Novel Alternative Splicings of BPAG1 (Bullous Pemphigoid Antigen 1) Including the Domain Structure Closely Related to MACF (Microtubule Actin Cross-linking Factor)*

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BPAG1 (bullous pemphigoid antigen 1) was originally identified as a 230-kDa hemidesmosomal protein and belongs to the plakin family, because it consists of a plakin domain, a coiled-coil rod domain and a COOH-terminal intermediate filament binding domain. To date, alternatively spliced products of BPAG1, BPAG1e, and BPAG1n are known. BPAG1e is expressed in epithelial tissues and localized to hemidesmosomes, on the other hand, BPAG1n is expressed in neural tissues and muscles and has an actin binding domain at the NH2-terminal of BPAG1e. BPAG1 is also known as a gene responsible for Dystonia musculorum (dt) neurodegeneration syndrome of the mouse. Another plakin family protein MACF (microtubule actin cross-linking factor) has also an actin binding domain and the plakin domain at the NH2-terminal. However, in contrast to its high homology with BPAG1 at the NH2-terminal, the COOH-terminal structure of MACF, including a microtubule binding domain, resembles dystrophin rather than plakins. Here, we investigated RNAs and proteins expressed from the BPAG1 locus and suggest novel alternative splicing variants, which include one consisting of the COOH-terminal domain structure homologous to MACF. The results indicate that BPAG1 has three kinds of cytoskeletal binding domains and seems to play an important role in linking the different types of cytoskeletons.

BPAG11 and BPAG2 are the autoantigens of bullous pemphigoid (BP), an autoimmune subepidermal skin blistering disease (1). Both are components of hemidesmosomes, and BPAG1 is a 230-kDa cytoplasmic protein and anchors keratin-containing intermediate filaments (IFs) to the inner plaque of hemidesmosomes (1–3). BPAG2 is a 180-kDa transmembrane protein with interrupted collagen domains in its extracellular part (1–3). BPAG1 belongs to the plakin family, including plakin, desmoplakin, envoplakin, and perilamin, which associate with IFs in various tissues (4, 5). Plakins have a common structure consisting of an amino (NH2)-terminal plakin domain, a central coiled-coil domain, and a carboxyl (COOH)-terminal domain containing the IF binding domain (4, 5). In addition, BPAG1 and plectin give rise to alternative splicing variants, which have an actin binding domain (ABD) at the NH2 terminus (6–11). BPAG1 with ABD expressed in nervous tissues and muscles are distinguished as BPAG1n from BPAG1e that has no ABD and localizes to the hemidesmosome in the epithelial tissue (11–13). Consistent with the expression and localization, BPAG1-deficient mice show skin blistering and neurodegeneration (12). Also, the mouse mutant, Dystonia musculorum (dt), which shows neurodegeneration but normal skin, is known to result from loss of BPAG1n but normal expression of BPAG1e (12).

Mammalian protein MACF (microtubule actin cross-linking factor) and Drosophila protein Kakapo are classified as members of the plakin family (13–15). Human MACF was also reported as ABP620 or trabeculin-α (16, 17). They are a novel type of plakin proteins, because their COOH-terminal structures containing microtubule binding domain are completely different from the classical plakins. They consist of long spectrin repeats, following two EF-hand calcium binding motifs and a growth arrest-specific 2 protein (GAS2)-related region, which resembles dystrophin rather than plakin (15–17). However, their NH2-terminal ABD and plakin domain are very similar to that of typical plakins. In particular, the ABD and the plakin domain of MACF are closely related to those of BPAG1 not only by sequence but also by their alternative splicing pattern of the NH2-terminal portion of the ABD (11, 13, 18).

In the present study, we analyzed the genomic organization and sequence of human BPAG1 and characterized novel alternative splicing products. Several alternatively spliced variants of BPAG1 were detected by RT-PCR, and their expression in tissues and cultured keratinocytes were examined at the RNA and the protein level. The results revealed new features of the BPAG1 gene. The detected alternatively spliced variants included one consisting of the same domain structure as MACF and indicated that BPAG1 and MACF are closely related not only at the 5′-located exons but also across their entire gene organization.

