Expression and characterization of a B cell growth promoting polypeptide derived from the 12 kDa B cell growth factor gene (*BCGF 1*)

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Abstract The expression and partial purification of recombinant 12 kDa B cell growth factor are reported. The polypeptide was derived from the genomic sequence of the gene (*BCGF 1*) which is here shown to be a single copy gene that localizes to human chromosome 16. When expressed as a glutathione *S*-transferase fusion protein in *E. coli*, the protein appears as a 38 kDa polypeptide in Western blot analysis using a peptide antibody. The purified fusion protein stimulates the proliferation of activated human B cells in a dose-dependent manner, and the active site resides within the 104 carboxy-terminal amino acids. The availability of biologically active recombinant 12 kDa B cell growth factor will enable its evaluation in B cell growth regulation, and provides a new means of in vitro culturing of human B lymphocytes.

Key words: Recombinant 12 kDa BCGF; *BCGF 1*; Gene; B cell proliferation; Chromosome 16

1. Introduction

Mature B lymphocytes express at least three different types of growth associated receptors, including surface immunoglobulins, transmembrane glycoproteins like CD40, and cytokine receptors. Some of these receptors, such as surface immunoglobulins and CD40, are constitutively expressed on B cells, whereas cytokine receptors are normally transiently expressed upon activation. In general, B cells require at least two stimuli for sustained growth. The first priming signal may be delivered by cross-linking surface immunoglobulin by antigen or anti-immunoglobulin antibodies, or by ligation of CD40 with its counter-receptor on activated T lymphocytes [1]. The second signal is mediated by cytokines. The cytokines that stimulate proliferation of activated B cells include IL-2, IL-4, IL-10, IL-13 and IL-14 [2–5]. Most studies concerning growth regulation of human B cells by cytokines have been performed with well-characterized recombinant proteins; however, impure cytokines have also been used, leaving the biological significance of some of these factors open.

One of the first cytokines that was shown to stimulate growth of activated mature B lymphocytes was a 12 kDa B cell growth factor (12 kDa BCGF) [6]. B cell growth factor activity was first demonstrated in 12,000–13,000 mol. wt. fractions of supernatants of lectin-activated T lymphocytes [6,7]. Later, the active protein (12 kDa BCGF) was purified to homogeneity [8]. The amino acid sequence of the protein was not determined, but using an expression cloning strategy Sharma and co-workers cloned the cDNA for 12 kDa BCGF [9]. A number of functions have been suggested for 12 kDa BCGF. This cytokine may stimulate growth of activated normal B lymphocytes, serve as a growth factor in B cell precursor acute lymphoblastic leukemia and in low-grade B cell lymphomas, or act as an autocrine growth factor in some cases of chronic lymphatic leukemia and hairy cell leukemia [7,10–16]. 12 kDa BCGF has also been suggested to co-stimulate with IL-2 the proliferation of lymphokine activated killer cells from children with acute lymphoblastic leukemia and normal natural killer cells [17–19]. However, most of the data regarding 12 kDa BCGF has been obtained using commercial semi-purified preparations of the cytokine, and have not been verified with a recombinant factor.

To obtain recombinant 12 kDa BCGF for functional studies we used PCR cloning to isolate the coding sequence for 12 kDa BCGF. During this attempt we identified a genomic open reading frame that is almost identical to the published cDNA of the 12 kDa BCGF. The observed nucleotide changes cause a reading frame shift in 12 kDa BCGF polypeptide which results in a novel 37 amino acid amino-terminus. The nucleotide differences are in concordance with those recently reported [20]. We have utilized this sequence data to further characterize the genomic sequence by localizing the gene (*BCGF 1*) to chromosome 16 and by expressing the encoded polypeptide as a glutathione *S*-transferase fusion protein. The recombinant protein stimulates the growth of activated B cells, suggesting that a bacterial expression system may prove useful in producing recombinant 12 kDa BCGF for biological assays.

