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Biochemical and Biophysical Research Communications 303 (2003) 160–169
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Characterization of alternatively spliced and truncated forms of the Arf guanine nucleotide exchange factor GBF1 defines regions important for activity

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Received 24 January 2003

Abstract

Analysis of multiple transcripts for the Arf-specific guanine nucleotide exchange factor GBF1 identified three positions displaying small in-frame deletions and insertions. Sequencing of genomic DNA for CHO GBF1 and analysis of the human gene established that those variations were consistent with alternate splicing events. RT-PCR analysis of CHO mRNA confirmed that these small in-frame deletions occurred at significant and similar frequencies in both WT and BFA resistant CHO cells. These splice variants behaved like GBF1 in biological assays based on the observation that GBF1 is cytotoxic at high levels but will confer resistance to BFA when moderately overexpressed. Comparison of variants with larger deletions defined regions of 75 (exons 5–7) and 412 (exons 31–39) amino acid residues that were required for cell killing but were dispensable for promoting BFA resistance.

Keywords: ADP-ribosylation factors; Golgi complex; Guanine nucleotide exchange factors; Brefeldin A; Protein traffic

Protein traffic is an essential cellular process that plays a critical role in establishing the temporal and spatial organization of eukaryotic cells [1,2]. A large number of accessory proteins and factors coordinate anterograde and retrograde pathways that facilitate the forward movement of cargo against the constant recycling of enzymes and transport components between Golgi cisternae and back to the endoplasmic reticulum (ER) [3,4]. Transport intermediates coated with cytoplasmic coats perform a key role in both processes. Small GTPases regulate the recruitment of those soluble coat elements to the donor membranes, which then trigger the concentration of cargo and eventually the budding of vesicles [5,6].

COPI is one of the better studied coat proteins and its recruitment to Golgi membranes is dependent on the activation of small GTPases of the Arf family. Arfs are found in the cytosol in the inactive (GDP) form and their activation by exchange of GDP for GTP is linked to a conformational change that exposes a short myristoylated amphipathic N-terminal helix and dramatically increases their affinity for membranes [7–9]. This activation is regulated by a family of Arf-specific guanine nucleotide exchange factors (GEFs). This large family of proteins is characterized by a highly conserved central region, termed the Sec7 domain, that is both necessary and sufficient for GEF activity in vitro [10,11]. Despite a very high degree of conservation of the Sec7d among family members, different GEFs exhibit preference for different Arf substrates. Outside of the Sec7d, the GEFs display highly divergent peptide sequences and can be grouped in two broad categories. The first group is composed of three subfamilies of small GEFs (ARNOs, EFA6, and GEP100) that seem primarily involved in PM-endosomal traffic with Arf6 as their primary substrate. The large GEFs can be subdivided in two subfamilies, Sec7/BIG and Gea/GBF/GNOM, both involved in ER-Golgi transport utilizing class I and II Arfs.

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doi:10.1016/S0006-291X(03)00316-4

Brefeldin A (BFA) is a small fungal metabolite that has become an important tool to investigate protein traffic. BFA blocks coat recruitment and intra-Golgi transport by preventing Arf activation by a subset of sensitive GEFs [12,13]. Interestingly, BFA is an uncompetitive inhibitor that forms an inactive Arf-GDP-GEF intermediate [14,15]. To better investigate the biological effects of this drug, we previously generated several BFA-resistant CHO cell lines (BFY1-24) by mutagenesis [16]. Analysis of mRNA transcripts from one of these lines, BFY1, yielded GBF1, an Arf-GEF whose overexpression promotes resistance to BFA [17]. GBF1 is a 206 kDa protein with a BFA resistant GEF activity towards Arf5 in vitro. Analysis of extracts from WT and BFY1 cells established that GBF1 is expressed at identical levels in both cell lines. Furthermore, preliminary examination of full-length transcripts indicated that the GBF1 isoforms produced in WT and BFY1 cells were similar. These observations suggested that changes in GBF1 did not account for the BFA resistance observed in BFY1 cells.

To further investigate the mechanism of resistance in BFY cells and analyze the GBF1 function, we isolated and characterized several additional mRNA variants from both mutant and WT CHO cells. Here we report that most of these variants presented small in-frame deletions or insertions relative to the original GBF1 isolate that arise from alternative splicing events. Quantitative RT-PCR analysis of mRNAs from WT, BFY1, and BFY2 cells confirmed that these cells produce similar types and amounts of mRNA variants. Finally, characterization of two additional variants with large in-frame deletions uncovered different biological properties that help define domains important for GBF1 function.

Materials and methods

Reagents. Unless otherwise indicated, all chemicals were obtained from Sigma Chemicals (St. Louis, MO, USA). BFA was stored at −20 °C as a stock solution of 10 mg/ml (36 mM) in DMSO. Total RNA and genomic DNA were isolated using Trizol (Gibco-BRL, Gaithersburg, MD, USA) as per manufacturer's instructions. mRNA isolation reagents, proteinase K, cDNA synthesis kits, as well as reagents for RT-PCR analysis were obtained from Gibco-BRL. PCR on genomic DNA was performed using the Expanded High Fidelity PCR system (Roche Diagnostics, Mannheim, Germany). The transformation reagent FuGene 6 and protease inhibitors were obtained from Roche Diagnostics (Mannheim, Germany). Plasmid DNA, DNA gel purification, and PCR purification kits were from Qiagen (Santa Clarita, CA, USA). Restriction enzymes, T4 DNA ligase, alkaline phosphatase, linker-adapters, and *Taq* and Klenow DNA polymerases were from New England BioLabs (Beverly, MA, USA). The pCEP4 plasmid and the TOP10 *E. coli* strain were from Invitrogen (Carlsbad, CA, USA). Random primer labeling kit was from Stratagene (La Jolla, CA, USA). Protein quantitation reagents, protein size markers, and secondary antibodies were from BioRad (Hercules, CA, USA). The radioactive nucleotide [α-^{32}P]GTP was from NEN (Mississauga, ON, Canada). Nitrocellulose membranes and filters were from MSI (Westborough, MA, USA).

