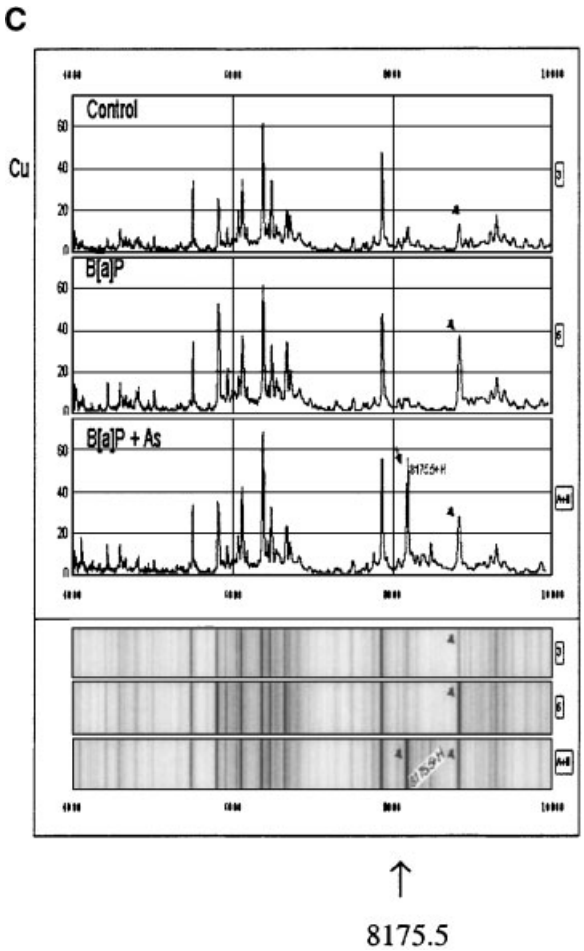

Fig. 2. (*Continued*)

identical in three groups of LEC cells, except one prominent protein peak with molecular mass at 8,180.9 Da dramatically increased in As-transformed cells. This protein mass is very close to the one (8,175.5 Da) detected in Cu-affinity chip (Fig. 2A). Possibility of these two proteins is the same protein will be further investigated.

In addition to the detection of difference in the frequency of individual protein peak between control and As-transformed cells, regional differences in the mass spectra were also observed. Figure 3B shows two protein cluster regions between 17,300–17,450 and 18,187–18,438 Da that demonstrated differences in the control and experimental groups. The level of protein cluster regions 17,300–17,450 increased in LEC cells treated B[a]P. However, the protein cluster region between 18,187–18,438 increased dramatically in As-transformed LEC cells.

Processing on a strong anion exchanger chip surface (a SAX2 chip), LEC cellular proteins in cell extract were resolved up to 100 protein peaks below the 20 kDa mass range. Figure 4 is a representative protein spectrum showing protein peaks with molecular masses between 4,000 and 12,500 Da of a single extract sample. As shown in this figure, the SELDI technology was particularly effective in resolving the low molecular weight (<10 kD) protein and polypeptide.

Analysis of cellular protein profiles from control, B[a]P-treated, and As-induced transformed LEC cells, revealed that one prominent protein peak was preferentially expressed in control cells. Representative mass spectra and gel views of this protein is shown in Figure 4A. This protein peak has a molecular mass of 4,694.4. A protein peak with mass at 4,839 was also decreasing in As-induced transformed cells in a less drastically manner. In contrast to this, we have identified protein peak around at 4,806 that increased or appeared in transformed LEC cells induced by B[a]P plus arsenic (Fig. 4B). Interestingly, using SAX2 chip, we were able to detect several protein peaks that increased their expression in LEC cells treated only with B[a]P. These protein peaks have molecular mass around 5,811.2, 7,960.8, 8,310.1, 8,978, 9,133.3, and 10,631.9 (Fig. 4A and C).

To search for a hint of the protein identities of three transformed cell-associated proteins, 4,099.3; 8,175.5; and 8,180.9, and one normal control cell-associated protein 4,694.4, we used the TagIdent tool from the ExPASy molecular server [Hochestrasser et al., 1995]. By entering the mass of an unknown protein, this tool will search in the SWISS-PROT and TrEMBLE protein databases for proteins that will match with the requested mass. We found that of all the entries in the rat protein database, the cytochrome B6-F complex subunit V matches the protein peak with a mass of 4,099.3 (Fig. 2A). The function of this protein is to transport electrons across the cellular membrane. Ubiquinol-cytochrome C reductase complex with a mass of 8,185.66 was identified from the database that matches both protein peaks of 8,175.5 (Fig. 2C) and 8,180.9 (Fig. 3A).

