Protein chemical identification and characterization of the human variants of far upstream element binding protein in medulloblastoma DAOY cell line

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Abstract. The assembly of *trans*-acting proteins on sequence-specific DNA *cis*-elements is crucial in the regulation of eukaryotic gene expression. Far upstream element binding proteins (FAB) are proteins that regulate the expression of the c-*myc* oncogene by binding to the far upstream element of the c-*myc* gene. The present study unambiguously identified the two human variants of FAB (FAB1, FAB2) in the medulloblastoma DAOY cell line and characterized their structure for the first time by tandem mass spectrometry independent of antibody availability and specificity. The study also tentatively assigned the third variant (FAB3) at the level of mass spectrometry, although tandem mass spectrometric analysis failed to corroborate the result. These findings open up an exciting possibility for discerning the cellular roles of FAB in tumor biology.

Introduction

In addition to conventional transcription factors bearing DNA binding and effector domains, several sequence-specific single-stranded DNA-binding proteins have been suggested to regulate gene expression (1). Among these, far upstream element (FUSE)-binding proteins (FBP) comprise a family of homologous gene regulatory proteins capable of tethering a powerful activation motif to specific sequences in single-stranded DNA (2-4). So far, three variants are known in humans (FBP1, FBP2 and FBP3), which have three distinct functional domains that display strong primary sequence and predicted secondary structure homology. The N-terminus domain represses transcription in *cis* and in *trans*. By contrast, the C-terminus domain confers transactivation through multiple repeats of a powerful tyrosine-rich activation motif. The central domain binds single-stranded nucleic acids of specific sequences and is composed of four distinct K homology (KH) motifs, each followed by an amphipathic helix (3-5).

Medulloblastoma is the most frequent malignant brain tumor in children and is considered to be of neuroectodermal origin. The medulloblastoma precursor cells are bipotential, able to differentiate into neuronal or glial cell types depending on stimulatory signals from the environment (6). Two main representative cell lines, DAOY and D283, are widely used in studies of medulloblastoma, as they show expression of glial and neuronal elements. In an attempt to identify novel and known proteins that could serve as candidate markers and/or therapeutic targets for medulloblastoma, we generated proteome maps of both cell lines, consisting of a series of proteins already linked to malignancy (7). In the present study, we analyzed the DAOY cell line, as it expresses both neuronal and glial elements, by two dimensional gel electrophoresis (2-DE) coupled to matrix-assisted laser desorption/ionization (MALDI) mass spectroscopy (MS) followed by tandem mass spectroscopy (MS/MS) because the specificity of MS/MS-based protein identification is much higher than that of MS (8). We report for the first time expression of all three variants of human FBPs at the protein level in the DAOY cell line.

Materials and methods

Cell culture and sample preparation. The DAOY cell line [ATCC: HTB-186; (9)] was cultivated according to specific ATCC guidelines (http://www.lgcpromochem-atcc.com/SearchCatalogs/lor). Harvested cells were washed three times with 10 ml PBS (phosphate-buffered saline) (Gibco BRL, Gaithersburg, MD, USA) and centrifuged for 10 min at 800 x g at room temperature. The supernatant was discarded and the pellet was suspended in 1.0 ml of sample buffer consisting of 40 mM Tris, 7 M urea (Merck, Darmstadt, Germany), 2 M thiourea (Sigma, St. Louis, MO, USA), 4% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate) (Sigma), 65 mM 1,4-dithioerythritol (Merck), 1 mM EDTA (ethylenediaminetetraacetic acid) (Merck), protease inhibitors

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complete® (Roche, Basel, Switzerland) and 1 mM phenylmethylsulfonyl fluoride. The suspension was sonicated for ~30 sec. After homogenization, samples were left at room temperature for 1 h and centrifuged at 14,000 rpm for 1 h. The supernatant was transferred into Ultrafree-4 centrifugal filter units (Millipore, Bedford, MA) for desalting and concentrating proteins. The protein content of the supernatant was quantified by the Bradford protein assay (10). The standard curve was generated using bovine serum albumin and absorbance was measured at 595 nm.

Two-dimensional gel electrophoresis (2-DE). 2-DE was performed in triplicate as reported elsewhere (11). Protein (500 μg) was applied on immobilized pH 3.0-10.0 non-linear gradient strips in sample cups at their basic and acidic ends. Focusing was started at 200 V and the voltage was gradually increased to 5000 V at a rate of 3 V/min and then kept constant for a further 24 h (~180,000 Vh in total). After the first dimension, strips (18 cm) were equilibrated for 15 min in the buffer containing 6 M urea, 20% glycerol, 2% SDS and 2% 1,4-dithioerythritol and then for 15 min in the same buffer containing 2.5% iodoacetamide instead of 1,4-dithioerythritol. After equilibration, strips were loaded on 9-16% gradient SDS gels for second-dimensional separation. Gels (180x200x1.5 mm) were then run at 40 mA per gel. Immediately after the second dimension run, gels were fixed for 18 h in 50% methanol, containing 10% acetic acid, the gels were then stained with Colloidal Coomassie Blue (Novex, San Diego, CA) for 12 h on a rocking shaker. Molecular masses were determined by running standard protein markers (Bio-Rad Laboratories, Hercules, CA) covering the range of 10-250 kDa. pI values were used as given by the supplier of the immobilized pH gradient strips (Amersham Bioscience, Uppsala, Sweden). Excess of dye was washed out from the gels with distilled water and the gels were scanned with ImageScanner (Amersham Bioscience). Electronic images of the gels were recorded using Adobe Photoshop and Microsoft Power Point software.

Matrix-assisted laser desorption/ionization mass spectroscopy/mass spectroscopy (MALDI/MS/MS). Spots were excised with a spot picker (Proteineer sp™, Bruker Daltonics, Germany) and placed into a 384-well microtiter plate. In-gel digestion and sample preparation for MALDI analysis were performed by an automated procedure (Proteineer dp™, Bruker Daltonics) (12). Briefly, spots were excised and washed with 10 mM ammonium bicarbonate and 50% acetonitrile in 10 mM ammonium bicarbonate. After washing, gel plugs were shrunk by addition of acetonitrile and dried by blowing out the liquid through the pierced well bottom. The dried gel pieces were re-swollen with 40 ng/μl trypsin (Promega, Madison, USA) in enzyme buffer (consisting of 5 mM octyl ß-D-glucopyranoside and 10 mM ammonium bicarbonate) and incubated for 4 h at 30˚C. Peptide extraction was performed with 10 μl of 1% trifluoroacetic acid in 5 mM octyl ß-D-glucopyranoside. Extracted peptides were directly applied onto a target (AnchorChip™, Bruker Daltonics) that was loaded with α-cyano-4-hydroxy-cinnamic acid (Bruker Daltonics) matrix thinlayer. The mass spectrometer used in this work was an Ultraflex™ TOF/TOF (Bruker Daltonics) operated in the reflector mode for MALDI-TOF peptide mass fingerprint (PMF) or LIFT mode for MALDI-TOF/TOF with a fully automated mode using FlexControl™ software. An accelerating voltage of 25 kV was used for PMF. Calibration of the instrument was performed externally with $[M+H]^+$ ions of angiotensin I, angiotensin II, substance P, bombesin, and adrenocorticotropic hormones (clip 1-17 and clip 18-39). Each spectrum was produced by accumulating data from 200 consecutive laser shots. Those samples which were analyzed by PMF from MALDI-TOF were additionally analyzed using LIFT-TOF/TOF MS/MS from the same target. A maximum of three precursor ions per sample were chosen for MS/MS analysis. In the TOF1 stage, all ions were accelerated to 8 kV under conditions promoting metastable fragmentation. After selection of jointly migrating parent and fragment ions in a timed ion gate, ions were lifted by 19 kV to high potential energy in the LIFT cell. After further acceleration of the fragment ions in the second ion source, their masses could be simultaneously analyzed in the reflector with high sensitivity. PMF and LIFT spectra were interpreted with Mascot software (Matrix Science Ltd., London, UK). Database searches, through Mascot, using combined PMF and MS/MS datasets were performed via BioTools 2.2 software (Bruker). A mass tolerance of 25 ppm and 1 missing cleavage site for PMF and MS/MS tolerance of 0.5 Da were allowed and oxidation of methionine residues was considered. The probability score calculated by the software was used as criterion for correct identification. The algorithm used for the determination of the probability of a false positive match with a given mass spectrum is described elsewhere (13).