Tissue culture. Media, culture reagents, and hygromycin B were purchased from Life Technologies (Gaithersburg, MD, USA). Disposable plasticware and culture dishes were purchased from Falcon (Lincoln Park, NJ, USA). The CHO^{pro-5} and BHK-21 cell lines (American Type Culture Collection; Rockville, MD, USA), the mutant BFY-1 line [16], and the 293-EBNA line (Invitrogen, Carlsbad, CA, USA) were maintained as previously described [17].

cDNA library preparation. Total RNA was extracted from 5×10^7 cells (2 g) of the parental line (CHO^{pro-5}) from which BFY cell lines were derived [16]. Poly(A)$^+$ mRNA (yield of 12 μg) was purified over oligo(dT) columns (Life Technologies) and used for cDNA synthesis according to manufacturer's instructions (Superscript II; Life Technologies). First strand synthesis was primed using the provided oligo(dT)/*Not*I primer-adapter, and a partially duplex *Hin*dIII–*Sal*I adapter with 5′ overhang (pAGCTCGAAGGGGTTCG; New England Biolabs) was blunt-end ligated to cDNAs after second strand synthesis. The largest cDNAs recovered after gel chromatography were treated with *Not*I and ligated into pCEP4 vector pre-digested with *Hin*dIII and *Not*I. The resulting libraries were transformed in *E. coli* TOP10 and greater than 2.5×10^5 colonies displayed on LB plates were harvested, pooled, and stored in aliquots at −70 °C. Analysis of several individual isolates established that the majority (> 90%) of plasmids contain inserts ranging in size from 1 to 4 kb, with a median of 2 kb.

Cloning of GBF1 from wild-type cDNA library. Library aliquots were thawed, added to 1.5 l of warm LB media, and grown to a final OD_{600} of 0.4 (2–3 generations). Approximately 5×10^5 clones from the CHO^{pro-5} cDNA library were displayed on LB plates (about 2×10^4 colonies per 15 cm plate) and screened by colony hybridization using standard techniques [18]. The [^{32}P]dCTP labeled, random primed probe was generated using a Stratagene kit and the *Nhe*I–*Sca*I DNA fragment of GBF1, encompassing the first 2 kb at the 5′ end of the cDNA. Plugs from regions corresponding to putative positives were transferred to 5 ml LB/amp and grown overnight for preparation of plasmids. Southern blotting of *Not*I/*Nhe*I restriction digests of plasmids recovered from a fraction of the overnight culture identified several plugs containing inserts complementary to GBF1. Further analysis of plugs with putative GBF1 cDNAs larger than 4 kb yielded five candidates which were then sequenced using primers previously obtained for analysis of GBF1 [17].

RT-PCR analysis. Total RNA from BFY-1 and CHO^{pro-5} cells was used to synthesize first strand cDNA according to manufacturer's instructions (Superscript II; Life Technologies). The 625/628 nt fragments encompassing position 1863 were amplified using primers +6 [^{1706}ATACCACACACCTACTGTCC$_{1725}$] and −7bis [^{2333}TCATCA AGCCGTAGACCCTG$_{2314}$], while the 639/651 nt fragments for position 4479 were recovered with primer pair +8a [^{3956}AAGTCTACA CTGACCATGGC$_{3975}$] and −2A [^{4606}TGCGTGAGTCAGCTTCAA TC$_{4587}$]. PCR products were labeled by including [^{32}P]CTP in the reaction. Loss of 3 nt at position 1864 eliminates a *Sca*I site. In contrast, loss of 12 nt at position 4479 creates an *Alw*NI site. Nucleotide positions are numbered relative to the AUG of the original GBF1 clone 32 [17]. The presence of these variants in the original RNA population was determined by exhaustive treatment of RT-PCR products with the appropriate restriction enzyme, followed by agarose electrophoresis and phosphorImager analysis on a Storm system (Molecular Dynamics).

Genomic sequencing. Genomic DNA was extracted from CHO^{pro-5} cells as before. The genomic fragment encoding the 3 nt variant at position 1864 was recovered by PCR (Expanded High Fidelity PCR system, Roche) using primers +6 (^{1706}ATACCACACACCTACTG TCC$_{1726}$) and −7 (^{2018}TCTCGCTCCAGTGATCAGC$_{2034}$). A 3.2 kb genomic fragment encoding the 12 nt variant at position 4479 (Fig. 1) was recovered using primers +8a (^{3956}AAGTCTACACTGACCAT GGC$_{3985}$) and −2a (^{4606}TGCGTGAGTCAGCTTCAATC$_{4587}$). Sequencing with primer +8a revealed two introns of 109 and 100 nt at positions 4038 and 4285 but did not extend to position 4479. Sequencing with a new primer, +8aa1 (^{4356}ACCGAAGGAGGGCTCAGTGC$_{4376}$), identified the expected intron. Similar analysis confirmed the presence of an

Table 1
Intron–exon junctions of the CHO GBF1 gene identified by sequencing of genomic DNA

Intron position	Intron length	(5' exon #) Splice junction sequence (3' exon #)
1011|1012	390	(8) CAG|GTAtGA.....agCAG|G (9)
1011| -	387	CAG|GTAtGA.....AG|cagg
1867|1868	228	(14) acagta|GTtAGA...CAG|c (15)
1864|1868	231	Aca|GTAgtT......CAG|c
4038|4039	109	(30) gtG|GTGAGT.....CAG|G (31)
4285|4286	100	(31) gtG|GTAgGT.....CAG|G (32)
4491|4492	>2.5kb	(32) caggtcagtcaggAc|GTAAGT......CAG|t (33)
4479|4492	>2.5kb	CAG|GTcAGTcaggacgtaagt......CAG|t
4875|4876	231	(35) AAG|GTActg.....tAG|G (36)

The genomic sequence of putative splice junctions of CHO GBF1 gene was determined as described in Materials and methods. Numbers are nucleotide positions in the original GBF1 cDNA (clone 32) with initial AUG defined as position 1. The splice junction sequence corresponds to the last 3 nt of the exon followed by partial sequence of the intron and the next exon. Vertical bars denote exon–intron junctions. The intron sequences are underlined. Bases that match the consensus sequence are represented in uppercase. Corresponding exon numbers in human gene are given in parentheses.

intron with two alternate 3′ splice junctions at position 1011. Sequencing of clone 5 identified an additional intron at position 4875 that was not spliced in this variant. Results are summarized in Table 1.