EXPERIMENTAL PROCEDURES

Cells—The DJM-1 cell line, isolated from a human squamous cell carcinoma (19), was the kind gift of Dr. Kitajima (Gifu University, Gifu, Japan). The cells were maintained in Eagle's minimal essential medium supplemented with 10% fetal calf serum, 20 ng/ml EGF, 400 ng/ml hydrocortisone, 84 ng/ml cholera toxin, and 2 mM glutamine.

RT-PCR and Sequencing—Total RNA from DJM-1 cells was prepared using QuickPrep® Total RNA Extraction Kit (Amersham Biosciences, Inc.), according to the manufacturer's protocol, and converted to...
Novel Alternative Splicings of BPAG1

Rodless Variant of BPAG1e

The rodless variant of BPAG1e, encoded by alternative splicing, contains a complete COOH-terminal globular domain but lacks the rod domain. This variant is expressed in human kidney and skeletal muscle tissues, indicating its potential role in cell adhesion and signaling.

Results

Novel Alternative Splicing Variants Detected by RT-PCR

The rodless variant of BPAG1e was detected in human kidney and skeletal muscle tissues, suggesting its possible involvement in cell adhesion and signaling. This variant is encoded by alternative splicing and extends the diversity of BPAG1 isoforms.
large ORF described above, showed a high degree of sequence identity to the spectrin repeat domain of MACF (GenBank™ accession number AF141968 or AB029290). Furthermore, we analyzed a cDNA clone KIAA0728 in the HUGE data base of Kazusa DNA Research Institute, which consists of a spectrin repeat domain, two calcium binding EF-hand motifs, and a GAS2 domain. GenBank™ accession number KIAA0728 is very similar to the COOH-terminal region of MACF and maps on the same chromosome 6 as BPAG1 (26), whereas MACF maps on chromosome 1 (16). Considering these facts, we designed several primers that bind to BPAG1e, spectrin repeat region of GenBank™ accession number AL096710 or KIAA0728, and performed RT-PCR with total RNA of DJM-1 cells. Actually, expected cDNA fragments were amplified, and the variant combining these cDNA fragments is shown as BPAG1eA in Fig. 1B. The entire domain structure is nearly the same as those of MACF and Kakapo, BPAG1eA; however, it does not have an ABD. The sequence from the region encoding spectrin repeats to that coding for COOH-terminal domains showed 72% similarity to that of MACF and 51% to the Kakapo protein at the amino acid level, respectively.

**RNA Blot Analysis**

To investigate the tissue distribution of each variant, RNA blot analyses of human tissues with four probes were performed. The probe-N, corresponding to the plakin domain, showed a ~15-kb band in some tissues, such as heart, placenta, liver, and most strongly in skeletal muscle, and weakly in kidney, and a slightly smaller band in brain, liver, kidney, and pancreas. In liver and kidney, the two bands were detected. No clear band, but a smear signal, was detected in other tissues, for example in lung, spleen, thymus, prostate, and testis (Fig. 2A). The probe-A corresponding to the spectrin repeat region also showed a very strong but smear 7~15-kb signal in skeletal muscle and a smear signal in several tissues that we examined. In addition, a 10~11-kb band was weakly detected in testis, ovary, and weakly in small intestine (Fig. 2B). The probe-B corresponding to the large ORF, the second COOH-terminal tail, also showed a strong smear signal in skeletal muscle and a smear signal in heart (Fig. 2C). The probe-C corresponding to the COOH-globular domain gave no signal in any tissues tested (data not shown), but a strong ~9-kb band, the size of known BPAG1e (24), was detected in total RNA of DJM-1 cells (Fig. 2D). This ~9-kb band was also recognized by the probe-N. The probe-A showed two bands, ~10 and ~15 kb, and the probe-B detected no signal in DJM-1 RNA (Fig. 2D). These results indicate that the BPAG1 gene is most active in skeletal muscles and cultured keratinocytes. To determine whether the smear signal indicates ubiquitous expression following rapid turnover of mRNA or bad condition of RNA preparations needs further analyses.