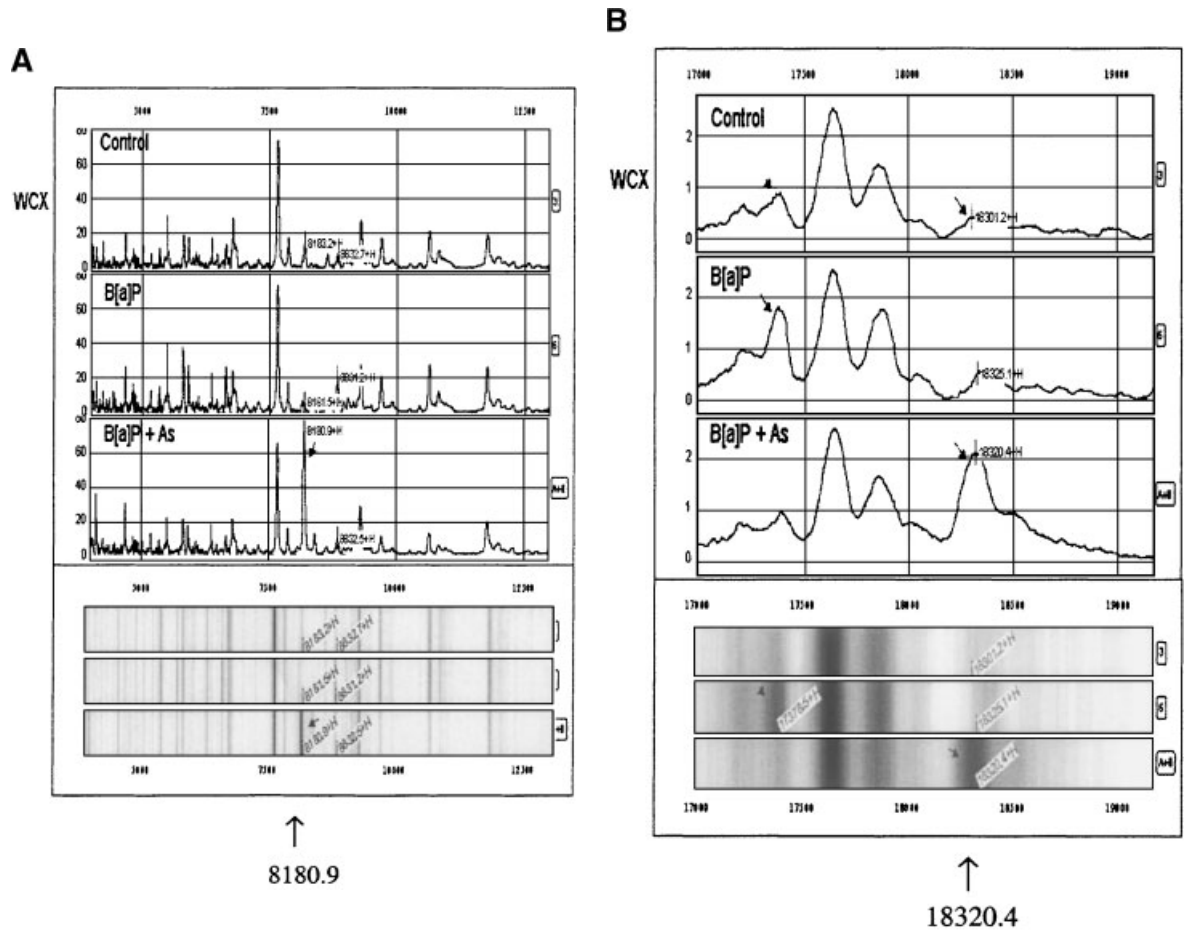

Fig. 3. Representative protein spectral (**upper panel**) and gel views (**lower panel**) of SELDI analysis of cellular proteins bound to WCX ProteinChip arrays. Cellular extract of control LEC cells, cells treated with B[a]P, or treated with B[a] + arsenite were spotted onto WCX-ProteinChip arrays and processed as described in the Methods. The spectra and gel views show the presence of proteins with MW of 8,180.9 and 18,320.4 in the As-transformed LEC cells.