Determination of hGBF1 gene structure. Comparison of the recently reported sequence of BACs RP11-118I14, RP11-242B12, and RP11-346A7 with that of the human GBF1 cDNA allowed us to identify 28 introns varying in size from 45 to 4498 nt. These results were later confirmed with the chromosome 10 genomic structure (entry NT 008804) available on the NCBI web site. The length of two introns remains unconfirmed due to gaps in the genomic DNA sequence.

Overexpression of GBF1 cDNAs. 293-EBNA cells were grown in T-75 flasks to ~60% density and transformed with 10 μg of purified plasmid using FuGENE 6 as per manufacturer's instructions. Unless indicated, 2 μg GBF1 clone plasmid DNA was diluted 4-fold by mixing with 8 μg of empty pCEP4 to avoid toxic effects [17]; some GBF1 clones were also transformed undiluted for specific tests, see text below. The next day the medium was replaced with complete DME containing 0.3 mg/ml hygromycin B and cells were cultured for 36 h to select transformants. Loose cells were then removed and survivors were trypsinized and transferred to new flasks with complete DME containing 0.3 mg/ml hygromycin B. After 24 h, hygromycin-sensitive cells that failed to reattach were removed and the surviving successful transformants (10–20% of initial population) were recovered by trypsinization. These cells were plated on Primaria dishes (Falcon) in complete DME with 0.3 mg/ml hygromycin B plus 0.4 μM BFA to select BFA resistant transformants. The medium was replaced every 24–36 h and BFA pressure was maintained until no survivors remained among control cells transformed in parallel with empty vector (typically 4–5 days). Transformants were maintained in DME containing 0.2 μM BFA.

Toxicity and BFA resistance assays. For toxicity assays, cells were transformed as described above with pCEP4 (empty plasmid), or GBF1, clone 2, clone 14, and clone 15 DNA, either undiluted or diluted 4-fold with the pCEP4 plasmid. Twenty four hours post-transfection, 0.3 mg/ml hygromycin B was added and the cultures were grown for 36 h to select transformants. Loose cells were then removed and survivors were trypsinized and transferred to new flasks with complete DME containing 0.3 mg/ml hygromycin B. After 24 h, hygromycin-sensitive cells that failed to reattach were removed and the surviving successful transformants were recovered by trypsinization. Cell suspensions were counted and 5×10^5 cells of each culture were plated in triplicates on six-well plates in the presence of 0.3 mg/ml hygromycin B. The cultures were incubated for 72 h and the density of surviving cells was determined by the MTT dye method (Sigma) as per manufacturer's instructions.

To determine the level of BFA resistance conferred by the GBF1 variants, 293-EBNA cells were transformed with the various GBF1 clones (diluted 4-fold with empty vector) as described above. After standard hygromycin B selection of transformants, cultures were grown for an additional 1–3 weeks in the presence of 0.4 μM BFA and trypsinized and the cell suspensions were counted. 10^6 cells from each culture were plated in six-well Primaria plates (Falcon) in triplicate in the presence of 0, 0.04, 0.18, 0.36, 0.72, and 1.8 μM BFA. After 48 h the surviving cell density was determined by the MTT dye method. Maintaining transformed cell populations in BFA for 1 or 3 weeks prior to analysis had no significant impact on the levels of either GBF1 expression or resistance to BFA.

Preparation of extracts and immunoblots. Detergent extracts were prepared either by incubating washed 150 cm^2 monolayers with 2.5 ml ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM PMSF, and 1× protease inhibitor cocktail), or by resuspending washed cell pellets in 4 volumes of lysis buffer. After 5 min on ice, the lysed cells were passed 10 times through a 23 gauge needle and spun for 10 min at 1000*g* at 4 °C. Supernatants were stored at −70 °C. Immunoblots were carried out essentially as described [19]. For determination of GBF1 levels, 100 μg of each sample was transferred to nitrocellulose and probed with polyclonal antibody 9D4, raised against recombinant protein containing residues 513–873 of human GBF1. Rabbit antibodies were detected by the enhanced chemiluminescence method using HRP-conjugated goat anti-rabbit IgG (Bio-Rad, Hercules, CA, USA). GBF1 levels between different extracts were normalized using calnexin as a control. The results were quantified using a ChemiImager (Canberra-Packard Canada, Mississauga, ON, Canada) as per manufacturer's instructions.

Results

Cloning of full length WT cDNAs for GBF1 identifies several mRNA variants

A cDNA library was constructed from the parental CHO pro-5 line as described in Materials and methods. A total of 2.5×10^5 colonies were screened by colony hybridization using a 2 kb probe derived from the 5′ end of the original GBF1 cDNA [17]. Plasmids recovered from 32 agar plugs containing potential candidates were

