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Natural N-terminal fragments of brain abundant myristoylated protein BASP1

Vladislav V. Zakharov[a,*], Jean-Paul Capony[b], Jean Derancourt[b], Ekaterina S. Kropolova[a], Vera A. Novitskaya[a], Marina N. Bogdanova[a], Mark I. Mosevitsky[a]

[a] *Molecular and Radiation Biophysics Division, Petersburg Nuclear Physics Institute of Russian Academy of Sciences, 188300 Gatchina, Leningrad District, Russia*

[b] *Centre de Recherches de Biochimie Macromoleculaire du Centre National de la Recherche Scientifique, INSERM U 249, BP 5051, 34033 Montpellier, France*

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Abstract

BASP1 (also known as CAP-23 and NAP-22) is a novel myristoylated calmodulin-binding protein, abundant in nerve terminals. It is considered as a signal protein participating in neurite outgrowth and synaptic plasticity. BASP1 is also present in significant amounts in kidney, testis, and lymphoid tissues. In this study, we show that BASP1 is accompanied by at least six BASP1 immunologically related proteins (BIRPs), which are present in all animal species studied (rat, bovine, human, chicken). BIRPs have lower molecular masses than that of BASP1. Similarly to BASP1, they are myristoylated. Peptide mapping and partial sequencing have shown that BIRPs represent a set of BASP1 N-terminal fragments devoid of C-terminal parts of different length. In a definite species, the same set of BASP1 fragments is present in both brain and other tissues. The sum amount of the fragments is about 50% of the BASP1 amount in a tissue. Obligatory accompanying of BASP1 by a set of specific fragments indicates that these fragments are of physiological significance.

Keywords: BASP1; CAP-23; NAP-22; Protein form; N-terminal fragment; Myristoylation

1. Introduction

Various proteins, in particular those participating in signal transduction, exist as families comprising several forms. These protein forms possess similar but not identical primary structures that provide a basis for variability in protein–protein interactions and functions. In neurons, this feature is a prerequisite for regulation of numerous signal processes occurring in nerve terminals. For example, the following protein families may be mentioned: protein kinase C (PKC), which is expressed as at least 12 isoforms (α, βI, βII, γ, δ, etc.) [1], MARCKS and MacMARCKS/F52 [2], SNARE proteins of the presynaptic exocytosis machinery, in particular, SNAP-25 (A and B), syntaxins (1A, 1B, 2, 3A–3E, 4–6), VAMP (I, II and cellubrevin), synaptotagmins (I–IX) and neurexins (Iα, Iβ, IIα and IIβ) [3]. Protein forms can be encoded by different genes (as in the cases of VAMP, synaptotagmins and syntaxins), can result from alternative splicing of RNA (as SNAP-25 forms), as well as can be formed by the both mechanisms (as PKC isoforms and neurexins). Distinct protein forms, often with autonomous physiological functions, can also be generated by site-specific proteolysis. For example, two forms of neuronal protein GAP-43 are produced by specific calcium-dependent proteolysis of this protein mediated by calpain [4,5].

Earlier we have isolated and characterized acid-soluble protein BASP1, which is abundant in nerve endings [6,7]. It is also known as CAP-23 [8] and NAP-22 [9]. BASP1 is a protein of 23 kDa with very acidic isoelectric point (p*I* 4.3–4.5). It is soluble in 5% perchloric acid, and shows an anomalous mobility on SDS polyacrylamide gel electrophoresis (SDS-PAGE). BASP1 can be phosphorylated by PKC on Ser-5, and binds calmodulin in a calcium- and phosphorylation-dependent manner [10]. In spite of high hydrophilicity, BASP1 possesses some hydrophobic properties due to the presence of myristic acid residue on its N terminus. [7,11]. N-myristoylation seems to be important for BASP1 binding to a lipid membrane [7,11] as well as for interaction with calmodulin and PKC [12]. Fatty acylation

* Corresponding author. Tel.: +7-812-328-2802; fax: +7-812-713-2303.
E-mail address: zakharov@vz5518.spb.edu (V.V. Zakharov).