Results

2-DE coupled to MALDI/MS/MS was used to analyze the expression of the DAOY medulloblastoma cell line in an attempt to elucidate marker proteins and identified an interesting protein family, FBP, that is relevant to tumor biology. 2-DE analysis of the DAOY medulloblastoma cell line revealed a total of eight spots, of which two spots represented FBP1, five spots represented FBP2 and one spot represented FBP3 (Fig. 1) with theoretical pI range of 7.2-8.0, and theoretical molecular weight range of 67-73 kDa (Table I). For the purpose of simplicity, the two spots of FBP1 are designated as FBP1a and FBP1b (a, b from left to right in Fig. 1) and that of FBP2 as FBP2a-e (d, c, b, a, e from left to right). There appeared to be a shift in observed pI as well as molecular weight from the theoretical values. Whilst a decreasing trend was noted in observed pI, molecular weight was increased for all spots of different variants of FBPs (Table I).

Subjecting the spots to PMF followed by MS/MS analysis produced significant MS/MS results for the two identified spots of FBP1. Data obtained from MS analysis are summarized in Table I. As shown in Table I, whilst PMF analysis of the spot of FBP1a revealed sequence coverage of 45%, that of FBP1b showed 43% (Fig. 2a). Following identification by PMF, the workflow control software automatically selected two peaks for FBP1a (m/z 1336.74 and m/z 1539.81) and three peaks (m/z 931.56, m/z 1336.72 and m/z 1539.80) for FBP1b (Fig. 3a and b) from the MS spectrum to generate

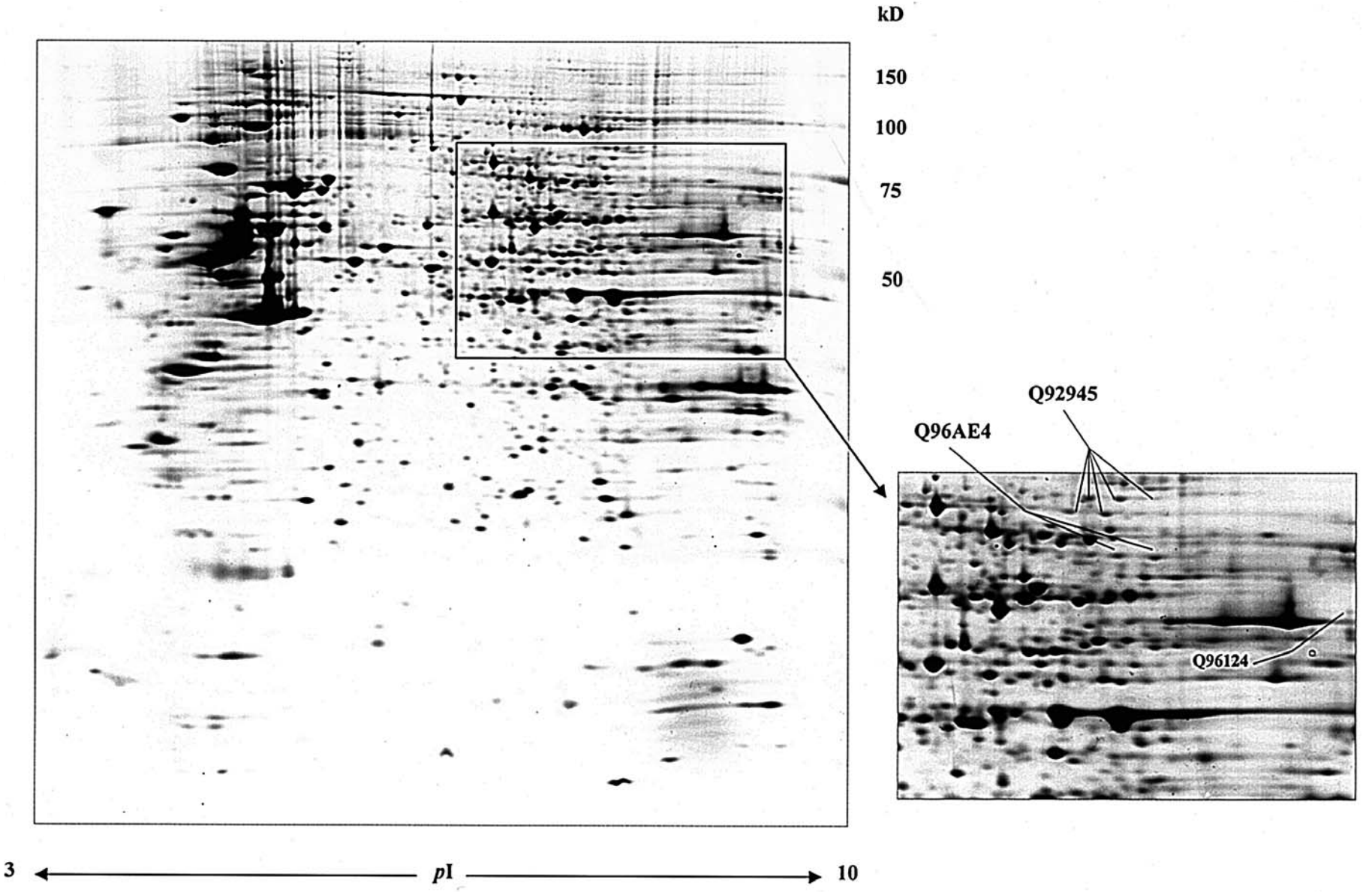

Figure 1. 2-DE gel image of variants of FBPs. Proteins were extracted and separated on an immobilized pH 3.0-10.0 non-linear gradient strip followed by separation on a 9-16% gradient polyacrylamide gel. Gels were stained with Coomassie blue and spots were analysed by MALDI-MS and MS/MS.