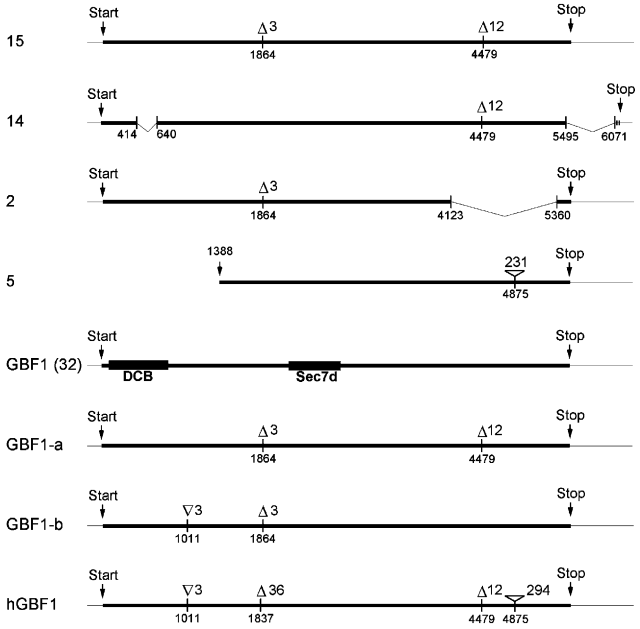

Fig. 1. Comparison of the cDNA isolates recovered from the wt and mutant CHO libraries. Diagram indicating the position and size of insertions/deletions in the GBF1 variants. GBF1-a and GBF1-b are additional forms of GBF1 obtained by colony hybridization from the original CHO BFY1 library that yielded clone 32. The hGBF1 model summarizes the variations observed among published isolates of human GBF1 cDNAs. All nucleotide positions are relative to the initiating codon (AUG) of the cDNA first recovered from CHO BFY1 cells (clone 32) defined here as GBF1. The nucleotide position for small deletions (Δ) and insertions (∇) corresponds to the last residue conserved before the modification. The number next to the symbol represents the size of the deletion/insertion. For large deletions, the numbers correspond to the last and first nucleotide conserved before and after the deletion, respectively. The vertical bars at the end of clone 14 correspond to a novel C-terminal sequence created in the 3′ UTR by the deletion. Clones 15, 14, 2, and GBF1-b were completely sequenced. Regions of clone 5 and GBF1-a corresponding to potential variations were sequenced, yielding coverages of about 85% and 50%, respectively.

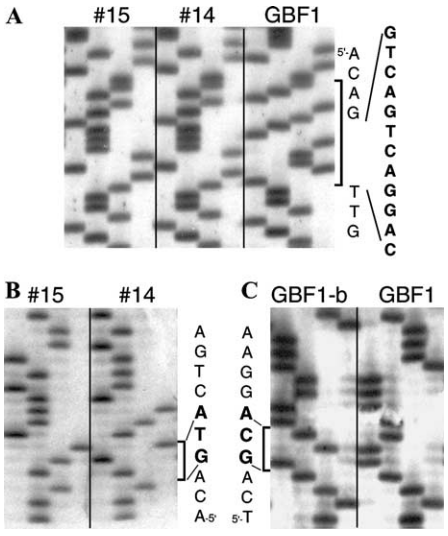

Fig. 2. Several cDNA isolates show discrete sequence differences. Panels show sections of sequencing gel corresponding to discrete deletions and insertions of isolates relative to GBF1. The plasmid templates used in the sequencing reaction are shown at the top. Gels were loaded with sequencing reactions in the order A, G, C, and T. The sequence listed on the side corresponds to the coding strand with differences between GBF1 and isolates printed in bold. Note that in the case of panel A, the primer used yielded the sequence of the complementary strand, which was converted and shown on the side as coding sequence for consistency.

analyzed by Southern blot to identify those containing complementary inserts larger than 4 kb. This analysis ultimately yielded five candidate plasmids containing inserts ranging from 4.8 to 6.0 kb.

Initial sequencing demonstrated that all clones except for clone 5 contained intact 5′ and 3′ UTRs. Further sequencing showed that with the exception of deletions and insertions illustrated in Fig. 1, the sequences of all inserts recovered from the WT cDNA library were identical to each other and to that of the GBF1 cDNA (clone 32) first recovered from the mutant BFY1 line. The sequence of the longest insert, clone 15, was identical to that of GBF1 but for the loss of 3 and 12 nt at positions 1864 and 4479 (Figs. 1 and 2A and B). These two in-frame deletions occurred independently in several of the clones analyzed. As indicated in Fig. 1, the inserts in clone 2 and 14 contained additional and more extensive in-frame deletions of 1236 and 225 nt, respectively. On the other hand, clone 5 lacked those deletions and contained instead a 231 nt insertion corresponding to an unexcised intron at nt 4875 (see below). Importantly, no sequence variation was observed in any of the cDNAs within the Sec7 domain (residues 2119–2631). All variants should therefore display Arf-GEF activity, since this domain by itself efficiently catalyzes the nucleotide exchange on Arfs [14,20].

To determine whether the identified sequence variations were unique to WT cDNAs, we analyzed several full length cDNAs independently recovered from cDNA libraries prepared from mutant BFY-1 and BFY-2 cells (see Materials and methods). As observed with the WT library, those cDNAs had sequences identical to that of the original GBF1 cDNA but for short in-frame sequence variations, including a 3 nt insertion at position 1011 in GBF1-b (Figs. 1 and 2C). We conclude that small sequence variations occur independently and are not unique to WT cells.

mRNA variants are produced at similar frequencies in WT and mutant cells

The analysis described above demonstrated that WT and BFA-resistant lines express similar GBF1 mRNAs but did not eliminate the possibility that mutant cells produce higher levels of a particular variant that could be responsible for BFA resistance.

To test this possibility, we established an RT-PCR assay to measure the relative levels of mRNA variants in those cells. In the case of the 3 and 12 nt deletions, this assay takes advantage of the fact that these result in deletion of a *Sca*I site and creation of an *Alw*NI site, respectively. The presence of larger deletions in clones 2 and 14 could be readily detected by clear size differences.

The results shown in Fig. 3 established that WT and mutant lines express the two main mRNA variants at similar frequencies. Quantitative analysis of phosphorimager data from three independent PCR demonstrated that in WT cells, $62 \pm 3\%$ and $44 \pm 4\%$ of mRNAs had in-frame deletions at positions 1864 and 4479, respectively. Those values in mutant BFY-1 cells were $62 \pm 1\%$ and $46 \pm 3\%$, respectively. Similar analysis for detection of large deletions at positions 414 and 4123 established that those splice variants are not present at detectable levels in the mRNA population of either cell lines (data not shown), and therefore might represent aberrant mRNAs or RT products. More importantly, we conclude that preferential expression of a particular mRNA variant is very unlikely to account for the BFA resistance phenotype of BFY-1 cells.