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results in BASP1 retardation in the course of acetic acid/urea PAGE in the presence of Triton X-100 [6,7]. By a number of properties, BASP1 is similar to neuronal protein GAP-43 [13,14] and prominent protein kinase C substrate MARCKS [2]. BASP1, GAP-43 and MARCKS are thought to play similar and synergistic roles in neurite outgrowth and synaptic plasticity [15–20]. In contrast with exclusive presence of GAP-43 in nervous system, BASP1 is also present in considerable amounts in some non-nervous tissues—spleen, thymus, kidney and testis, where its functions have not yet been studied [7].

In the present study, we have discovered that in all tissues, where BASP1 is present, it is accompanied with a few immunologically related proteins (BIRPs) possessing lower molecular masses. We have shown that these proteins represent BASP1 fragments devoid of C-terminal parts of different length. Similarly to BASP1, the fragments are N-terminally myristoylated. We have revealed similar sets of BASP1 fragments in rat, bovine, human and chicken species. This finding indicates that they have physiological significance. Some part of this work has been previously presented in abstract form [21].

2. Materials and methods

2.1. Chemicals and antibodies

Complete™ EDTA-containing protease inhibitor cocktail and pepstatin were from Boehringer Mannheim. All electrophoretical reagents of analytical grade were from Serva. Protein A conjugated with horseradish peroxidase was from the Pasteur Institute for Microbiology (St. Petersburg, Russia). Methylated trypsin of sequencing grade was from Promega. Other reagents were from Sigma. Polyclonal antibodies were raised in rabbits against rat, bovine, human and chicken BASP1. Brain and other tissue samples were kept in liquid nitrogen from the moment of their removal until use.

2.2. Protein isolation

Protein isolation was performed as described in Refs. [6,7]. Briefly, tissue samples (of about 5 g) were rapidly defrosted at 0 °C and homogenized in 4 volumes of ice-cold 10 mM Tris–HCl (pH 7.4) in the presence of protease inhibitors (Complete™ EDTA-containing Protease Inhibitor Cocktail and 1 μM pepstatin) by 5–10 strokes in a hand homogenizer. Triton X-100 and perchloric acid were added to tissue homogenate to reach final concentrations of 1% and 5%, respectively, and the mixture was incubated for 30 min at 0–4 °C with continual stirring. Then the suspension was centrifuged at 8000 × *g* for 10 min. Proteins present in supernatant were precipitated by addition of 6 volumes of cold (−20 °C) acetone acidified by 0.1 M HCl. Twelve to sixteen hours later, the proteins were pelleted by centrifugation at 8000 × *g* for 15 min at −20 °C, washed with acetone and dried under vacuum.

2.3. Polyacrylamide gel electrophoresis (PAGE)

Protein pellets were solubilized in acetic acid/urea sample buffer (0.9 M acetic acid, 6 M urea, 10% [w/v] glycerol, 2% 2-mercaptoethanol, 0.005% methyl green). PAGE was performed in 12% gels in 0.9 M acetic acid/2.5 M urea [22]. The gels were stained with Coomassie R-250.

Two-dimensional PAGE was performed in 12% gels using acetic acid/urea system for the first dimension and 0.9 M acetic acid/2.5 M urea/8 mM Triton X-100 system [23] or common SDS system [24] for the second one. The strips of the first dimension gel containing protein tracks were excised and incubated in appropriate sample buffer for 10 min. Then the strips were placed on the top of the second dimension gel, fixed with melted 1% agarose in the same sample buffer, and the proteins were resolved in the second dimension.

2.4. Immunoblotting

The immunoblots were prepared using rabbit polyclonal antibodies raised against rat, bovine, human and chicken BASP1. The blots were treated by protein A conjugated with horseradish peroxidase using a standard chromogenic procedure. The antiserum dilution used (1:300–1:500) was well within the linear range for semi-quantitative BASP1 detection on immunoblots. The relative amounts of BASP1 and immunologically related proteins (BIRPs) were estimated by scanning densitometry of the immunoblots using ScanDens computer software.