a. Upper panel for FBP1a and lower panel for FBP1b

```
  1 MADYSTVPPP SSGSAGGGGG GGGGGGVNDA FKDALQRARQ IAAKIGGDAG
 51 TSLNSNDYGY GGQKRPLEDG DQPDAKKVAP QNDSFGTQLP PMHQQQRSVM
101 TEEYKVPDGM VGFIIGRGGE QISRIQQESG CKIQIAPDSG GLPERSCMLT
151 GTPESVQSAK RLLDQIVEKG RPAPGFHHGD GPGNAVQEIM IPASKAGLVI
201 GKGGETIKQL QERAGVKMVM IQDGPQNTGA DKPLRITGDP YKVQQAKEMV
251 LELIRDQGGF REVRNEYGSR IGGNEGIDVP IPRFAVGIVI GRNGEMIKKI
301 QNDAGVRIQF KPDDGTTPER IAQITGPPDR CQHAAEIITD LLRSVQAGNP
351 GGPGPGGRGR GRGQGNWNMG PPGGLQEFNF IVPTGKTGLI IGKGGETIKS
401 ISQQSGARIE LQRNPPPNAD PNMKLFTIRG TPQQIDYARQ LIEEKIGGPV
451 NPLGPPVPHG PHGVPGPHGP PGPPGPGTPM GPYNPAPYNP GPPGPAPHGP
501 PAPYAPQGWG NAYPHWQQQA PPDPAKAGTD PNSAAWAAYY AHYYQQQAQP
551 PPAAPAGAPT TTQTNGQGDQ QNPAPAGQVD YTKAWEEYYK KMGQAVPAPT
601 GAPPGGQPDY SAAWAEYYRQ QAAYYAQTSP QGMPQHPPAP QGQ
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```
  1 MADYSTVPPP SSGSAGGGGG GGGGGGVNDA FKDALQRARQ IAAKIGGDAG
 51 TSLNSNDYGY GGQKRPLEDG DQPDAKKVAP QNDSFGTQLP PMHQQQRSVM
101 TEEYKVPDGM VGFIIGRGGE QISRIQQESG CKIQIAPDSG GLPERSCMLT
151 GTPESVQSAK RLLDQIVEKG RPAPGFHHGD GPGNAVQEIM IPASKAGLVI
201 GKGGETIKQL QERAGVKMVM IQDGPQNTGA DKPLRITGDP YKVQQAKEMV
251 LELIRDQGGF REVRNEYGSR IGGNEGIDVP IPRFAVGIVI GRNGEMIKKI
301 QNDAGVRIQF KPDDGTTPER IAQITGPPDR CQHAAEIITD LLRSVQAGNP
351 GGPGPGGRGR GRGQGNWNMG PPGGLQEFNF IVPTGKTGLI IGKGGETIKS
401 ISQQSGARIE LQRNPPPNAD PNMKLFTIRG TPQQIDYARQ LIEEKIGGPV
451 NPLGPPVPHG PHGVPGPHGP PGPPGPGTPM GPYNPAPYNP GPPGPAPHGP
501 PAPYAPQGWG NAYPHWQQQA PPDPAKAGTD PNSAAWAAYY AHYYQQQAQP
551 PPAAPAGAPT TTQTNGQGDQ QNPAPAGQVD YTKAWEEYYK KMGQAVPAPT
601 GAPPGGQPDY SAAWAEYYRQ QAAYYAQTSP QGMPQHPPAP QGQ
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b. matched peptides to FBP2a, FBP2b, FBP2c, FBP2d & FBP2e

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  1 MSDYSTGGPP PGPPPPAGGG GGAGGAGGGP PPGPPGAGDR GGGPCGGGP
 51 GGGSAGGPSQ PPGGGGPGIR KDAFADAVQR ARQIAAKIGG DAATTVNNST
101 PDFGFGGQKR QLEDGDQPES KKLASQGDSI SSQLGPIHPP PRTSMTEEYR
151 VPDGMVGLII GRGGEQINKI QQDSGCKVQI SPDSGGLPER SVSLTGAPES
201 VQKAKMMLDD IVSRGRGGPP GQFHDNANGG QNGTVQEIMI PAGKAGLVIG
251 KGGETIKQLQ ERAGVKMILI QDGSQNTNVD KPLRIIGDPY KVQQACEMVM
301 DILRERDQGG FGDRNEYGSR IGGGIDVPVP RHSVGVVIGR SGEMIKKIQN
351 DAGVRIQFKQ DDGTGPEKIA HIMGPPDRCE HAARIINDLL QSLRSGPPGP
401 PGGPGMPPGG RGRGRGQGNW GPPGGEMTFS IPTHKCGLVI GRGGENVKAI
451 NQQTGAFVEI SRQLPPNGDP NFKLFIIRGS PQQIDHAKQL IEEKIEGPLC
501 PVGPGPGGPG PAGPMGPFNP GPFNQGPPGA PPHAGGPPPH QYPPQGWGNT
551 YPQWQPPAPH DPSKAAAAAA DPNAAWAAYY SHYYQQPPGP VPGPAPAPAA
601 PPAQGEPPQP PPTGQSDYTK AWEEYYKKIG QQPQQPGAPP QQDYTKAWEE
651 YYKKQAQVAT GGGPGAPPGS QPDYSAAWAE YYRQQAAYYG QTPGPGGPQP
701 PPTQQGQQQA Q
```

```
  1 MSDYSTGGPP PGPPPPAGGG GGAGGAGGGP PPGPPGAGDR GGGPCGGGP
 51 GGGSAGGPSQ PPGGGGPGIR KDAFADAVQR ARQIAAKIGG DAATTVNNST
101 PDFGFGGQKR QLEDGDQPES KKLASQGDSI SSQLGPIHPP PRTSMTEEYR
151 VPDGMVGLII GRGGEQINKI QQDSGCKVQI SPDSGGLPER SVSLTGAPES
201 VQKAKMMLDD IVSRGRGGPP GQFHDNANGG QNGTVQEIMI PAGKAGLVIG
251 KGGETIKQLQ ERAGVKMILI QDGSQNTNVD KPLRIIGDPY KVQQACEMVM
301 DILRERDQGG FGDRNEYGSR IGGGIDVPVP RHSVGVVIGR SGEMIKKIQN
351 DAGVRIQFKQ DDGTGPEKIA HIMGPPDRCE HAARIINDLL QSLRSGPPGP