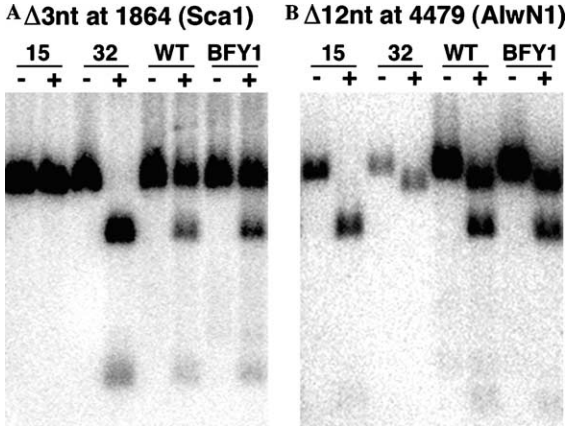

Fig. 3. Small deletions are present at the same frequency in WT and BFA-resistant CHO cells. RT-PCR analysis was performed as described in Materials and methods. As control for the diagnostic digestion with restriction enzymes, reactions were performed with PCR products obtained using clone 15 (lacks *Sca*I site) and GBF1 (lacks *Alw*NI site) plasmids as templates. A phosphorimage of a representative agarose gel is shown. The source of mRNA or DNA template is shown above the gels. The symbols + and − indicate the presence or absence of restriction enzyme during incubation of the PCR products. (A) shows analysis for the presence or absence of 3 nt deletions at position 1864 detected by digestion with *Sca*I. (B) shows similar analysis for the 12 nt deletion at 4479 with *Alw*NI. Analysis of control PCR products confirmed the specificity of the reaction and established that digestion was complete. Note that the presence of salts in the *Alw*NI preparation caused a reproducible slight shift in mobility most apparent for the undigested 639/651 nt fragment.

Sequencing of genomic fragments suggests sequence variants arise from alternate splicing

Alternative splicing can yield proteins with distinct biological functions and may act as a regulatory mechanism [21,22]. To determine whether the GBF1 mRNA variants arose from such splicing events, we sequenced fragments containing the putative splice junctions that were generated by PCR of CHO genomic DNA (see Materials and methods for details). This analysis identified six introns (sequence underlined), all of which show consensus splice junction sequences (Table 1). Importantly, for all sites of variation except for the large deletion at position 4123 in clone 2, we identified not only intron junctions, but also alternative splice sites that accounted for the observed variations. At position 1864, for example, the sequence of the 5′ junction contains two potential acceptor splice sites and is therefore consistent with the alternative splicing events yielding GBF1/clone 32 (acagta|GTtAGA . . . CAG|c) and clone 15 (Aca|GTAgtT . . . CAG|c). As illustrated in Table 1, the presence of alternative 5′- or 3′-splice junctions can similarly account for sequence variations at positions 1011 and 4479.

Examination of the intron/exon structure of the human GBF1 gene yielded further insights into the origin of observed GBF1 variants. Comparison of genomic and cDNA sequences identified 28 introns varying in size from 45 to 4498 nt (Table 2). Interestingly, the human gene contains introns at the positions corresponding to the six introns identified in the CHO gene (Table 2), suggesting that the exon structures of the human and CHO genes are highly conserved. Furthermore, the boundaries of the deletion in clone 14 (positions 414 and 640; see Fig. 1) correspond to the 3′ and 5′ splice junctions of exons 4 and 8 in the human gene, respectively (Table 2). These results strongly suggest that the large deletion in clone 14 resulted from exon skipping during the splicing reaction leading to loss of exons 5–7.

The various forms of GBF1 promote different levels of BFA resistance

The availability of several mRNA variants allowed us to investigate in more detail the impact that the small and large sequence variations may have on the ability of GBF1 to promote resistance to BFA and thus shed light on GBF1 function. We expected that the relatively large deletions of clones 2 and 14 might encompass domains important for protein activity. For example, the three missing exons in clone 14 encode the C-terminal half of a domain (Fig. 1) implicated in GBF1 dimerization and cyclophilin 5 binding, termed DCB [23]. Similarly, we could not rule out the fact that the small 3 and 12 nt changes affect biological properties. Since full-length proteins cannot be readily purified and characterized, we