The alternative splicing variants suggested here cannot explain the range of mRNAs detected in tissues examined. For example, the probe-N could not detect all variants shown by the other three probes (Fig. 2, A and D), but detected other smaller mRNA. By RT-PCR, a fragment consisting of a part of a rod domain followed by spectrin repeats was also amplified (data not shown). It has been reported that there are alternative splicing variants with and without 300 amino acids at the similar site in Kakapo (27). These data indicated the existence
of additional splice variants not reported here. In any case, it is difficult to estimate their exact sizes and entire structures because of their large size.

Immunofluorescence Microscopy and Immunoblot Analysis

To investigate the expression of these variants at protein level, we used several antibodies against each domain of BPAG1. mAb-N46 recognizes the plakin domain of BPAG1. mAb-N619 also recognizes that domain, but cross-reacts with the corresponding domain of MACF. mAb-R815 and mAb-C319 recognize the rod domain and the COOH-terminal globular domain of BPAG1e, respectively (Fig. 3). mAb-1A16 also reacted with the rod domain of BPAG1e (data not shown). pAb-BPB and pAb-BPA are the mouse antisera, which recognize the large ORF domain and the spectrin repeat domain of BPAG1, respectively. However, pAb-BPA also recognized the corresponding domain of MACF weakly (Fig. 3). All the antibodies did not react with the plakin domain or the COOH-terminal globular domain of human plectin (data not shown).

By immunofluorescence microscopy, basement membrane zone of human skin, where hemidesmosomes localize to the basement membrane zone, was clearly stained with mAb-N46, -R815, and -C319, but pAb-BPB showed no clear staining, and the pAb-BPA stained the entire living cell layer weakly instead of the basement membrane zone (Fig. 4). The epithelial cells were also stained with the concentrated N46 (Fig. 4A’). DJM-1 cells show very characteristic distribution of hemidesmosomes (28). When DJM-1 cells were stained with these antibodies, mAb-N46, -R815, and -C319 showed hemidesmosomal pattern clearly, whereas pAb-BPB evidenced no clear staining and pAb-BPA stained the entire cytoplasm weakly (Fig. 5). By immunoblot analysis of DJM-1 cell extract, mAb-N46, -1A16, and -C319 reacted with the ~230-kDa band of BPAG1e, but pAb-BPB and pAb-BPA showed no reactivity (Fig. 6). mAb-N619, which cross-reacts with MACF, detected not only the band of ~230 kDa but also higher molecular mass polypeptides (Fig. 6). These bands could not be detected by mAb-N46 and pAb-BPA and are expected to be MACF or its fragments.

**Fig. 3. Reactivities of antibodies to each domain of BPAG1.** Each domain of BPAG1 or MACF was immunoblotted. N46, N619, R815, and C319 are monoclonal antibodies; pBPB and pBPA are mouse sera. BPN, BPR, BPC, BPB, and BPA are the pET fusion proteins with the plakin domain, rod domain, COOH-terminal globular domain, large ORF domain, and the spectrin repeat region of BPAG1, respectively. MAN and MAA are the GST fusion proteins with the plakin domain and the spectrin repeat region of MACF, respectively.

**Fig. 4. Immunofluorescence microscopy of human skin.** A, stained with mAb-N46; A’, with concentrated mAb-N46; B, mAb-R815; C, mAb-C319; D, mouse serum pBPB; E, pBPA. Bar, 40 μm.

**Fig. 5. Immunofluorescence microscopy of DJM-1 cells.** A, stained with mAb-N46; B, mAb-R815; C, mAb-C319; D, mouse serum pBPB; E, pBPA. Bar, 40 μm.

**Fig. 6. Immunoblot analysis of DJM-1 cells.** S, soluble fraction; P, insoluble fraction. Each sample was blotted by the antibodies indicated above the lanes. Markers are at the left, and the arrow indicates the ~230-kDa band of BPAG1e. Dots indicate the bands that are presumably MACF.

DISCUSSION

Both BPAG1n and MACF belong to the plakin family. They are very similar in the NH2-terminal ABD and plakin domain, with 48% identity in amino acid level, but their downstream structures are completely different. BPAG1n has a coiled-coil rod domain and a COOH-terminal globular domain, like other plakins (4, 5). MACF, on the other hand, has long spectrin repeats, two EF-hand motifs and a GAS2 region, resembling dystrophin rather than plakins (15–17).