2.5. Purification of BASP1 and BIRPs

The proteins were purified by preparative electrophoresis in 12% polyacrylamide gels reversibly cross-linked by 0.15% *N*,*N*′-diallyltartardiamide (DATD) using acetic acid/urea/Triton X-100 system. After electrophoresis, the gels were faintly stained with Coomassie R-250. The strips with protein bands were excised, ground by glass stick and incubated with about equal volume (0.5–1 ml) of 3% periodic acid at 0 °C for 20 min (till gel swelling). Then the gel pieces were ground by glass stick to homogeneity. Polyacrylamide was precipitated by addition of 2 volumes of cold (0 °C) acetone and removed by centrifugation at 8000 × *g* for 10 min. Proteins present in supernatant were precipitated by the addition of still 4 volumes of acetone. The proteins were additionally purified by reversed-phase HPLC (RP HPLC) using a Pharmacia Smart System on μRPC C2/C18 column (2.1 × 100 mm) filled with 3 μm diameter beads. The gradient was from 5% to 95% of solvent B (95% acetonitrile, 0.075% TFA) in solvent A (0.1% TFA) for 60 min. Flow rate was 100 μl/min. The eluted samples were lyophilized.

2.6. Peptide maps

Protein samples were dissolved in 10 μl 0.1 M *N*-ethylmorpholine (pH 8) and digested overnight at room temperature with 0.5 μg of methylated trypsin. Peptides were resolved by running the digests on microbore RP HPLC columns (1 × 100 mm). Solvent A was 0.1% TFA, solvent B was 80% acetonitrile, 0.08% TFA. Flow rate was 20 μl/min. The following gradient was used: 5% B up to 15 min; 5–10% B from 15 to 45 min; 10–22% B from 45 to 100 min; 22–34% B from 100 to 128 min. The peaks (peptides) were collected separately and partially sequenced from the N-terminus through 5–10 residues using a gas phase sequencer model 470 A coupled to a PTH analyzer model 120 A from Perkin-Elmer. Molecular masses of the peptides were determined on a BioTech Fisions Trio 2000 mass spectrometer equipped with an electrospray source. These data were used to dispose the peptides on BASP1 amino acid sequence.

3. Results and discussion

3.1. Existence of BIRPs in rat brain

After treating rat brain homogenate with 1% Triton X-100 and 5% perchloric acid, a limited number of proteins, including BASP1, remain soluble [6,7]. Immunoblots reveal, in addition to BASP1, a number of immunologically related to BASP1 proteins (BIRPs), that is, the proteins recognized by polyclonal antibodies raised against rat BASP1 (Fig. 1A). The pattern of minor immunostained protein bands (BASP1-a, -b, -c, -d, -e, -f) is shown in Fig. 1A. This pattern is highly reproducible in independent protein preparations derived from the whole brain or from its different regions (forebrain, cerebellum, spinal cord, etc.) under stringent conditions in the presence of complex inhibitor cocktail against main types of protease activity (serine-, cysteine-, aspartic- and metalloproteases). Therefore, appearance of these proteins cannot be ascribed to BASP1 degradation during tissue homogenization and protein isolation. Moreover, after incubation of whole rat brain homogenate (or isolated brain synaptosomes) for 5 h at +37 °C in the absence of protease inhibitors, no proteolytic conversion of BASP1 to BIRPs was detected (Fig. 2A). Under the same conditions, neuronal protein GAP-43 proved to be substantially converted into two big proteolytic fragments devoid of 4 and 40 N-terminal residues (Fig. 2B, see also Ref. [4]). Therefore, we conclude that BIRPs coexist with BASP1 in brain in vivo. To finally exclude the possibility of BIRPs appearance as products of BASP1 artificial proteolysis during protein isolation, we have performed a short-cut protein isolation experiment. Rat brain tissue was defrosted and homogenized in 4 volumes of 2% SDS at 0 °C. After centrifugation, the proteins present in supernatant were precipitated with cold acetone (see Materials and methods). Pelleted proteins were solubilized in SDS sample buffer and subjected to SDS-PAGE. The immunoblots (data not shown) revealed a set of BIRPs similar to that observed in the standard experiments described above (Fig. 1A).