Table 2
Size and position of exons and introns in the human GBF1 gene

	Exon cDNA position (from AUG)	Exon length	Intron length	Splice junction sequence
1	-10:96	106	1015	CtG|GTAAGT...CAG|G
2	97:163	67	(5789)	CAG|GTAAGT...CAG|A
3	164:295	132	(6944)	tAG|GTAAGA...CAG|A
4	296:414	119	468	CAG|GTAAGc...tAG|G
5	415:523	109	506	gtG|GTAgGT...CAG|A
6	524:584	61	1081	AAG|GTAAac...tAG|G
7	585:639	55	4428	AAG|GTAtGA...CAG|c
8	640:787	148	319	CtG|GTGAGT...CAG|G
9	788:1011	224	540	CAG|GTAtGg...CAG|G
10	1012:1177	166	748	AAG|GTGgGT...CAG|G
11	1178:1389	212	626	CAG|GTAAGA...CAG|c
12	1390:1488	99	597	gAG|GTGAGT...CAG|A
13	1489:1683	195	562	AAG|GTGctg...CAG|A
14	1684:1873	190	591	Ata|GTGAGA...CAG|c
15	1874:2014	141	313	ggG|GTGgGT...TAG|c
16	2015:2103	89	1589	AAG|GTAcat...CAG|c
17	2104:2306	203	783	gAG|GTGAGg...TAG|c
18	2307:2430	124	578	AtG|GTGAGT...CAG|A
19	2431:2556	126	267	gAG|GTAAGc...TAG|G
20	2557:2639	83	657	CAa|GTGAGT...CAG|G
21	2640:2876	237	283	CAG|GTAgcc...CAG|G
22	2877:2970	94	378	gAG|GTGAGc...gAG|t
23	2971:3147	177	323	gAG|GTAAtt...CAG|G
24	3148:3212	65	85	CcG|GTAAGA...CAG|A
25	3213:3336	124	189	AAG|GTAAcT...TAG|c
26	3337:3408	72	138	AAG|GTAAag...CAG|G
27	3409:3503	95	228	CAG|GTAAGa...CAG|G
28	3504:3657	154	4498	CAG|GTAAGc...CAG|G
29	3658:3883	226	687	CcG|GTAAGc...TAG|G
30	3884:4044	161	127	gtG|GTGAGT...CAG|G
31	4045:4285	241	134	gcG|GTGgGT...CAG|G
32	4286:4491	206	2143	gAc|GTAAGT...TAG|t
33	4492:4642	151	86	AgG|GTAAac...CAG|G
34	4643:4773	131	201	AAG|GTGgGA...CAG|G
35	4774:4875	102	294	AAG|GTActg...TAG|G
36	4876:4989	114	143	CtG|GTAtGT...CAG|A
37	4990:5170	181	440	AgG|GTAgGg...CAG|A
38	5171:5299	129	800	gtG|GTGAGT...CAG|A
39	5300:5577	278		

Size and position of exons and introns in the human GBF1 gene. All numbers are nucleotide positions in the cDNA (with initial AUG as position 1) and the genomic DNA clones. The colons in the cDNA sequence separate the first and last nucleotide of each exon. The splice junction sequence corresponds to the last 3 nt of the exon followed by partial sequence of the intron. The reported size of introns 2 and 3 is tentative due to gaps in the genomic sequence. Bases that match the consensus sequence are represented in uppercase.

chose to test these possibilities in a cellular context using biological assays. We first compared the ability of several splice variants to allow growth over a wide range of BFA concentrations.

The results, shown in Fig. 4A, established that 293-EBNA cells overexpressing clone 15 (with both Δ3 and Δ12 deletions) produced BFA resistance levels that were slightly lower than those observed with GBF1/clone 32 but significantly higher than those observed with clone 14. Similar results were observed in several independent experiments. This observation confirmed and extended our previous demonstration that overexpression of clone 15 and GBF1 produced similar number of viable transformants at the single BFA concentration of 0.4 μM [17], but suggests that clone 15 may have slightly reduced activity or expression levels. Overexpression of clone 14 produced marginal BFA resistance, while on the other hand that of clone 2 promoted a level of BFA resistance in vivo, significantly higher than that observed with either clone 15 or GBF1. The large truncation in the C-terminal region of clone 2 apparently had minimal impact on activity in this assay. The greatly reduced activity of clone 14 could have resulted from either loss of a domain critical for GEF function and/or from protein instability, leading to lower levels of expression.

The various forms of GBF1 have different effects on cell survival

We used another in vivo test to assess the relative cytotoxicity of the various splice forms as a further measure of their functional integrity. Previous studies revealed that few if any transformants are recovered when 293-EBNA cells are transfected with vectors containing full length GBF1. For this reason, we routinely dilute GBF1 clones with a 4-fold excess of empty vector to allow transformed cells to adjust the level of GBF1 expression by asymmetric plasmid segregation during division [17]. From such observations, we concluded that GBF1, although able to confer BFA resistance at moderate levels, caused cell death when overexpressed, possibly through hyper-activation of reactions regulated by GBF1 and consequent disruption of the homeostatic balance between various transport pathways. Here we

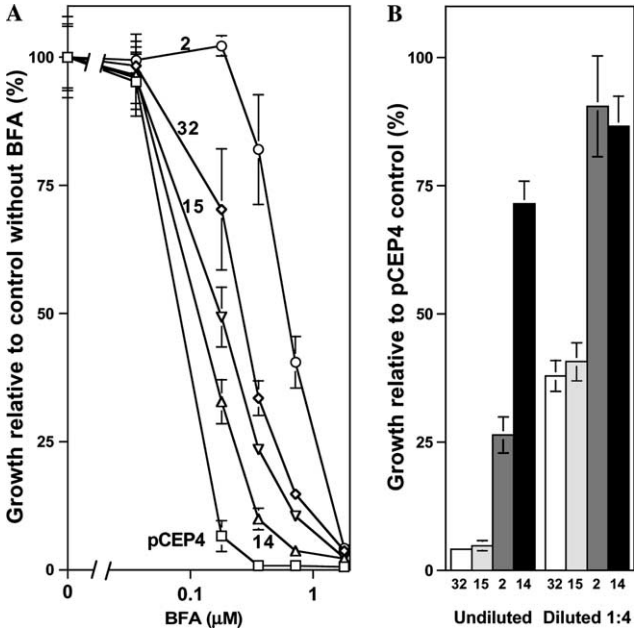

Fig. 4. Comparison of splice variants in biological assays. (A) Overexpression of various splice forms of GBF1 promotes different levels of BFA resistance. 293 cells were transfected with GBF1 variants (clones 2 (○), 32 (◇), 15 (▽), and 14 (△)) or empty pCEP4 vector (□) and selected with hygromycin B and BFA as described in Materials and methods. An equal number of transfectants were grown in the presence of the indicated amounts of BFA for 3 days before determining the number of survivors. Values are the average of triplicate measurements ± SD and are expressed as a percentage of surviving cells compared to control cells grown in the absence of the drug. Similar results were observed in several independent experiments. (B) GBF1 variants display different toxic effects. Cells were transfected with the GBF1 variants either undiluted or diluted 4× with pCEP4 and selected with hygromycin B as described in Materials and methods. An equal number of cells were plated and the number of survivors was scored 3 days later. Values for the indicated splicing forms are the average of triplicate measurements ± SD and are normalized against cells transformed with empty vector. Similar results were observed in several independent experiments.

tested the impact of the various GBF1 isolates on cell survival, by measuring cell density after transformation with the GBF1 cDNAs either undiluted, or diluted with empty pCEP4 vector.