In light of the diversity of other plakin family members, we...
wondered whether BPAG1 genomic and cDNA sequences represented novel alternatively spliced products of BPAG1. In fact, we could detect their mRNAs by RT-PCR and RNA blotting. In certain tissues, the probe-N, corresponding to the plakin domain, detected mRNAs of larger size than ~9 kbp of BPAG1e mRNA. Since they were also detected by the probe-A and -B, but not by the probe-C, they are thought to represent BPAG1-eA and -eB. However, the signals obtained by probe-A and -B appeared as a smear. This could indicate rapid turnover of these mRNAs or may be dependent on the quality of mRNA samples. In total RNA of the keratinocyte, DJM-1 cell, probe-N, and -C clearly detected a ~9-kbp mRNA, coding the BPAG1e. The probe-N also detected some other smaller mRNAs, although the probe-A detected two bands of large size mRNA. These results do not coincide with our suggestion (Fig. 1). In addition, Leung et al. (29) have reported BPAG1-b of mouse recently, which has the BPAG1eA-like form with the ABD at NH2 terminus and the large ORF domain between the recently, which has the BPAG1eA-like form with the ABD at NH2 terminus and the large ORF domain between the linker domain and the spectrin repeat region, although our RT-PCR system did not detect such a product. These differences may depend on a difference between species or the specificity and sensitivity of each probe or more likely suggest more alternatively spliced variants. In fact, many alternative splicing variants have been reported on plakins. BPAG1, MACF, Kakapo, and plectin have splicing variety at the 5'-region of AB (8–11, 13, 18, 27). Desmplakin and plectin have rodless forms like the BPAG1eS (7, 30, 31). In particular, Kakapo have many alternative splicing patterns at both the NH2- and COOH-terminus, like the BPAG1 suggested here (27). Therefore, our RNA blot data could indicate the existence of alternative alternative splicing products of BPAG1. BPAG1 expressed in neural tissue most likely include BPAG1eA with the ABD, like BPAG1-a reported in mouse (29).

Immunofluorescence microscopy of DJM-1 cells and skin sections with mAb-N46, -R815, and -C319 clearly showed the typical hemidesmosomal staining pattern (28). In addition, mAb-N46 and pAb-BPA stained the entire cytoplasm of keratinocytes and DJM-1 cell, in contrast to the clear signal of 230-kDa BPAG1eA and -eB. However, the signals obtained by RNA blotting, the corresponding mRNA. Since they were also detected by the probe-A and -B, we could think that mRNA may not be translated, we could think that the similarity between BPAG1 and MACF might produce a cross-reacting antibody. In fact, several mAbs including mAb-N619, which we produced against the plakin domain of BPAG1, reacted very strongly with the corresponding domain of MACF (Fig. 3). Therefore, it is especially important to use highly specific monoclonal antibodies for further investigation.

Plakins have a conserved exon organization. The ABD and the plakin domain are distributed to many exons, in contrast to the rod domain and the COOH-globular domain encoded in one exon, respectively (6, 25, 33–36). MACF does not have the rod and COOH-globular domain but has a spectrin repeat region, GAS2 domain, and EF-hand motif (15–17). The organization of corresponding exons is very similar among the genes encoding BPAG1 and MACF (data not shown). So, the alternative splicing pattern may also be conserved. Actually, mAb-N619 detected at least three large polypeptides in immunoblotting, which were not recognized by mAb-N46 and expected to be MACF (Fig. 6). They might be alternative splicing variants of MACF.

Recently, GenBankTM accession number KIAA0728, which corresponds to the COOH-terminal fragment cDNA clone of BPAG1eA, was reported as MACF2, and its mouse homologue has a functional microtubule binding domain (37). This means that BPAG1 has three cytoskeleton binding domains, i.e. for actin, IFs, and microtubules, and all of them are alternatively spliced and expressed in a complex splicing pattern. BPAG1 would thus be a multifunctional cytoskeletal linking factor, in addition to its function at the hemidesmosome.

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