401 PGGPGMPPGG RGRGRGQGNW GPPGGEMTFS IPTHKCGLVI GRGGENVKAI
451 NQQTGAFVEI SRQLPPNGDP NFKLFIIRGS PQQIDHAKQL IEEKIEGPLC
501 PVGPGPGGPG PAGPMGPFNP GPFNQGPPGA PPHAGGPPPH QYPPQGWGNT
551 YPQWQPPAPH DPSKAAAAAA DPNAAWAAYY SHYYQQPPGP VPGPAPAPAA
601 PPAQGEPPQP PPTGQSDYTK AWEEYYKKIG QQPQQPGAPP QQDYTKAWEE
651 YYKKQAQVAT GGGPGAPPGS QPDYSAAWAE YYRQQAAYYG QTPGPGGPQP
701 PPTQQGQQQA Q
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1 MSDYSTGGPP PGPPPPAGGG GGAGGAGGGP PPGPPGAGDR GGGGPCGGGP
51 GGGSAGGPSQ PPGGGGPGIR KDAFADAVQR ARQIAAK**IGG DAATTVNNST**
101 **PDFGFGGQK**R QLEDGDQPES K**KLASQGDSI SSQLGPIHPP PRTSMTEEYR**
151 **VPDGMVGLII GR**GGEQINKI QQDSGCK**VQI SPDSGGLPER** SVSLTGAPES
201 VQKAKMMLDD IVSRGR**GGPP GQFHDNANGG QNGTVQEIMI PAGK**AGLVIG
251 KGGETIKQLQ ERAGVK**MILI QDGSQNTNVD KPLR**IIGDPY K**VQQACEMVM**
301 **DILRERDQGG FGDRNEYGSR IGGGIDVPVP R**HSVGVVIGR SGEMIKKIQN
351 DAGVRIQFKQ DDGTGPEK**IA HIMGPPDR**CE HAAR**IINDLL QSLRSGPPGP**
401 **PGGPGMPPGG** RGR**GRGQGNW GPPGGEMTFS IPTHK**CGLVI GRGGENVK**AI**
451 **NQQTGAFVEI SR**QLPPNGDP NFKLFIIRGS PQQIDHAKQL IEEKIEGPLC
501 PVGPGPGGPG PAGPMGPFNP GPFNQGPPGA PPHAGGPPPH QYPPQGWGNT
551 YPQWQPPAPH DPSKAAAAAA DPNAAWAAYY SHYYQQPPGP VPGPAPAPAA
601 PPAQGEPPQP PPTGQSDYTK AWEEYYK**KIG QQPQQPGAPP QQDYTKAWEE**
651 **YYKKQAQVAT GGGPGAPPGS QPDYSAAWAE YYRQQAAYYG QTPGPGGPQP**
701 PPTQQGQQQA Q

1 MSDYSTGGPP PGPPPPAGGG GGAGGAGGGP PPGPPGAGDR GGGGPCGGGP
51 GGGSAGGPSQ PPGGGGPGIR K**DAFADAVQR** ARQIAAK**IGG DAATTVNNST**
101 **PDFGFGGQK**R QLEDGDQPES K**KLASQGDSI SSQLGPIHPP PR**TSMTEEYR
151 **VPDGMVGLII GR**GGEQINKI QQDSGCK**VQI SPDSGGLPER** SVSLTGAPES
201 VQKAKMMLDD IVSRGR**GGPP GQFHDNANGG QNGTVQEIMI PAGKAGLVIG**
251 **KGGETIK**QLQ ERAGVK**MILI QDGSQNTNVD KPLR**IIGDPY K**VQQACEMVM**
301 **DILRERDQGG FGDRNEYGSR IGGGIDVPVP RHSVGVVIGR** SGEMIKKIQN
351 DAGVRIQFK**Q DDGTGPEKIA HIMGPPDR**CE HAAR**IINDLL QSLRSGPPGP**
401 **PGGPGMPPGG R**GRGR**GQGNW GPPGGEMTFS IPTHK**CGLVI GRGGENVK**AI**
451 **NQQTGAFVEI SR**QLPPNGDP NFKLFIIRGS PQQIDHAKQL IEEKIEGPLC
501 PVGPGPGGPG PAGPMGPFNP GPFNQGPPGA PPHAGGPPPH QYPPQGWGNT
551 YPQWQPPAPH DPSKAAAAAA DPNAAWAAYY SHYYQQPPGP VPGPAPAPAA
601 PPAQGEPPQP PPTGQSDYTK AWEEYYK**KIG QQPQQPGAPP QQDYTKAWEE**
651 **YYKKQAQVAT GGGPGAPPGS QPDYSAAWAE YYR**QQAAYYG QTPGPGGPQP
701 PPTQQGQQQA Q

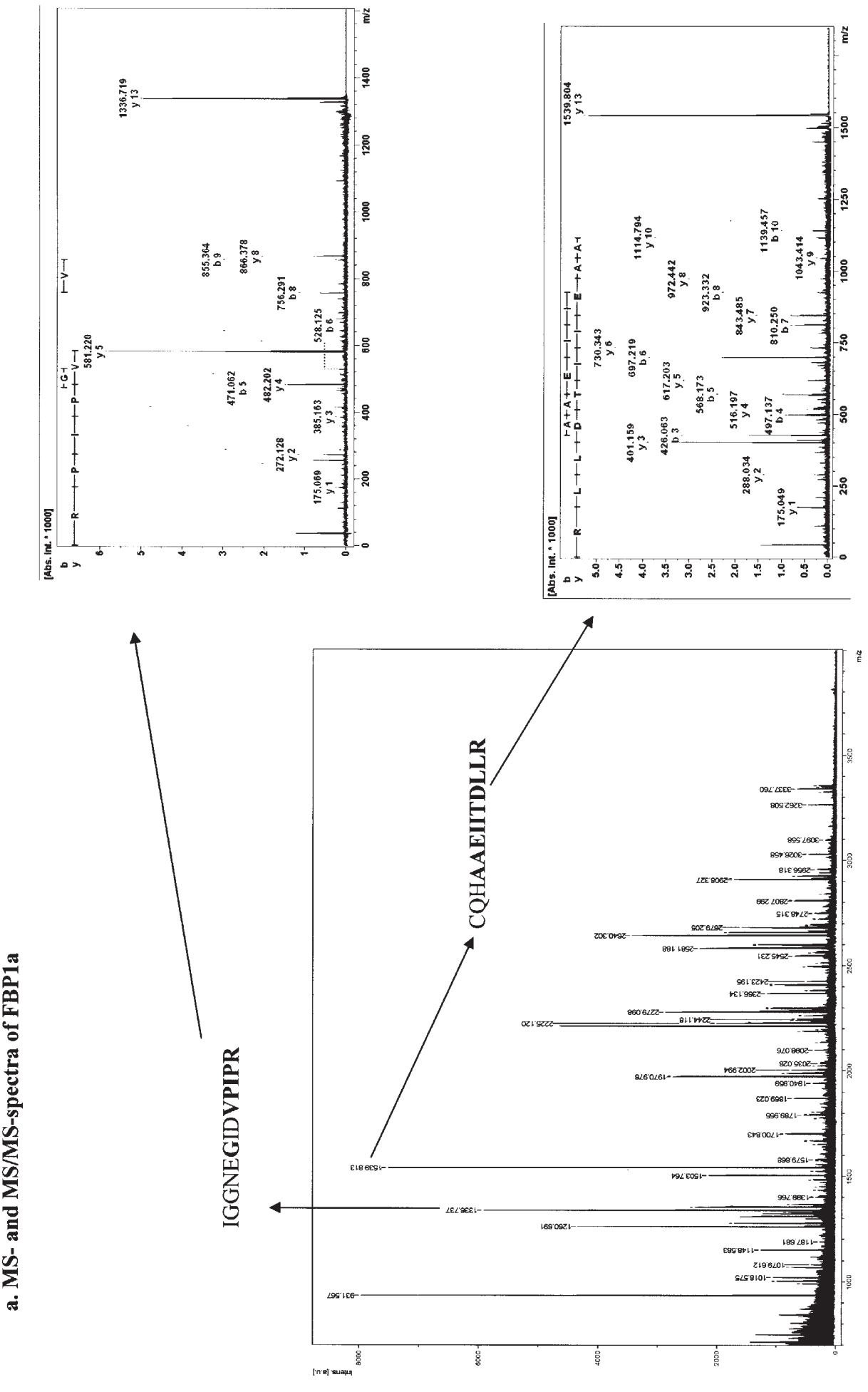
1 MSDYSTGGPP PGPPPPAGGG GGAGGAGGGPPPGPPGAGDRGGGGPCGGGP
51 GGGSAGGPSQ PPGGGGPGIR KDAFADAVQR ARQIAAK**IGG DAATTVNNST**
101 **PDFGFGGQKR** QLEDGDQPES K**KLASQGDSI SSQLGPIHPP PR**TSMTEEYR
151 VPDGMVGLII GRGGEQINKI QQDSGCKVQI SPDSGGLPER SVSLTGAPES
201 VQKAKMMLDD IVSRGRGGPP GQFHDNANGG QNGTVQEIMI PAGKAGLVIG
251 KGGETIKQLQ ERAGVK**MILI QDGSQNTNVD KPLR**IIGDPY KVQQACEMVM
301 DILRER**DQGG FGDRNEYGSR IGGGIDVPVP R**HSVGVVIGR SGEMIKKIQN
351 DAGVRIQFKQ DDGTGPEK**IA HIMGPPDRCE HAARIINDLL QSLRSGPPGP**
401 **PGGPGMPPGG R**GRGRGQGNW GPPGGEMTFS IPTHKCGLVI GRGGENVK**AI**
451 **NQQTGAFVEI SRQLPPNGDP NFKLFIIR**GS PQQIDHAKQL IEEKIEGPLC
501 PVGPGPGGPG PAGPMGPFNP GPFNQGPPGA PPHAGGPPPHQYPPQGWGNT
551 YPQWQPPAPH DPSKAAAAAA DPNAAWAAYY SHYYQQPPGP VPGPAPAPAA
601 PPAQGEPPQP PPTGQSDYTK AWEEYYKK**IG QQPQQPGAPP QQDYTK**AWEE