As shown by SDS-PAGE (second dimension in Fig. 1B), all BIRPs possess lower molecular masses in comparison with BASP1. Their apparent molecular masses range from 30 to 50 kDa, while apparent molecular mass of BASP1 under the same conditions is of about 58 kDa (Fig. 1B). As known, BASP1 shows an anomalous migration on SDS-PAGE and its real molecular mass is equal to about 23 kDa [6,8,9]. Since BIRPs also have an anomalous behaviour on

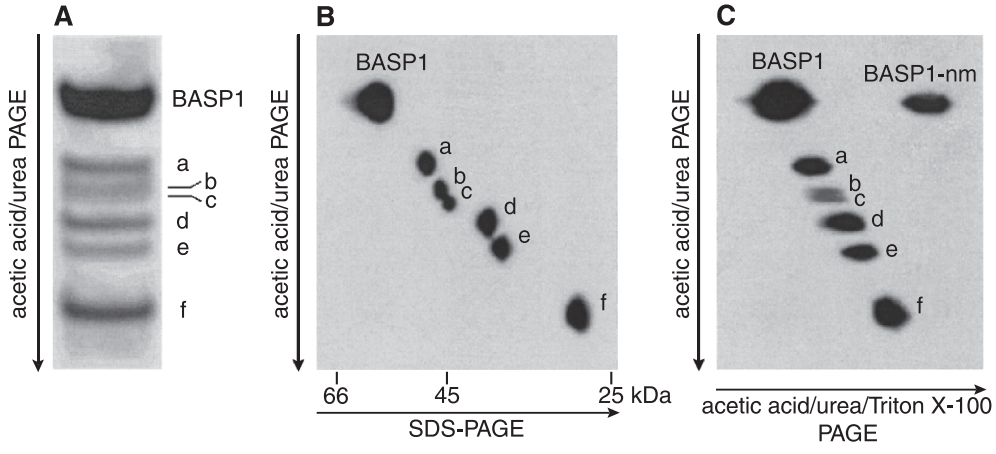


Fig. 1. Rat brain BIRPs as revealed by immunoblotting. Proteins extracted with 1% Triton X-100 and 5% perchloric acid were subjected to 12% PAGE in different electrophoretic systems. BASP1 and BIRPs were detected by antibodies raised against rat BASP1 in rabbits. (A) PAGE in acetic acid/urea. (B) 2D-PAGE in acetic acid/urea (first dimension, see A) and in SDS (second dimension). Positions of molecular mass markers in SDS-PAGE are shown. (C) 2D-PAGE in acetic acid/urea (first dimension, see A) and in acetic acid/urea/Triton X-100 (second dimension). Designations: BASP1-a, -b, -c, -d, -e, -f—myristoylated BIRPs; BASP1-nm—non-myristoylated BASP1.