The ubiquinol-cytochrome C reductase complex is part of the mitochondrial respiratory chain. Our findings are interesting for the reason that all transformed cells need more energy to support their rapid growth. The expressions of enzymes involved in the mitochondrial respiratory chain and ATP production are therefore, expected to increase in transformed cells.

We have also found that the Bax protein with molecular weight (MW) 4,678.22 matches with the protein peak of 4,694.4 which only appears in control cells and not in carcinogen-treated cells (Fig. 4A). The Bax protein is the gene product that is regulated by the tumor suppressor gene *p53*. When p53 detects that cellular DNA mutation is beyond repair, it induces cell apoptosis through activation of the Bax protein. In this study, we demonstrated that this protein production was strongly suppressed after treatment with carcinogen B[a]P (Fig. 4A). Whether the suppression of Bax protein synthesis is a precursor for As-induced cell transformation is under investigation.

In conclusion, we have demonstrated by a ProteinChip-based technology that several specific proteins are significantly elevated in transfomed LEC cells induced by sodium arsenite. The emergence of new technologies for the

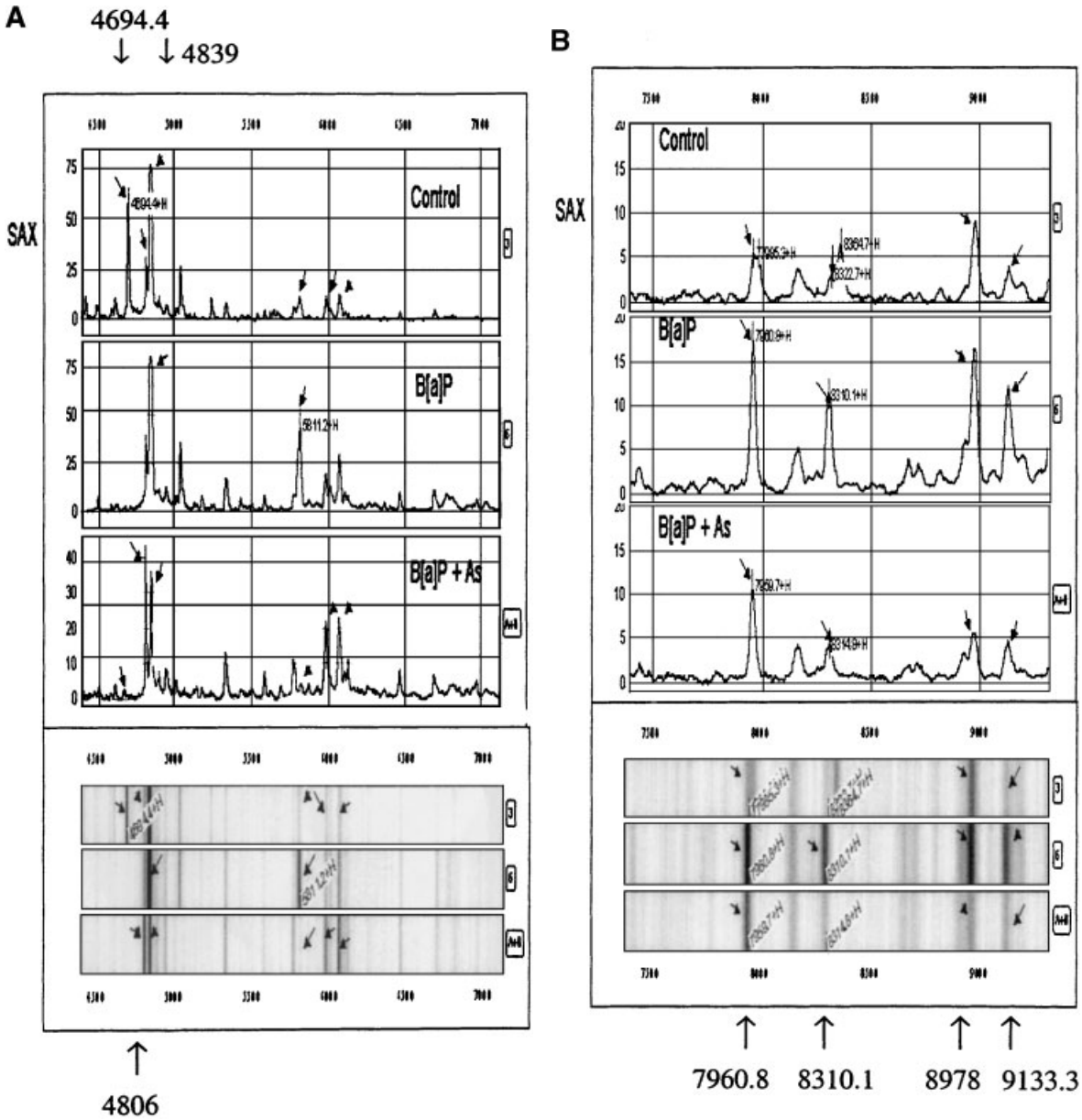

Fig. 4. Detection of differentially expressed protein samples bound to SAX2 ProteinChip arrays. Cellular extract of control LEC cells, cells treated with B[a]P, or treated with B[a] + arsenite were spotted onto SAX2-ProteinChip arrays and processed as described in the Methods. Mass spectra (**upper panel**) and gel views (**lower panel**) show the presence of a prominent protein peak with MW of 4,694.4 in the control LEC cells, but not in B[a]P- and B[a]P + As-treated LEC cells. Several protein peaks were specifically captured by SAX2 ProteinChip that are dominantly present in the LEC cells treated with B[a]P. These protein peaks are:4,806; 7,960.8; 8,310.1; 8,978; 9,133.3; and 10,631.9 Da.

identification of unknown proteins from mass spectrometry profiles is expected to accelerate the identification and characterization of these proteins that will reveal the molecular basis of As-induced cell proliferation and provide insight into the mechanisms by which arsenic-induced carcinogenesis.

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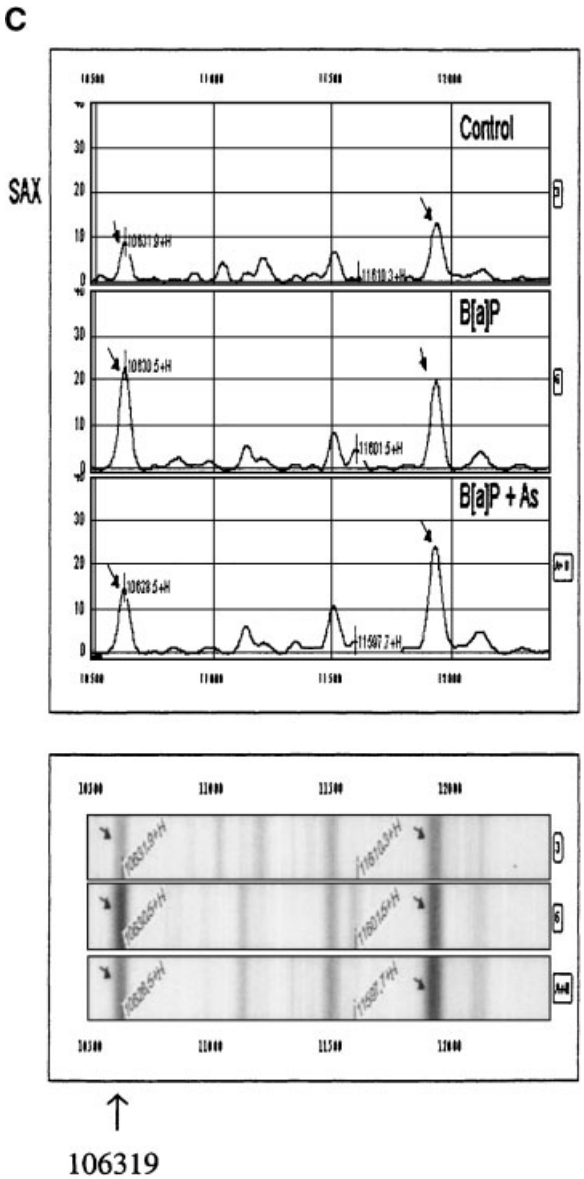

Fig. 4. (*Continued*)

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