651 YYKKQAQVAT GGGPGAPPGS QPDYSAAWAE YYRQQAAYYG QTPGPGGPQP
701 PPTQQGQQQA Q

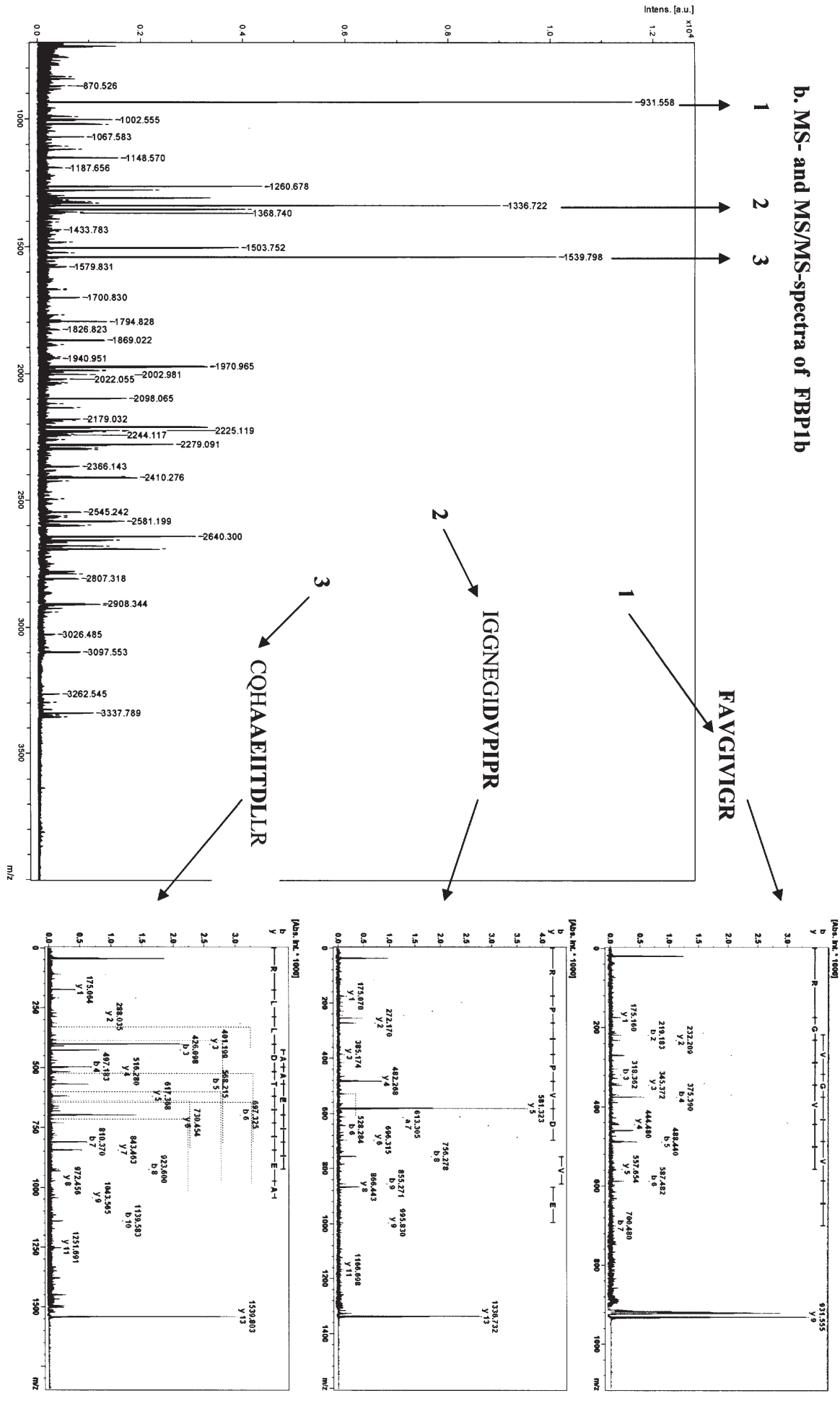
c Matched peptides to FBP3

1 MAELVQGQSA PVGMK**AEGFV DALHRVR**QIA AK**IDSIPHLN NSTPLVDPSV**
51 **YGYGVQKRPL DDGVGNQLGA LVHQR**TVITE EFKVPDKMVG FIIGRGGEQI
101 SRIQAESGCK **IQIASESSGI PERPCVLTGT PESIEQAKR**L LGQIVDRCRN
151 **GPGFHNDIDS NSTIQEILIP ASK**VGLVIGR GGETIKQLQE RTGVKMVMIQ
201 DGPLPTGADK PLR**ITGDAFK VQQAR**EMVLE IIREKDQADF RGVRGDFNSR
251 MGGGSIEVSV PR**FAVGIVIG RN**GEMIKK**IQ NDAGVRIQFK PDDGISPER**A
301 AQVMGPPDR**C QHAAHIISEL ILTAQERDGF GGLAAAR**GRG RGRGDWSVGA
351 PGGVQEITYT VPADKCGLVI GK**GGENIKSI NQQSGAHVEL QRNPPPNSDP**
401 **NLRRFTIRGV PQQIEVAR**QL IDEK**VGGTNL GAPGAFGQSP FSQPPAPPHQ**
451 **NTFPPR**SSGC FPNMAAKVNG NPHSTPVSGP PAFLTQGWGS TYQAWQQPTQ
501 QVPSQQSQPQ SSQPNYSKAW EDYYKKQSHA ASAAPQASSP PDYTMAWAEY
551 **YRQQVAFYGQ TLGQAQAHSQ EQ**

Figure 2. Protein sequence of FBP with peptides matched (bold letters) demonstrating sequence coverage.

an MS/MS spectrum, as MS/MS analysis of one or more available peptides can provide unambiguous identification of a protein in question. Indeed, the generated LIFT-TOF/TOF spectra of m/z 1336.74 and m/z 1539.81 were significantly matched to peptide IGGNEGIDVPIPR and peptide CQHAA EIITDLLR, respectively (Fig. 3a). Likewise, the generated


a. MS- and MS/MS-spectra of FBP1a
IGGNEGIDVPIPR
CQHAAEIITDLLR
[Abs. Int. * 1000]
m/z
Intens. [a.u.]


b. MS- and MS/MS-spectra of FBP1b
1
2
3
FAVGIVIGR
IGGNEGIDVPIPR
CQHAAEIITDLLR
Intens. [a.u.]
m/z
870.526
931.558
1002.555
1067.583
1148.570
1187.656
1260.678
1336.722
1368.740
1433.783
1503.752
1539.798
1579.831
1700.830
1794.828
1826.823
1869.022
1940.951
1970.965
2002.981
2022.055
2098.065
2179.032
2225.119
2244.117
2279.091
2366.143
2410.276
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2581.199
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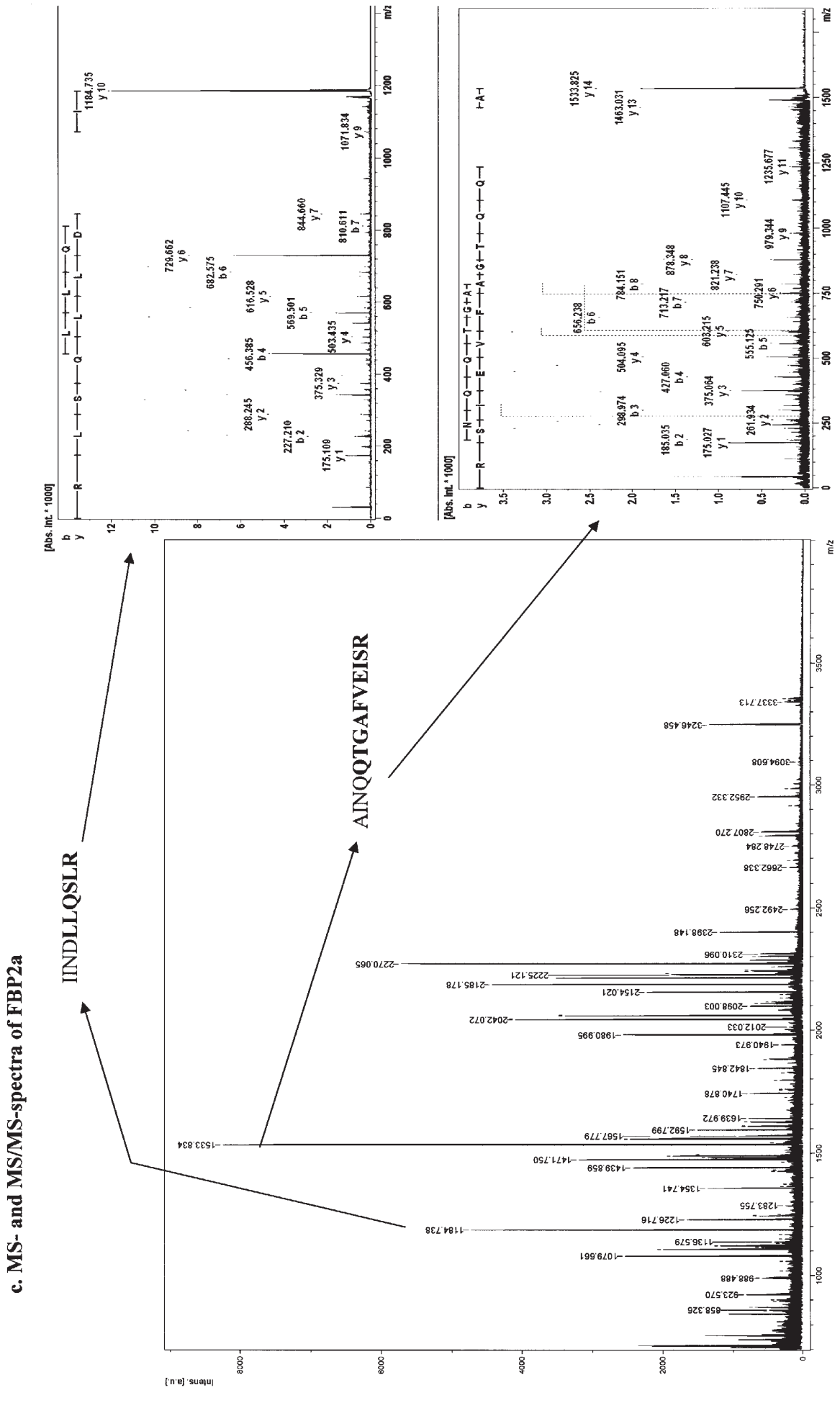
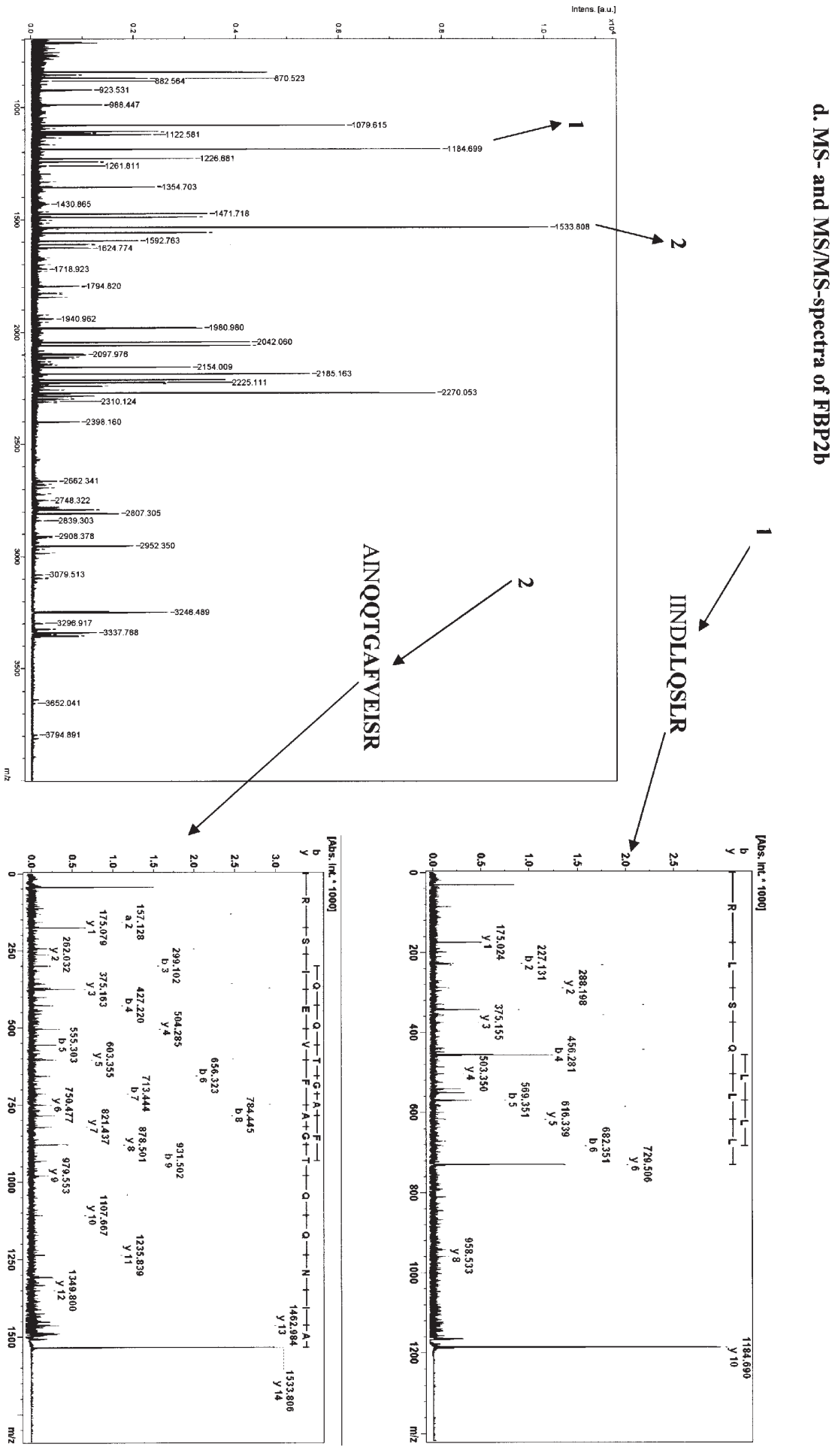
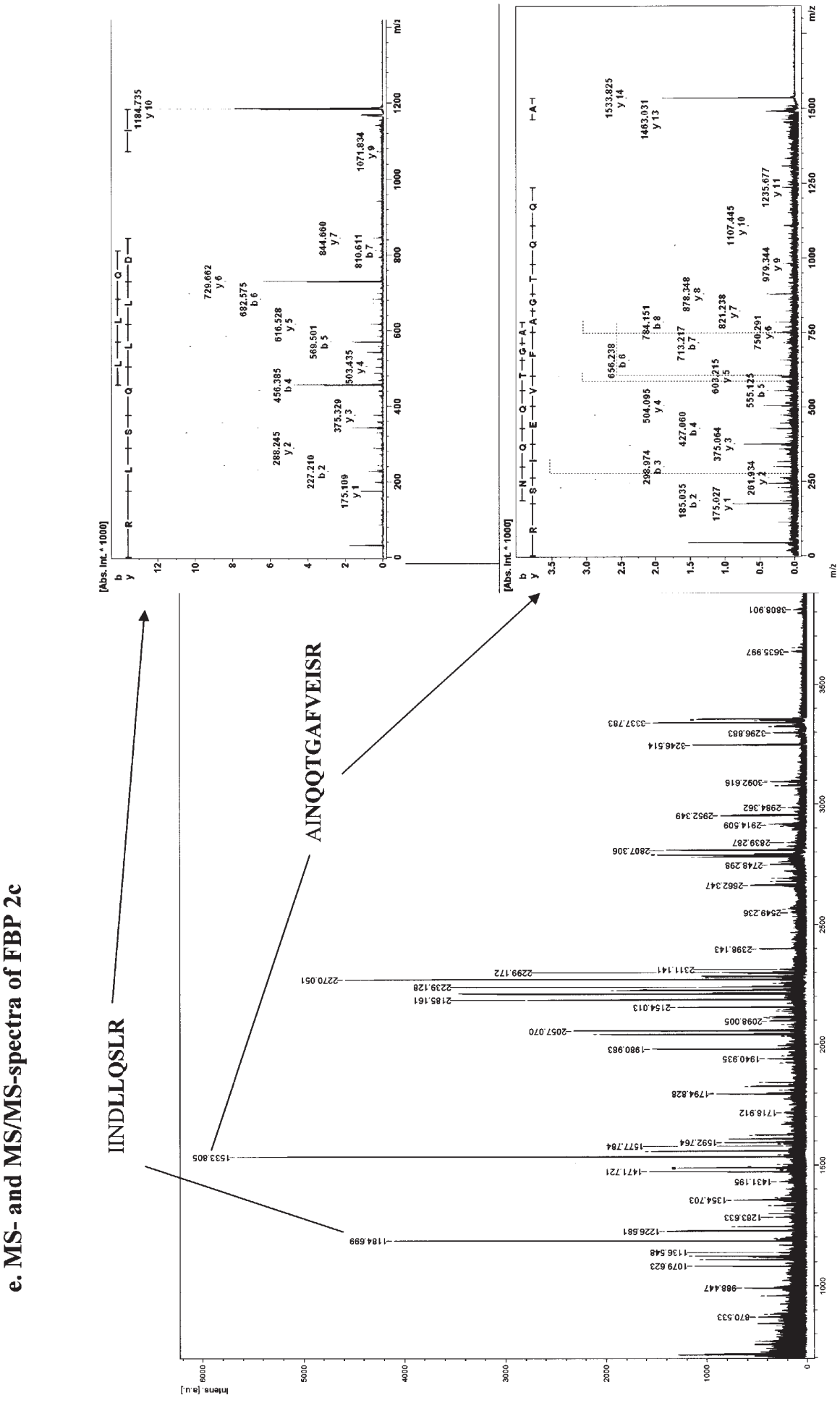
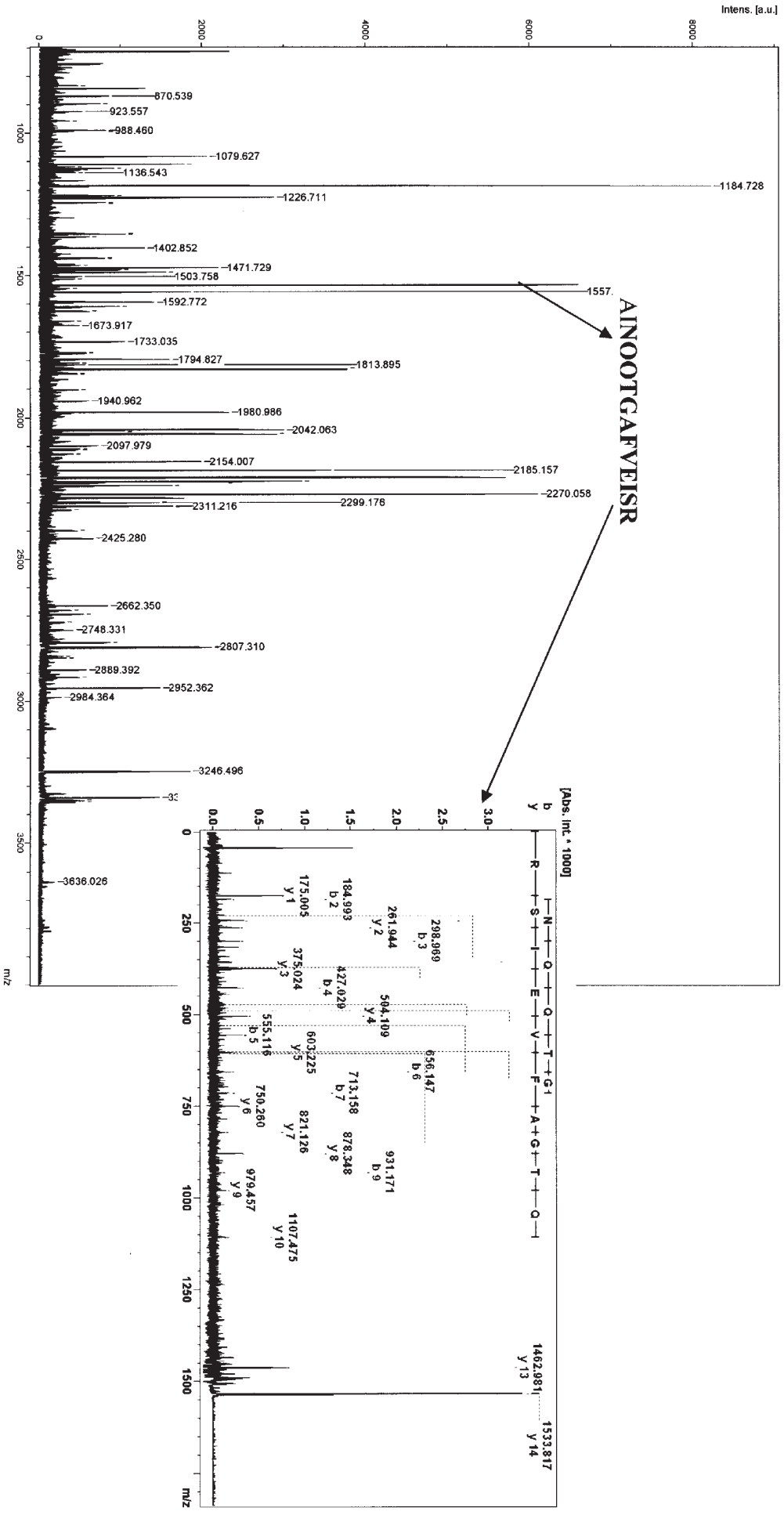
3337.789


c. MS- and MS/MS-spectra of FBP2a
IINDLLQSLR
AINQQTGAFVEISR
Intens. [a.u.]
m/z
[Abs. Int.* 1000]
b
y

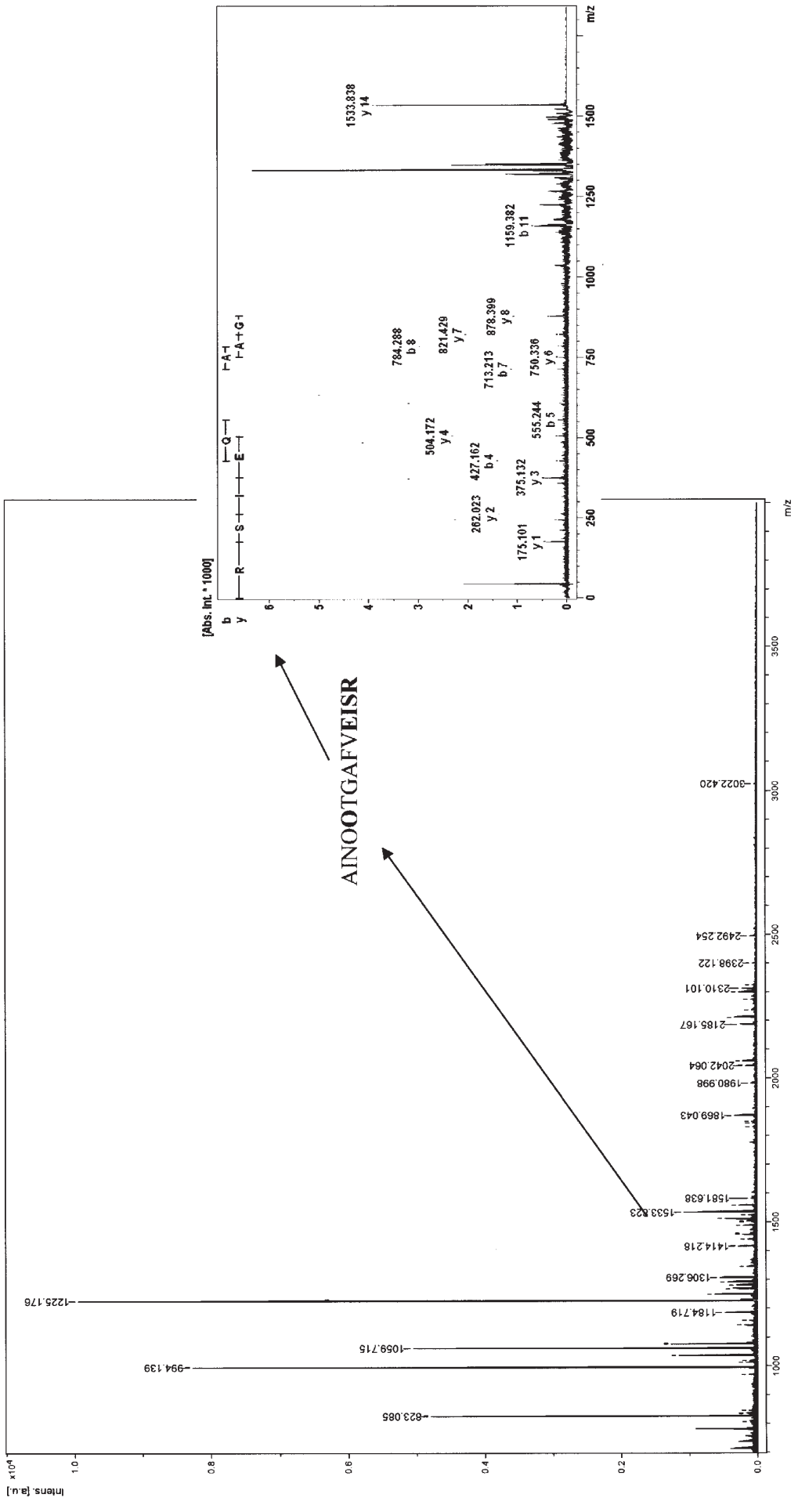

d. MS- and MS/MS-spectra of FBP2b
Intens. [a.u.]
m/z
1
2
AINQQTGAFVEISR
IINDLLQSLR
[Abs. Int. * 1000]



e. MS- and MS/MS-spectra of FBP 2c



f. MS- and MS/MS-spectra of FBP2d



g. MS- and MS/MS-spectra of FBP2e

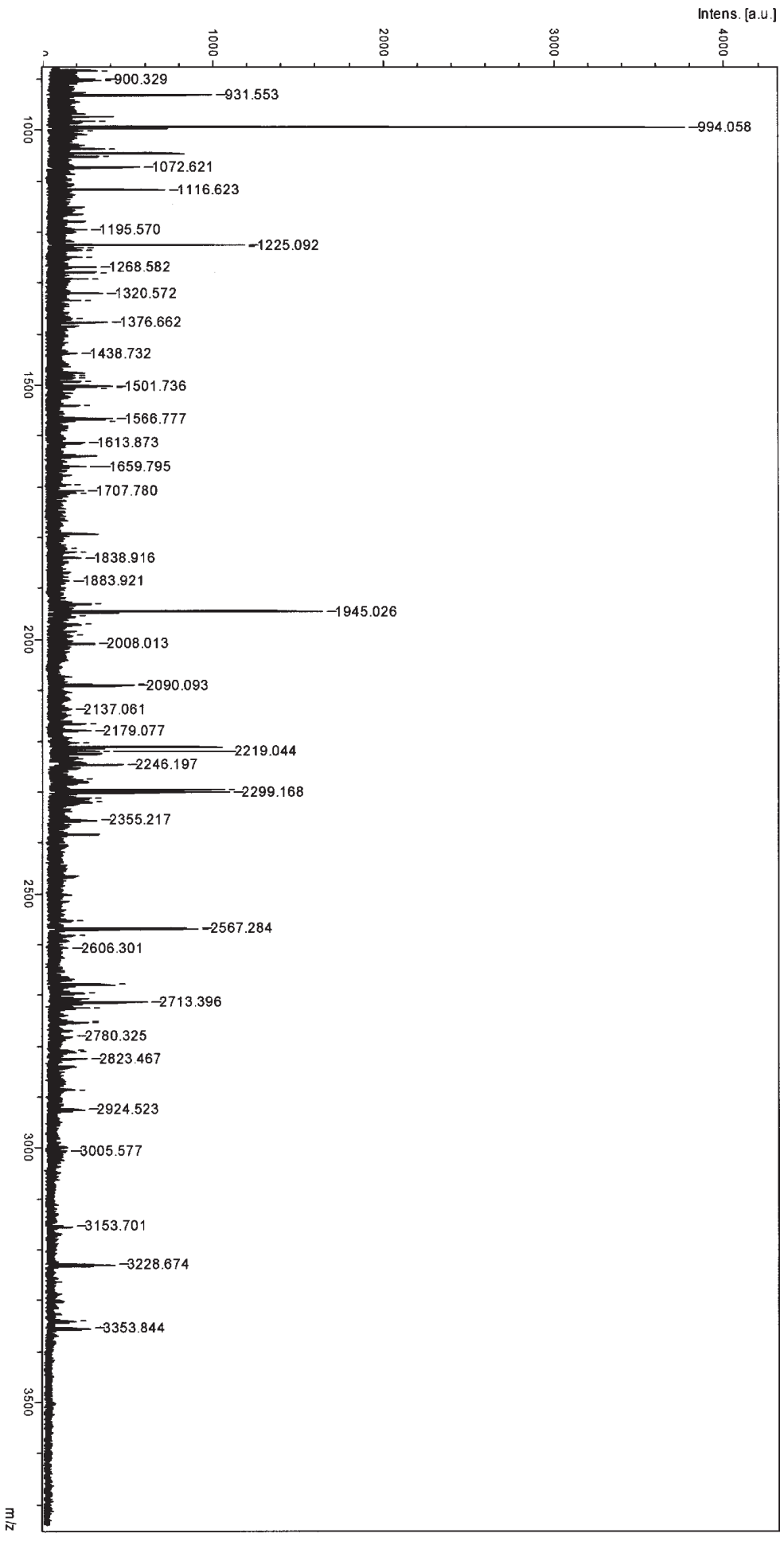


Figure 3. Peptide mass fingerprint (PMF) and MS/MS analysis of FBPs. Picks were automatically selected from the MS spectra by the computer software and the peptide sequences were matched with the spectra generated by MS/MS analysis.

Table I. Mass spectrometrical identification of FBPs in medulloblastoma cell line (DAOY).

						Mascot-search results (combined MS and MS/MS)					MS/MS results		
Acc. no.	Protein name	No. of identified spots	TMW[a]	TIP[b]	OIP[c]	OMW[d]	Score	No. of matched peptides	Sequence coverage (%)	Expectation	Score	Significant matched peptides	Peptide sequence