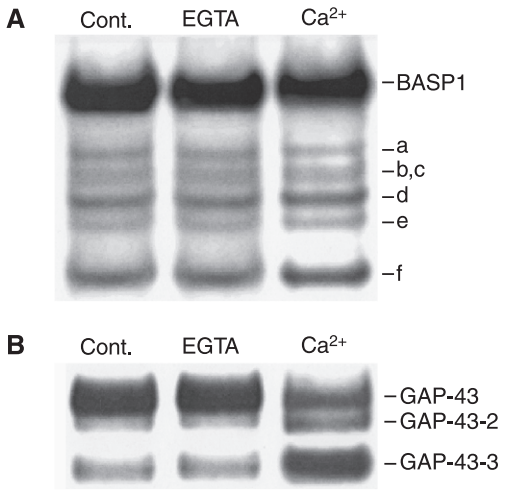


Fig. 2. Failure to detect accumulation of BIRPs during incubation of whole rat brain homogenate. Rat brain homogenate was prepared without any protease inhibitors and incubated for 5 h at +37 °C with 1 mM EGTA (lane 2) or 1 mM Ca^{2+} (lane 3). Lane 1: proteins isolated from non-incubated rat brain homogenate (control). Proteins were electrophoresed in 12% gel in acetic acid/urea. Total amount of protein loaded was 40–60 μg per line. The same picture was observed in three different brain homogenate preparations. (A) BASP1 and BIRPs detected by anti-rat-BASP1 antibodies. (B) Neuronal protein GAP-43 and its proteolytic fragments detected by anti-rat-GAP-43 antibodies. GAP-43-2 and GAP-43-3 are GAP-43 fragments devoid of 4 and 40 N-terminal residues, respectively.

SDS-PAGE (data not shown), their actual molecular masses may range from 12 to 19 kDa.

3.2. Myristoylation of BIRPs

Due to N-terminal myristoylation, BASP1 exhibits retardation during gel electrophoresis in acetic acid/urea system in the presence of Triton X-100 [6,7]. Fig. 1C shows that under these conditions, all BIRPs (BASP1-a, -b, -c, -d, -e, -f) exhibit similar retardation. Indeed, BIRPs and myristoylated BASP1 line up in a single diagonal, while non-myristoylated and, therefore, not retarded BASP1 (BASP1-nm, see below) moves outside. Note that in the absence of Triton X-100 (first dimension), myristoylated and non-myrystoylated BASP1 molecules moved as a single band. We conclude that similarly to the main part of BASP1 molecules, BIRPs (a–f) are myristoylated.

3.3. BIRPs are present in non-nervous tissues. Comparison of rat, bovine and human BIRPs

We showed earlier that besides the brain, BASP1 is present in significant amounts in rat testis, kidney and lymphoid tissues [7]. BIRPs (a–f) are also present in these tissues. In all tissues studied, the overall content of BIRPs comprises 50–60% of the content of BASP1. Furthermore, their relative amounts in different tissues are similar (Fig. 3A–C). This is not the case for many other protein families. For example, different forms of protein kinase C are characteristic for different tissues: ε-PKC is localised in brain only, γ-PKC—in brain and spinal cord, θ-PKC—in skeletal muscle and hematopoietic cells, etc. [1]. Similarly, the isoforms of synaptic plasma membrane protein SNAP-25 (A and B) are characteristic for nervous tissue, but exhibit anatomically distinct patterns of expression [25].

BIRPs isolated from the tissues and cultured cells originating from other species (bovine and chicken brain, human HeLa cells) show electrophoretic patterns similar to the patterns of BIRPs present in the rat tissues (Fig. 3D,E). Species specificity of the patterns reflects the species specificity of primary structures of BIRPs. It should be noted that electrophoretic mobilities of rat, bovine and human BASP1 are also slightly different due to their imperfect amino acid sequence similarity (70–80%) [6,7]. Compulsory expression of all BIRPs in different tissues as well as preservation of BIRPs in evolution certifies its physiological significance.