2. Materials and methods

2.1. Identification of the genomic sequence (BCGF 1 gene)

Genomic high molecular weight DNA was extracted from white blood cells of two healthy donors and from the NK line 3.3 using standard techniques. PCR reactions were performed using primers P1 and P2 with overhanging *Bam*HI sites (Table 1). The sequences of these primers were deduced from the published sequence for 12 kDa BCGF [9]. The reactions were carried out according to the manufacturer's instructions (Perkin-Elmer/Roche Molecular Systems Inc., Branchburg, NJ, USA) under the following conditions: 95°C for 1 min, 55°C for 1 min and 72°C for 2 min for 30 cycles followed by 10 min of extension at 72°C. The reaction products were ethanol precipitated, and subsequently electrophoresed in 2% low-melting point agarose gels. The approximately 360 bp bands were excised from the gel, purified and digested with *Bam*HI. The purified fragments were ligated into the dephosphorylated *Bam*HI site of pBluescript SK− (Stratagene, CA,

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Abbreviations: BCGF, B cell growth factor; GST, glutathione *S*-transferase.

SSDI 0014-5793(95)00186-7

Table 1
List of synthesized primers

Primer	Sequence
P1	5′-TATTGGATCCAGGCATGGTAGTTTATG-3′
P2	5′-GATCGGATCCTTTTTAGTAGAGACAGGGTTC-3′
P3	5′-ATATTCTGCATCAGCCAGGC-3′
P4	5′-GATCGGATCCACGCGTGCCACTGCACCCAGCTAA-3′
P5	5′-TATTGGATCCATGCCTATAATCCCATCACTGTGG-3′
P6	5′-TATTGGATCCAAAATTCAAAGAACTATACA-3′
P7	5′-TATTGGATCCTGGCTGGGCCCAGTGGCT-3′

USA) and cloned into XL-1 Blue strain of *E. coli*. The plasmid DNAs were sequenced using the dideoxy method (Sequenase; USB, Cleveland, OH). The termination codon for the detected open reading frame (ORF) was ascertained by amplifying and sequencing PCR fragments that were generated using primers around the 3′ and 5′ ends of the ORF (P3 and P4).

2.2. Southern blot analysis

Genomic DNA from a healthy donor was restriction digested with *Bam*HI and *Hin*dIII. The digested DNA was size fractionated by agarose gel-electrophoresis and transferred onto a nitrocellulose filter according to Southern [21]. The filter was hybridized with a randomly primed *Mae*III–*Acc*I fragment of the sequence and washed under low-stringency conditions. The filter was exposed for autoradiography for 72 h.

2.3. Chromosomal assignment of BCGF 1 gene

Two DNA panels of interspecies-specific somatic cell hybrids were used for the chromosomal assignment. The panels were obtained from the Mutant Cell Repository of the Coriell Institute (Camden, NJ). The other panel was screened using PCR amplification and the reactions were carried out using primers P3 and P4. In each reaction 50 ng of template DNA was used and the cycle parameters were 95°C for 1 min, 60°C for 1 min and 72°C for 2 min for 30 cycles followed by 10 min at 72°C. The analysis was repeated four times. The other panel was screened using Southern blot analysis as described previously [22] using a randomly primed *Mae*III–*APA*I fragment as a probe.

2.4. Construction and purification of glutathione S-transferase fusion proteins

Three different fusion proteins were constructed by utilizing the predicted amino acid sequence of the genomic sequence of 12 kDa BCGF. All fusion proteins were constructed by cloning PCR amplified fragments with *Bam*HI overhangs into the *Bam*HI site of the pGEX-2T prokaryotic expression vector [23] (Pharmacia Biotech Norden AB, Sollentuna, Sweden). The primers synthesized for different fusion proteins were P5 and P2 for GST-I/III, P6 and P2 for GST-II/III and P7 and P2 for GST-III. All fusion constructs were sequenced and only unaltered clones were selected for fusion protein production.

The fusion proteins were purified essentially as suggested in the manufacturer's manual (Pharmacia) with minor modifications. The fusion proteins GST-I/III and -II/III were purified as follows: 80 ml of overnight bacterial cultures were incubated for 2 h in 4 l of prewarmed (37°C) LB media (ampicillin 50 μg/ml) with continuous shaking. The cultures were then cooled to 28°C and the fusion protein expression was induced for 10 min with 0.1 mM IPTG. The cells were chilled in an ice-water bath for 30 min, and then pelleted at 6000 × *g*, resuspended in 40 ml of 10 mM Tris buffer, pH 8, containing 100 mM NaCl and treated with lysozyme at 0°C for 30 min. The cell suspension was sonicated for 1 min and insoluble debris was removed by centrifugation (10,000 × *g*, 30 min). The supernatant was filtered through a 0.45 μm filter (Sartorius, Göttingen, Germany) and applied to a glutathione-Sepharose column (Pharmacia) which had previously been equilibrated with 10 vols. of Tris buffer. The column was washed with 15 vols. of 0.15 M NaCl, 0.01 M sodium phosphate (PBS), pH 7.4; 5 vols. of 1 M NaCl in PBS to remove bacterial LPS; and with 5 vols. of PBS. The fusion proteins were eluted with 5 vols. of 25 mM glutathione in PBS (pH 8). The presence of fusion proteins in the eluates was monitored by electrophoresis in 12% SDS-polyacrylamide gels. The free glutathione was removed and the samples were concentrated by ultrafiltration using Centricon 30 concentrators (Amicon, MA, USA). The concentrated samples were analyzed by SDS-PAGE and tested for B cell growth factor activity in a thymidine incorporation assay. The fusion proteins GST-III and GST were purified essentially as described above, but as they were significantly more soluble than the other fusion proteins, smaller culture volumes (500 ml) and longer induction times (1 h) were used.