Q96AE4	Far upstream element binding protein 1	2	67473	7.2	6.8	67603	282	35	45	6.6e-024	118	2 peptides	IGGNE**G**IDV**PIPR** CQH**AAEIITDLLR**
					6.9	67603	341	35	43	8.3e-030	174	3 peptides	**FAVGIVIGR** IGGNEGI**DVPIPR** CQH**AAEIITDLLR**
Q92945	Far upstream element binding protein 2	5	72709	8.0	6.8	73458	424	22	32	4.2e-038	321	2 peptides	IIND**LLQSLR** AINQ**QTGAFVEISR**
					6.8	73063	411	36	48	8.3e-037	196	2 peptides	IIND**LLQSLR** AIN**QQTGAFVEISR**
					6.7	73458	349	29	44	1.3e-030	178	2 peptides	IIND**LLQSLR** AI**NQQTGAFVEISR**
					6.6	73063	259	27	44	1.3e-021	88	1 peptide	AI**NQQTGAFVEISR**
					6.9	73458	119	13	25	1.3e-007	43	1 peptide	AINQ**Q**TGAFV**EISR**
Q96I24	Far upstream element binding protein 3	1	67473	7.2	8.3	61944	139	18	48	2.9e-009	-	-	-

Peptide sequences in bold show fragment of peptide sequence responsible for generating MS/MS-spectra. [a]Theoretical molecular weight. [b]Theoretical isoelectric point. [c]Observed isoelectric point. [d]Observed molecular weight.

MS/MS spectra of m/z 931.56 m/z 1336.72 and m/z 1539.80 were significantly matched to peptide sequences of 'FAV GIVIGR', 'IGGNEGIDVPIPR' and 'CQHAAEIITDLLR', respectively (Fig. 3b). Collectively, the data confirm unambiguous assignment of the two spots to FBP1 (accession no. Q96AE4).

Not unlike FBP1, all of the five spots of FBP2 that were identified by mass spectrometry had shown significant matching following MS/MS. PMF analysis showed a sequence coverage of 32, 48, 44, 44 and 25% for spot FBP2a-e, respectively (Table I, Fig. 2b). The computer then picked up a list of peptides for fragmentation and for a series of tandem spectrometry. Two peaks for spot FBP2a (m/z 1184.73 and m/z 1533.83) (Fig. 3c), spot FBP2b (m/z 1184.70 and m/z 1533.81) (Fig. 3d) and spot FBP2c (m/z 1184.70 and m/z 1533.81m) (Fig. 3e), and one for FBP2d (m/z 1533.82) (Fig. 3f) and FBP2e (m/z 1533.82) (Fig. 3g) were picked from the generated MS spectrum and found to significantly match with the peptide sequences given in Table I following MS/MS analysis (Fig. 3c-g), pointing to the unequivocal assignment of the spots to FBP2 (accession no. Q92945).

The single spot identified as FBP3 (accession no. Q96I24) by MS had a sequence coverage of 48% (Fig. 2c). Although the spectra obtained following MS analysis assigned the spot to FBP3 (Fig. 3h), the effort made to confirm by MS/MS was not successful due to several reasons.

Discussion

FBP1 was originally identified as a factor binding to FUSE, a positive *cis*-element of the human c-*myc* gene (3,14,15). In addition to their transcriptional role, FBPs have been reported to bind RNA and participate in various processes of RNA processing, transport and catabolism (16). Members of the *myc* family of oncogenes, including N-*myc*, c-*myc*, and L-*myc* have been implicated in the development of many human tumors. Myc forms a heterodimer with Max and binds to E-box elements in promoter and/or enhancer regions of target genes to modulate transcription (17).