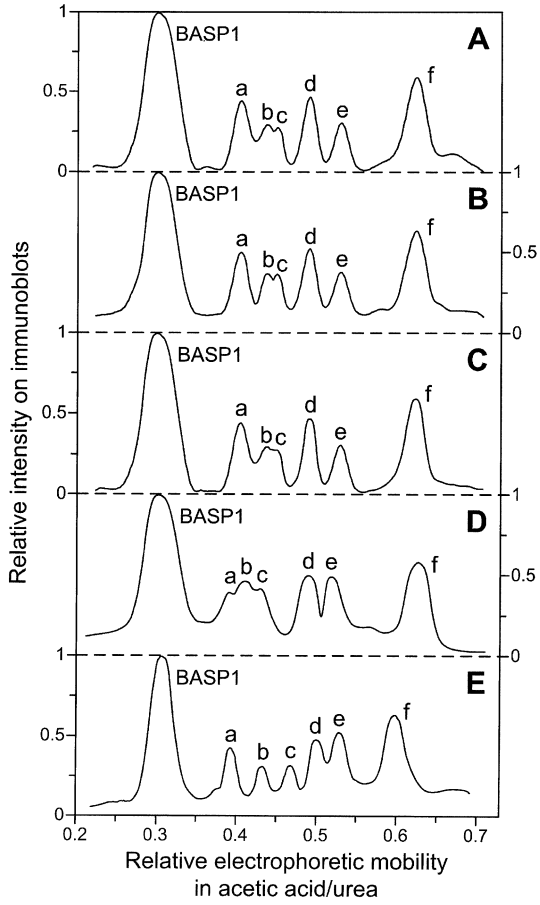


Fig. 3. BIRPs are present in rat brain (A), rat testis (B), rat kidney (C), bovine brain (D) and human HeLa cells (E). Proteins were electrophoresed in 12% gel in acetic acid/urea and immunostained by anti-rat-BASP1, anti-bovine-BASP1, or anti-human-BASP1 antibodies, each in their respective cases. Relative intensity of protein bands on immunoblots was measured by scanning densitometry. Abscissa axes represent the direction of electrophoresis. The curves are averaged data from three independent experiments (scatter of corresponding data in the repeated experiments did not exceed 10%).

3.4. BIRPs are BASP1 fragments devoid of C-terminal parts of different lengths

We attempted to purify bovine BIRPs by a combination of preparative electrophoresis in acetic acid/urea/Triton X-100 and RP HPLC. It proved impossible to obtain pure samples of individual BIRPs, because they eluted from RP HPLC column as a single broad peak. Purified mixture of BIRPs (a–f) and (separately) BASP1 from bovine brain were subjected to tryptic digestion and peptide mapping (Fig. 4). Comparison of the plots A and B in Fig. 4 shows that peptide map of BIRPs contains the same peptides as the map of BASP1 except for peptides 8, 9, and 9a, which are missing in the map of BIRPs. The peptides 8, 9, and 9a correspond to BASP1 C-terminal amino acid sequence 184–226 (Fig. 4C). Similar results were obtained, when endoproteinase Asp-N was used for protein digestion. In this case, the peptide map of BIRPs did not contain peptides originating after the residue 170 (data not shown). Therefore, we conclude that BIRPs are fragments of BASP1 molecule devoid of C-terminal parts of different length. The longest BIRP is devoid of 40–60 C-terminal residues.

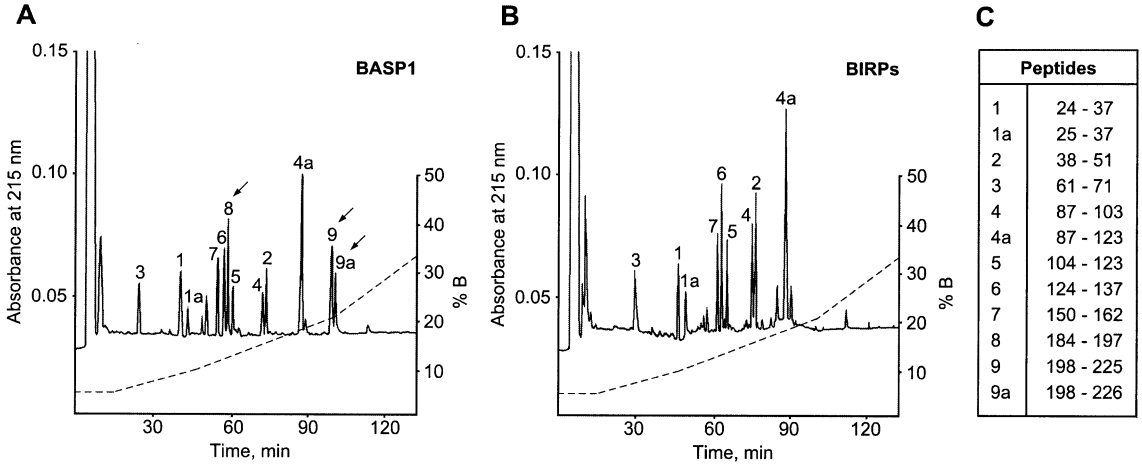
Peptide map of BASP1-nm was also made. It turned out to be the same as the map of BASP1. This supports our assumption that BASP1-nm is a non-myristoylated full-length form of BASP1.