2.5. B cell preparations and thymidine incorporation assays

Human peripheral blood B cells were purified from buffy coats (Finnish Red Cross Blood Transfusion Service) using magnetic CD19 beads, a magnetic particle concentrator and Detachabead reagent according to manufacturer's instructions (Dynal A.S., Norway). The purity of the B cell populations was determined by FACS analysis using monoclonal FITC-conjugated CD20 antibody (Becton-Dickinson, Erembogen, Belgium). Only over 96% pure B cell preparations were used in the growth assays. The B cell growth factor activity of different fusion proteins was tested using standard thymidine incorporation assays. The cells were cultured as triplicates on round bottom 96-well microtiter plates (1×10^5 cells/well) in RPMI medium with 5% fetal calf serum and antibiotics, and they were stimulated for 4 days with anti-IgM beads (100 μg/ml; Bio-Rad Laboratories, CA, USA) and different concentrations of fusion protein eluates (expressed as percentage of total culture volume). During the last 6 h of culture the cells were pulsed with radioactive thymidine (1 μCi/well; Amersham International, Buckinghamshire, UK) and then harvested on filter papers. The amount of incorporated radioactive thymidine was measured using a beta-counter.

2.6. Peptide synthesis

The peptide NNQPKNLCSHFSFPTTYIKKERL was chosen from the hydrophilic region of the polypeptide and synthesized by a solid-phase method using Fmoc chemistry on an Applied Biosystems 430 A instrument. Cleavage and deprotection were carried out in TFA in the presence of scavengers (thioanisole, 1,2-ethanedithiol, phenol). The peptide was purified on HPLC using a C_{18} reverse-phase column with an acetonitrile/TFA gradient. The structure was confirmed by FAB-mass spectrometry (Jeol SX-102 instrument).

2.7. Immunization, affinity purification of the antiserum and Western blot analysis

The peptide was coupled to a carrier protein (Keyhole limpet hemocyanin) and used for immunization of a New Zealand white rabbit. The immunization was carried out five times at 2-week intervals with Freund's adjuvant. Bleeding was done 2 weeks after the last injection. The antiserum was run through an affinity column containing the peptide coupled to thiopropyl-Sepharose 6B (Pharmacia). The column was eluted with 0.1 M glycine-HCl buffer, pH 2.5, and the pooled Ig

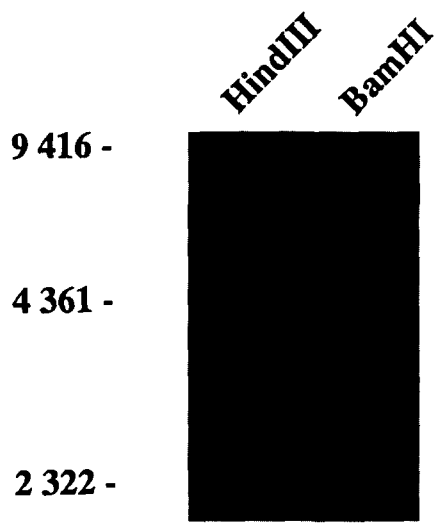

Fig. 1. Southern blot analysis of the genomic sequence of 12 kDa BCGF. Genomic DNA was digested with *Hin*dIII and *Bam*HI and hybridized with the *Mae*III–*Acc*I fragment of the gene.