The c-*myc* gene encodes an important member of the helix-loop-helix leucine zipper family of transcription factors and is involved in cell growth, proliferation, differentiation and apoptosis (1,3,14,15), thus its deregulation contributes to formation of a variety of tumors. Indeed, overexpression of c-*myc* was one of the first acquired genomic alterations found in medulloblastoma (18) and the expression of c-*myc* mRNA

by the tumor correlates with poor survival, suggesting that high c-*myc* expression produces a more aggressive tumor phenotype (19). Although this deregulation largely results from genomic amplification of c-*myc*, mechanisms independent of genomic amplification have also been suggested to lead to activation of c-*myc* in medulloblastoma cell lines (20). Regulation of the abundance or activity of proteins binding to the c-*myc* gene may thus provide an essential non-genomic mechanism for controlling c-*myc* expression. In this regard, although there are some techniques, including immunochemistry that can be of use, a robust and reliable analytical tool is lacking. In the present study, we systematically elaborated the protein profile of the DAOY medulloblastoma cell line by a high-throughput proteomic analysis and unambiguously identified FBPs, which opens up an exciting possibility to better understanding of tumor biology.

FBP mRNA has been shown to be expressed broadly, but to varying levels, in different tissues and cell lines (3,14). FBP1 protein is also detected in the HeLa cell line by immunoblotting and/or immunostaining (16,21). In our earlier study (7), FBP1 protein was detected only in D283, FBP2 in both D283 and DAOY cell lines, and FBP3 in none of the cell lines. Vindigni *et al* (22) purified DNA helicase V by affinity chromatography from a HeLa cell line and reported that this helicase is identical to FBP1. They showed that helicase V/FBP1 unwinds DNA with a 3' to 5' polarity in a strictly ATP-dependent fashion, although an accumulated body of evidence indicates that FBP1 binds to single-stranded or torsionally strained DNA and is devoid of inherent unwinding activity (1,23).

Here, we also detected several spots representing FBP1 and FBP2, probably indicating the existence of different splice variants, isoforms or a post-translationally modified form of the same variant. Indeed, two splice variants of FBP1 have been entered in the Swiss-Prot database (Q96AE4-1 and Q96AE4-2), although no experimental confirmation is available.

Though all the spots representing FBP1 and FBP2 produced significant MS and MS/MS results, this could not be replicated at the stage of tandem mass spectroscopy when it came to FBP3. It is well known that there are numerous sources of error associated with the collection and processing of tandem mass spectra including, but not limited to, errors attributed to the number of ion counting events (i.e., counting statistics), the inherently random nature of the fragmentation process, and errors attributed to centroiding (24). All of these sources might have contributed to the relative failure to characterize FBP3. The possibility that failure of identification by MS/MS could be