3.5. Origin of BIRPs

Partial sequences determined on the N-ends of the peptides prepared from BIRPs (Fig. 4B) comprise, totally, about 40% of the whole BASP1 molecule. They proved to be identical to corresponding sites of BASP1 molecule (no mutations were revealed). This result is in agreement with the data showing that only one *basp1* gene exists [8,26,27]. Therefore, BASP1 fragments are formed either due to processing of RNA (alternative splicing or mRNA editing) or by specific proteolysis of BASP1 protein.

An exploration of nucleotide genome sequences deposited in GenBank shows that human, mouse and rat *basp1* genes contain two exons. The complete protein-coding sequence is located in the second exon (V.V. Zakharov, unpublished data). Therefore, appearance of different forms of BASP1 is impossibly to be due to alternative splicing. Editing of mRNA can generate truncated protein forms (devoid of C-terminal parts) in the case of cytidine to uridine deamination in codons CAA (Gln), CAG (Gln), or CGA (Arg) resulting in appearance of a terminating codon [28–30]. In contrast to already known examples of such editing, BASP1 mRNA should contain several sites where such editing can occur, because several truncated BASP1 molecules (BIRPs) are observed.

In the case of BIRPs formation by site-specific proteolysis, the process should be restricted to a definite cell compartment. For example, it can occur shortly after BASP1 synthesis on ribosomes bound to endoplasmic reticulum. These or some other restrictions may ensure balanced production and, therefore, high similarity of the amounts of the fragments relatively to each other and to BASP1 in different tissues (Fig. 3A–C) as well as in repeated experiments. An example of such kind of specific proteolysis is cotranslational generation of p50 subunit of transcription factor NF-κB with participation of 26S proteasome [31]. We are not certain now that exactly one of these mechanisms is responsible for BASP1 N-terminal fragments (BIRPs) formation. One can expect that some another way not yet described may be used here. Localization of the C-ends of the BIRPs on BASP1 primary structure, and, therefore, the possibility to analyze the adjoining amino acid sequences will be helpful for elucidation of the real mechanism of N-terminal BASP1 fragments formation. This study is now in progress.



Peptides	
1	24 - 37
1a	25 - 37
2	38 - 51
3	61 - 71
4	87 - 103
4a	87 - 123
5	104 - 123
6	124 - 137
7	150 - 162
8	184 - 197
9	198 - 225
9a	198 - 226

Fig. 4. Peptide maps of bovine BASP1 and BIRPs obtained by tryptic digestion. BASP1 and mixture of BIRPs were purified by preparative electrophoresis and RP HPLC and digested by trypsin. Resulted peptides were resolved by HPLC and then subjected to N-terminal sequencing and molecular mass determination for identification. (A) Peptide map of bovine BASP1. (B) Peptide map of mixture of bovine BIRPs. (C) Peptides identified in A and B. Arrows indicate the peaks in A that are absent in B.

4. Conclusion

Membrane bound N-terminally myristoylated protein BASP1 is abundant in brain and some other tissues (testis, kidney, spleen) of mammals and birds. We have discovered that in all tissues, where BASP1 is expressed, it coexists with a number of immunologically related proteins (BIRPs). These proteins are BASP1 N-terminal fragments devoid of C-terminal parts of different length, but retaining myristic acid residue on their N-termini. The function of BASP1 fragments is not known, but their preservation in different species is a sign of their physiological significance. Further investigations of both mechanism of BASP1 N-terminal fragment formation and their functions are very promising.

Acknowledgements

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