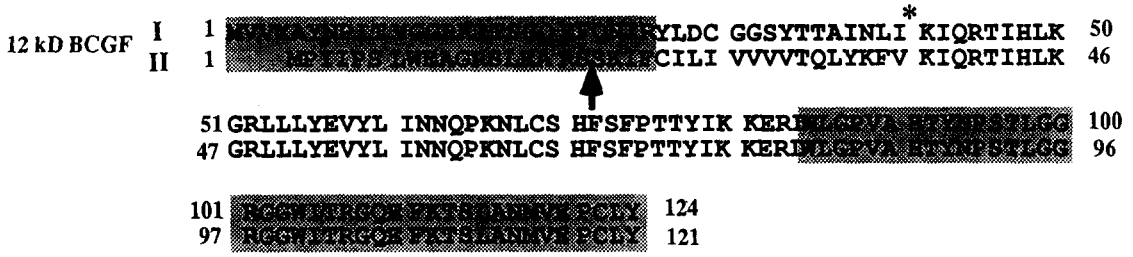

Fig. 2. The alignment of amino acid sequences of reported (lower, II) and previously published (upper, I) 12 kDa BCGF polypeptides. Vertical arrow indicates the cleavage site of the predicted signal peptide and asterisk marks the site where the amino acid sequences become identical. Shaded boxes indicate sequences derived from ALU-like elements. These sequence data are available from EMBL/Genbank/DDJB under accession number M15530.

fractions were neutralized and dialyzed against water. Western blot analysis was performed according to the manufacturer's instructions using chemiluminescense detection of proteins (BM Chemiluminescence Western blotting kit; Boehringer Mannheim, Germany).

3. Results and discussion

The cDNA coding for 12 kDa BCGF was published in 1987 [9], but data describing biological functions of the cytokine have accumulated slowly as the recombinant factor has not been available. Here we describe the bacterial expression and purification of biologically active 12 kDa BCGF by utilizing its genomic sequence. Using PCR and primers deduced from the published cDNA sequence of the 12 kDa BCGF we identified a genomic open reading frame with four base differences from the published sequence: two base substitutions (119 C→G, 143 G→T) and two single base deletions (176 C, 187 A). The first substitution creates a stop codon at position 26 of the predicted amino acid sequence. However, with the two base deletions, a novel 363 bp open reading frame is formed with an ATG start codon at nucleotide position 77 of the 12 kDa BCGF sequence. The possibility that the differences between our sequence and the published cDNA of 12 kDa BCGF would have resulted from PCR artefacts is negligible as the changes were consistent in three independent amplifications. Furthermore, during the final stages of this study the nucleotide sequence differences described here were also detected by another group, thus confirming the authenticity of our sequence [20].

To verify that the obtained sequence represents the genomic sequence of 12 kDa BCGF and not a pseudogene or a member of a novel gene family, we performed genomic Southern blot analysis and PCR analysis of cross-species somatic cell hybrids. The Southern blot shows only a single band (Fig. 1), and PCR analysis of a panel of 24 cross-species somatic cell hybrids yielded a positive signal only from a hybrid which contained 98% of human chromosome 16. The localization to human chromosome 16 was verified with Southern blot analysis of an other hybrid panel (Table 2). We are currently working on the exact localization of 12 kDa BCGF/*BCGF 1* which may turn out to be interesting as chromosome 16 also contains other B cell associated genes, including a B cell maturation factor (*BCMA*, 16p13.1), interleukin-4 receptor (16p12) and genes for leukocyte integrin α-chains CD11 a, b and c (16p11).

The polypeptide coded by the genomic sequence differs by 37 amino-terminal amino acids from the published amino acid sequence of 12 kDa BCGF (Fig. 2). The amino-terminus contains characteristics of a signal peptide, and the algorithm of von Heijne predicts a potential 18 amino acid signal peptide with a cleavage site between two serine residues [24]. However, as there are three other potential cleavage sites at positions 32, 33 and 35 (although significantly less probable), definite conclusions cannot be drawn. The 40 carboxy-terminal amino acids of the polypeptide seem to derive from translated ALU sequences. Additionally, there is also an ALU-like element at the amino-terminus. The presence of ALU-translated sequences in a protein is interesting as only a few other proteins have been