As shown in Fig. 4B, GBF1/clone 32 and clone 15 behaved similarly and yielded the smallest number of survivors, especially when transformed undiluted. Twenty-four hours post-transformation, these cells enlarged dramatically (2–4-fold) and displayed large multi-lobed nuclei as well as a significant number of vacuoles, consistent with apoptotic death; most of such transformants detached in the next 24 h. In contrast, clone 2 had markedly lower impact on cell survival. The large deletion in the C-terminal half apparently eliminated a domain involved in cytotoxicity while retaining the ability to confer BFA resistance. Surprisingly, clone 14 had the least impact of all. The low activity of clone 14 in both the BFA resistance and survival assays suggests that clone 14 is either not expressed or is inactive. More importantly, these results indicate that high toxicity and high BFA resistance levels can be uncoupled from each other.

The extent of resistance to BFA correlates with the relative expression levels of splice variants

To measure Arf-GEF levels, we took advantage of a new antiserum raised against the sec7 domains of GBF1 (see Materials and methods). Fig. 5 shows an immunoblot of various cell extracts with a quantitation of the fold-increase in total GBF1-signal over endogenous signal in control transformants. In order to examine a wider range of expression levels, we took advantage of the lack of toxicity of clones 2 and 14 and included in this analysis cells transformed with undiluted clones 2 and 14. New populations of cells were transformed with either diluted or undiluted clones 2 and 14, and selected as described in Materials and methods. Fig. 6 compares the growth of these transformants at several BFA concentrations.

Comparison of expression levels and sensitivity to BFA suggests that the different degrees of BFA resistance produced by the GBF1 splicing variants correlate well with their relative expression levels. For example, GBF1/clone 32 and clone 15 were moderately overexpressed (1.5- to 2-fold) and produced intermediate resistance. In contrast, clone 2 transformants (diluted) displayed both elevated expression (~7-fold) and resistance ($LD_{50} > 0.5\,\mu M$) levels. At the other extreme, clone 14 was poorly expressed relative to GBF1/clone 32 and transformants (diluted) showed marginal resistance ($LD_{50} < 0.15\,\mu M$). Increasing its expression to levels

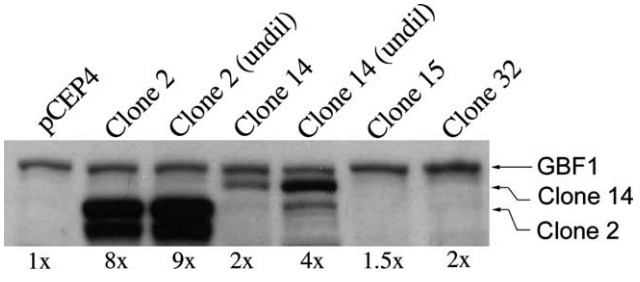

Fig. 5. Measurement of GBF1 levels in transformed 293 cells. 293 cells were transfected with the GBF1 variants. We took advantage of the lower toxicity of clones 2 and 14, and transfected cells with these clones undiluted to obtain higher expression levels. Unless otherwise indicated, the DNAs were diluted 4× with empty vector. Transformants were selected in the presence of hygromycin B and BFA as described in the text (hygromycin B only for pCEP4). Total cell lysates were prepared 2 weeks after transfection and 100 μg of each sample was resolved by SDS–PAGE, followed by immunoblot using anti-GBF1 (9D4) serum. Clone 15 was not included since its effects on growth and BFA resistance are similar to those of GBF1. Arrows mark the relative positions of proteins of interest. Clone 2 GBF1 levels are likely underestimated due to partial proteolysis of the overexpressed protein.

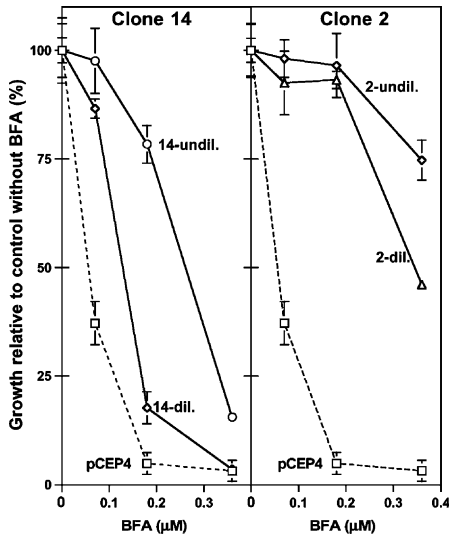

Fig. 6. Cellular BFA resistance induced by GBF1 splice forms. 293 cells were transfected with the GBF1 variants alone or in equimolar mixtures with clones 2 or 32 (without empty vector) and selected with hygromycin B and BFA as described in the text. Unless indicated, the GBF1 variant DNAs were diluted 4× with empty vector. All the determinations presented were carried out in parallel. The transformed populations were allowed to grow in the presence of 0.4 μM BFA and 0.3 mg/ml hygromycin B for a total of 30 days post-transfection. Cells (10^5) were seeded per well and grown in the presence of 0, 0.07, 0.18, and 0.36 μM BFA for 3 days before determining the number of survivors. The growth curve of pCEP4 is used as a reference value and is repeated on both panels (dashed line) for convenience. Values are the average of triplicate measurements ± SD and are expressed as a percentage of surviving cells compared to controls grown in the absence of the BFA.

closer to those for GBF1/clone 32 by performing transformations without empty vector (undiluted) led to a resistance level comparable to that of GBF1/clone 32. Interestingly, increasing clone 2 expression level in this manner also led to enhanced BFA resistance. Taken as a whole, these results further demonstrate that neither deletions abolish the ability of GBF1 to promote resistance to BFA, but rather impact its expression level. This effect could result from altered transcript levels or protein stability.