attributed to low abundance of the protein cannot be totally excluded. A growing body of evidence indicates that post-translational modifications such as phosphorylation, acetylation and fatty acid modification can shift the pI to lower values and increase the molecular weight to higher values (25). It is thus plausible that such modifications could explain the shift in pI and molecular weight in all FBPs. Indeed, database search predicting possible post-translational modifications revealed that phosphorylation (http://www.cbs.dtu.dk/services/NetPhos/) is the likely modification to occur with FBP.

The transcription of the human c-*myc* gene is affected by multiple *cis*-elements that are present both upstream and downstream of the promoter sites and a multitude of signals can affect a cell's decision to proliferate by acting through these *cis*-acting elements. Single-strand DNA in these *cis*-elements is induced by torsion and flexural strain exerted on the DNA during the course of transcription (5) and the recognition of single stranded cis-elements by transcriptional regulators provides a mechanism for the re-establishment of transcription after mitosis (26) and the tight control of oncogenes (23,27). FUSE, one of the *cis*-elements, is most active in undifferentiated cells and becomes inactive as differentiation is induced and transcription initiation is shut off (2,14). FBP1 is also shown to be highly expressed in dividing cells with a temporal and regulatory profile paralleling c-*myc*. However, no growth-regulated or differentiation-specific expression of FBP2 and FBP3 has been observed, implicating FBP1 as a candidate for regulating cell growth and differentiation (4). The lingering expression of FBP2 and FBP3 during differentiation while that of FBP1 is shut off suggests that FBPs may have other cellular activities apart from growth regulation, such as regulation of other molecules with short half-life where small changes in expression modify cellular activity (23). Thus, a protein analytical tool that allows expression of all the FBP proteins to be detected offers a fertile ground for discerning other cellular roles of the proteins.

Proteomics is a particularly rich source of biological information because proteins are involved in almost all biological activities and they also have diverse properties, which collectively contribute greatly to our understanding of biological systems (28). The high precision of mass spectrometric measurements can distinguish closely related species, and tandem mass spectrometry or MS/MS can provide structural information on molecular ions that can be isolated and fragmented within the instrument. Detection of the variants of FBP in the DAOY cell line using MS/MS indeed confirms the robustness of this method compared to PMF and presents an opportunity, without a need for antibody availability and specificity, for unambiguous assigning of identity to proteins as well as for manipulating the protein to further our understanding of tumor biology.

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