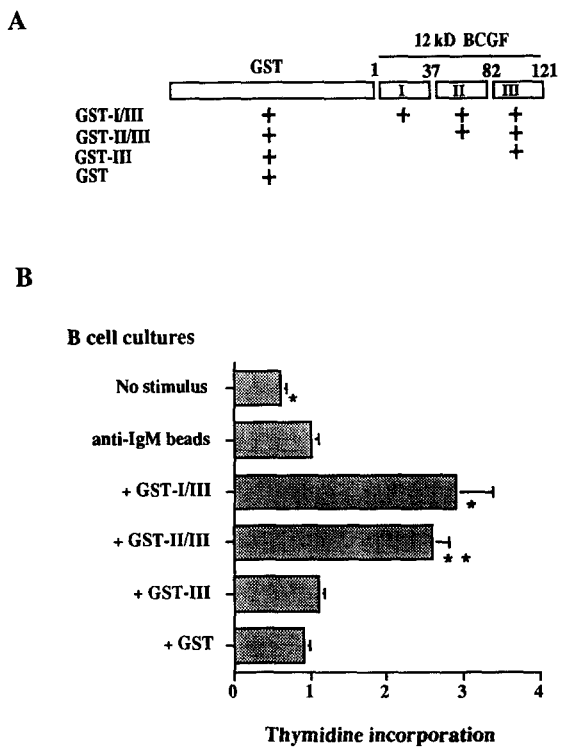

Fig. 3. B cell growth promoting activity of different fusion proteins derived from the genomic sequence of 12 kDa BCGF. (A) Schematic presentation of different fusion proteins. GST-I/III is the full-length polypeptide, GST-II/III is the polypeptide with 37 amino-terminal amino acids truncated, and GST-III contains the ALU-derived carboxy-terminus. (B) Relative thymidine incorporation of purified B cells cultured with anti-IgM-coupled beads (100 μg/ml) and with fusion protein eluates (10% of total culture volume). The amounts of fusion proteins were equalized by their λ280 absorbance. The data is the average of five separate experiments. The standard error is given and the asterisks represent significant differences in thymidine incorporation when compared to anti-IgM-stimulated B cells ($*P < 0.02$, $**P < 0.002$, paired Student's *t*-test).

suggested to contain translated ALU sequences. One of these is a splice variant of primary biliary glycoprotein and another is RMSA-1, a presumably chromosomal protein [25–27].

To study possible mitogenic effects of the polypeptide and to start mapping the active domains we constructed three different glutathione *S*-transferase fusion proteins and tested their effect on the growth of anti-IgM activated B lymphocytes: the full-length polypeptide (GST-I/III) and two truncation mutants (Fig. 3A). In the first mutant the 37 amino-terminal amino acids were truncated, leaving the part identical with the previously reported 12 kDa BCGF protein intact. The second mutant contains only the ALU-derived carboxy-terminal sequences (GST-III). As presented in Fig. 3B, both the full-length fusion protein (GST-I/III), and the amino-terminally truncated one (GST-II/III) significantly increased the thymidine incorporation of activated B cells when compared to B cells activated with anti-IgM beads alone ($P < 0.02$ and $P < 0.002$, respectively). On the other hand, the polypeptide derived from translated ALU sequences (GST-III) or GST alone did not induce B cell growth. Fig. 4B demonstrates the dose-dependent response of anti-IgM-activated B cells to full-length polypeptide, and the lack of response to ALU-derived GST-III when stimulated with the same concentrations of fusion protein (as estimated from Coomassie blue stained gels; Fig. 4A) The growth-promoting activity of GST-I/III was significant at a dilution of $\geq 1:100$ ($P < 0.007$), corresponding to a specific concentration of approximately 10 ng/ml. The fusion proteins GST-I/III and -II/III were relatively hydrophobic which resulted in co-purification of some bacterial proteins and endotoxin. The endotoxin content was reduced to a final concentration of < 2 ng/ml with 1 M NaCl and elution through a detoxigel column (Pierce) without affecting the growth factor activity (data not shown). It is also known that only relatively high concentrations of endotoxin are mitogenic to human B cells (≥ 100 μg/ml) [28]. Therefore, we concluded that the genomic sequence encodes a polypeptide with BCGF activity.

Table 1
Hybridization of human *BCGF 1* gene with human/rodent somatic cell hybrids

Hybrid	*BCGF 1*	Chromosomes present
NA07299	–	1,X
GM10880	–	1,13,14
NA10826B	–	2
NA10253	–	3
NA10115	–	4
NA10114	–	5
NA10629	–	6
NA10791	–	7
NA10156B	–	8
NA10611	–	9
NA10926B	–	10
NA10927A	–	11
NA10868	–	12
NA10898	–	13
NA10479	–	14
NA11418	–	15
NA10567	+	16
NA10498	–	17
NA11010	–	18
NA10449	–	19
NA10478	–	4,20
NA10323	–	21
NA10888	–	22
NA06318B	–	X
NA06317	–	Y

+ The presence of the *BCGF 1* gene in the hybrids.