Discussion

Further examination of GBF1 mRNAs produced by WT and BFA-resistant CHO lines led us to reinvestigate the mechanism through which overexpression of this GEF promotes BFA resistance. Analysis of multiple GBF1 transcripts revealed sequence variations in the form of small in-frame deletions and insertions that were consistent with alternative splicing. While small variations did not alter GBF1 activity in biological assays, clones with large deletions displayed substantially different characteristics.

Alternative splicing as a source of GBF1 variation

Sequencing of several cDNAs identified alternate splice forms of GBF1 with small in-frame insertions/deletions at positions 1010, 1864, and 4479 of the CHO cDNA. These small variations were neither generated during construction of the cDNA libraries nor had resulted from our use of cultured cell lines. First, an RT-PCR assay detected those variations at high frequency in several CHO cell lines. Second, genomic sequencing determined that those variations arose at exon–intron boundaries through the use of alternate 5′ or 3′ splice sites. Furthermore, variations similar to those reported here were identified in mRNAs and expressed sequence tags (ESTs) for human GBF1 (see Fig. 1 for summary). For example, comparison of two full-length cDNAs obtained from human tissue (Accession Nos. #AF 068755 and AK 025330) revealed alternate splices at the junctions between exons 9/10 and exons and 32/33. Genome-wide analysis of ESTs reported at http://www.bioinformatics.ucla.edu/HASDB/ [24] confirmed the occurrence of alternate splicing in hGBF1 at the third site, between exons 14 and 15, which leads to shortening of human exon 14 by 36 nt (Accession No. #BE706137).

The three positions identified in our study are likely the only ones showing such small variations since inspection of the GBF1 gene revealed no obvious potential alternative splice junctions within 60 nucleotides of each of the 76 intron–exon junctions. Analysis of the human EST database reported on the HASDB site mentioned above identified three cDNAs that like our clone 5 retained the intron between exons 35 and 36 (see Fig. 1). Retention of this intron introduces a stop codon that yields a GBF1 molecule lacking residues 1627–1859 but with an intron-encoded C-terminus of 41 residues. This novel sequence does not show any similarity to known proteins but we cannot exclude the possibility that it has a specific biological function. Lastly, analysis of the human EST database did not yield any evidence of alternate mRNAs resulting from either exon skipping and/or splicing of cryptic exons.

Our initial interest in this phenomenon was prompted by the growing evidence that alternative splicing plays a crucial role in increasing the diversity of expression in 40–60% of human genes and can yield protein isoforms with distinct function [25–27]. This diversity can result not only from the use of alternate exons, but also from the use of alternate 5′ or 3′ splice sites [22]. Relevant examples of this process include spectrin [28] and dynamin [29], whose multiple splice isoforms are targeted to different cellular compartments. ARNO represents an extreme case, where the introduction of a single glycine residue in the pleckstrin homology domain [30] dramatically alters the protein affinity for phosphatidylinositol-4,5-diphosphate [31] and its targeting to the

plasma membrane [32]. In the case of GBF1, the small 3 and 12 nucleotide variations resulting from the use of alternate 5′ or 3′ splice sites appeared to have minimal impact on function as measured by the effect of overexpression on cell survival or promotion of BFA resistance. However, these remain relatively crude assays of GBF1 function and we cannot exclude the possibility that these alternate splice forms differ in other important, yet untested, properties. Clearly, more specific tools than those currently available will be required to identify any alterations in GBF1 properties caused by these sequence modifications.

Analysis of clones 2 and 14 identifies domains responsible for cell death but dispensable for promoting BFA resistance

The GBF1 variants with large deletions provided an unexpected opportunity to initiate a functional dissection of GBF1. In the absence of a specific biological assay or GBF1-mutant cell line, we took advantage of the ability of moderate GBF1 overexpression to induce BFA resistance, and cell death at higher expression levels. Overexpression studies with clones 2 and 14 demonstrated that the large deletions in those variants caused significant reduction in toxicity but did not eliminate their ability to promote BFA resistance. In the case of clone 2, the high level of expression observed in transfectants clearly established its lack of toxicity. Furthermore, the relatively high BFA resistance of clone 2 transformants clearly demonstrated that the 412-residue region encoded by exons 31–39 is dispensable for this activity. Comparison of the expression level and survival rate of various transformants (Figs. 4–6) revealed that clone 14 similarly displayed reduced toxicity. More importantly, cells transformed with clone 14 (undiluted) expressed protein and displayed BFA resistance levels nearly identical to those of clone 32 (diluted) transformants. This established the fact that the domain encoded by exons 5–7 is not essential for promoting drug resistance.

These initial results suggest that while the domains altered in clones 2 and 14 are not required for promoting BFA resistance, they do play a role in cell death induced by GBF1 overexpression. The lethality mediated by these domains might result from their natural catalytic activity or more likely through interactions with their normal binding partners. Elevated expression of GBF1 would cause an increase of these interactions leading to cell death. This information could be potentially useful in the design of chimeras for differential two-hybrid screens or co-precipitation studies. Interestingly, the 3-exon deletion in clone 14 results in the loss of the C-terminal half of the DCB domain involved in protein binding [23]. Loss of this portion of the DCB domain completely abolished dimerization of the *A. thaliana* GBF1 orthologue GNOM, as well as binding to the interacting partner cyclophilin 5. It is therefore likely that clone 14 will exhibit equivalent defects that could explain its low steady-state expression levels.

Acknowledgments

We thank M. Hughes and H. Vandertol-Vanier for maintenance of cultured cells. We are particularly grateful to X. Zhao for characterization of anti-GBF1 sera. Finally, we thank Dr. T. Hobman and members of the Melançon lab for their helpful comments on the manuscript. This study was initiated with funds from the Alberta Heritage Foundation for Medical Research and supported by a grant (PM) from the Canadian Institutes of Health Research. Dr. A. Claude was supported by Grant DID-UACH S-2002-90 during the latter stages of the work.

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