In summary, we have characterized and mapped the genomic locus coding for the 12 kDa BCGF (*BCGF1*), a potential B cell

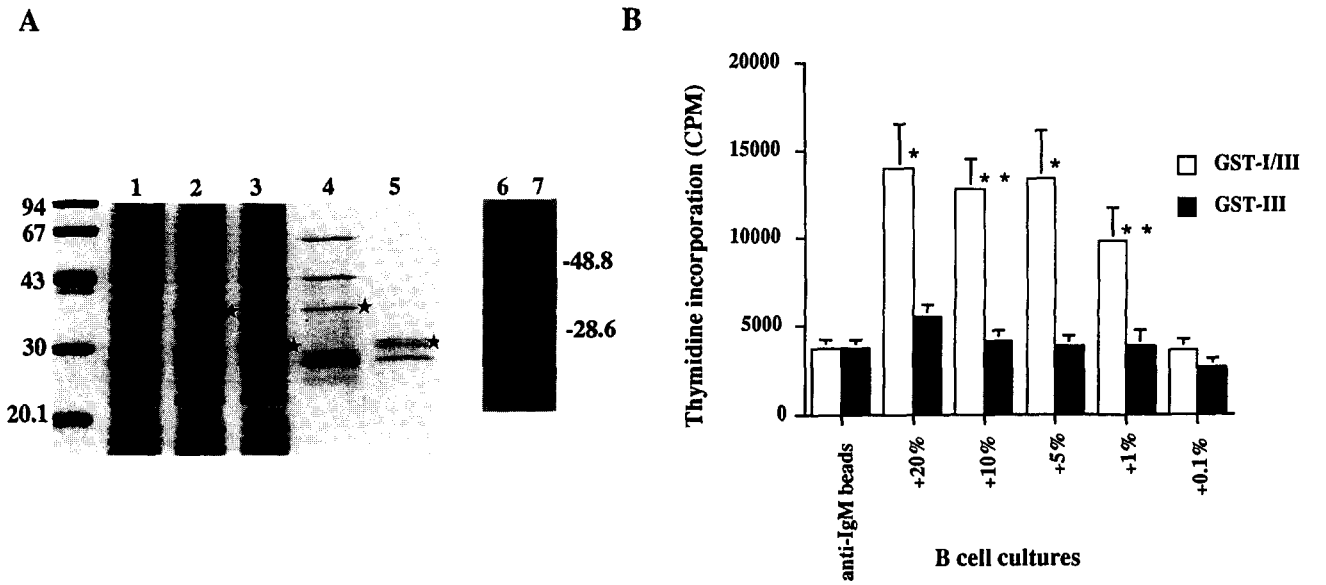

Fig. 4. Polyacrylamide gel analysis of fusion proteins GST-I/III and GST-III and their effect on the growth of activated B lymphocytes. (A) Coomassie-blue stained lysates of uninduced bacteria (lane 1) and IPTG-induced (1.5 h) bacteria expressing GST-I/III (lane 2) and GST-III (lane 3) and purified fusion protein eluates of GST-I/III (lane 4) and GST-III (lane 5). The corresponding fusion proteins are marked by asterisks. Lanes 6 and 7 show a Western blot analysis of the purified fusion proteins GST-I/III and GST-III, respectively. The peptide antibody was raised against a sequence present in GST-I/III but not in GST-III. 5 μl of eluates were loaded to the gels. (B) Thymidine incorporation of anti-IgM-activated B lymphocytes induced by different concentrations (% of total culture volume) of the same eluates. The results are presented as the average of five separate experiments with standard error given. The asterisks represent significant differences in thymidine incorporation between cultures. (*$P < 0.015$, **$P < 0.007$, paired Student's *t*-test).

mitogen and growth factor of neoplastic B cells. Furthermore we have expressed the polypeptide as a GST fusion protein and shown that the purified recombinant factor stimulates the growth of activated B lymphocytes. The reported expression system for recombinant 12 kDa BCGF will facilitate the study of human B cell proliferation and may prove useful in developing culture techniques of human B cells, for example for human antibody production.

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