A novel GTP-binding protein hGBP3 interacts with NIK/HGK

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Abstract A novel human guanylate-binding protein (GBP) hGBP3 was identified and characterized. Similar as the two human guanylate-binding proteins hGBP1 and hGBP2, hGBP3 has the first two motifs of the three classical guanylate-binding motifs, GXXXXGKS (T) and DXXG, but lacks the N (T) KXD motif. *Escherichia coli*-expressed hGBP3 protein specifically binds to guanosine triphosphate (GTP). Using a yeast two-hybrid system, it was revealed that the N-terminal region of hGBP3 binds to the C-terminal regulatory domain of NIK/HGK, a member of the group I GCK (germinal center kinase) family. This interaction was confirmed by in vitro glutathione-*S*-transferase (GST) pull-down and co-immunoprecipitation assays.

Key words: Human guanylate-binding protein 3; Nck-interacting kinase/HPK/GCK-like kinase; Yeast two-hybrid screening

1. Introduction

Guanine nucleotide-binding proteins play important roles in various basic cellular processes such as protein synthesis, signal transduction, intracellular protein transportation, cell proliferation and differentiation, and regulation of cytoskeleton [1,2]. Most known guanine nucleotide-binding proteins contain three classical motifs: GXXXXGK (S/T), DXXG and (N/T) KXD [3,4]. If present in a given protein sequence in an ordered fashion and with typical spacing, these motifs are highly indicative for associated guanine nucleotide-binding activity [3]. Structural analysis of P21-ras [5], elongation factor Tu [6–8] and human guanylate-binding protein 1 (hGBP1) [9] indicated that these motifs formed a part of the guanosine triphosphate (GTP)-binding pocket. Some guanine nucleotide-binding proteins including p21 ras [10–12] and heterotrimeric G proteins [13] harbor at their C-terminal an additional CAAX motif, which functions as an isoprenylation signal and thus ensures the proper anchoring of these proteins in a membrane [14,15].

Among the guanine-binding proteins, a group of proteins, named GTP-binding proteins (GBPs), are interferon-inducible and can be immobilized by agarose-guanylate nucleotide [16,17]. A characteristic feature of those GBPs is that they lack the third classical motif (N/T) KXD, and might also lack the C-terminal motif CAAX [3]. Thus far GBPs have been identified in human [14,18–21], mouse [20,21], rat [21] and chicken [23], as well as in *Drosophila* and *Caenorhabditis elegans* [24]. However, the physiological roles of these GBPs remain elusive. GBPs can be accumulated to high level in the interferon-treated cells and thus qualify as potential intracellular mediators of the interferon-induced antiviral and antiproliferation effect. Overexpression of hGBP1 or hGBP2 blocks the replication of nesiculul stomatitis virus and encephalomyocarditis virus in HeLa cells [25]. However, GBP1 knock-out mice and cells derived from such mice are not more susceptible to viral infections than their wild counterparts [24].

Human Nck-interacting kinase/HPK/GCK-like kinase (NIK/HGK) [4] is a member of the group I germinal center kinases (GCKs), a group of mitogen-activated protein kinase kinase kinase kinases (MAP4K) that activate the SAPK/JNK cascade in a variety of cell types [4,22,26–29]. At least five members of this group have been identified including GCK [30], GCK-related kinase (GCKR) [27], GCK-like kinase (GLK) [26], hematopoietic progenitor kinase 1 (HPK1) [22] and NIK/HGK [4]. Like other members of the group I GCKs, NIK/HKG is composed of an N-terminal kinase domain and a highly conserved C-terminal regulatory domain necessary for its kinase activity [4]. The C-terminal domain consists of a hydrophobic leucine-rich CHN domain, a 140–150 amino acid stretch, a C-terminal (CT) region [4], in addition to at least two proline/glutamic acid/serine/threonine (PEST) motifs and two polyproline consensus-binding sites for proteins containing Src homolog (SH-3) domains [4]. Human NIK/HGK was originally identified based on its interaction with Nck and homology to HPK and GCK [28,29]. It could activate JNK through TAK and/or MEKK1→MKK4 and MKK7→JNK kinase cascade [28,29].

As a part of our effort to search for brain-specific genes involved in signal transduction, we cloned a novel GBP homolog, hGBP3, and identified the CHN domain of NIK/HGK as its binding partner.

2. Materials and methods

2.1. Cloning of hGBP3 and database search

The 5′ rapid amplification of cDNA ends (5′-RACE) was performed using human brain Marathon cDNA library (Clontech) with

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Abbreviations: GBP, guanylate-binding protein; NIK/HGK, Nck-interacting kinase/HPK/GCK-like kinase; 5′-RACE, 5′ rapid amplification of cDNA ends

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a gene-specific primer 5′-TCA ACA TGA AGT CCA TC-3′ following the manufacturer's protocol. The polymerase chain reaction (PCR) product obtained was gel purified, and cloned into pcDNA3.1 (−)/Myc-His A vector (Novagen) and subsequently sequenced.

2.2. GTP-binding assay

The coding sequence of the hGBP3 gene was generated by PCR from a human Marathon cDNA library (Clontech) using the primers 5′-GGC G*GT CGA C*AT GGC CAA GAA CCT CAG G-3′ and 5′-GGC G*CT CGA G*CA TTT TTT TCT TTT CTG ATT GTT C-3′. The PCR product was cloned into the PET22b (+) (Novagen) between the *Sal*I and *Xho*I recognition sites. The resulted plasmid encoded hGBP3 protein tagged by $(His)_6$ at the C-terminal. The $(His)_6$-tagged hGBP3 protein was induced by 0.1 mM isopropyl beta-D-thiogalactoside (IPTG) in *Escherichia coli* BL21(DE3) at 30°C for 3 h and purified using TALon-Nx Metal Resin Column (Clontech) according to the manufacturer's protocol. Binding assays were carried out using 5 μg purified recombinant hGBP3 and GTP- and CTP-agarose (Sigma) as described [32]. Bound proteins were eluted and analyzed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and visualized by Coomassie blue staining. For the competition experiment, the reaction system was suspending 10 mM of competitor before adding the purified hGBP3.

2.3. Northern blot analysis

Northern blot analysis was carried out using Human 12-Lane MTN Blot membrane (Clontech) as per manufacturer's protocol. The hGBP3 probe derived from its 3′ untranslated region (base 1840–2300) was prepared by random primer labeling with [α^{32}P]dATP using a prime-a-gene labeling system kit (Promega). After prehybridization, hybridization and highly stringent washing, the membrane was exposed to an X-ray film (Eastman Kodak Co.) for 48 h at −80°C.

2.4. Yeast two-hybrid screening and mapping of hGBP3 for the region binding to NIK/HGK

The complete coding region of hGBP3 was inserted into pLexA (Clontech) and used as bait to screen a human adult brain cDNA library in pB42AD vector in a yeast strain EGY48 possessing the p8op-Laz reporter plasmid (Clontech). Approximately 2×10^6 yeast transformants were screened according to the manufacturer's instruction. For mapping the region of hGBP3 that mediated the interaction between hGBP3 and NIK/HGK, N- or C-terminal truncated hGBP3 mutants were fused with LexA protein in vector pLexA and the CHN domain of HGK was fused with B42 transactivation domain in vector pB42AD.

2.5. Glutathione-S-transferase (GST) pull-down and co-immunoprecipitation assays

A DNA fragment encoding the C-terminal 367 amino acids of NIK/HGK was inserted into pGEX-2T (Amersham Pharmacia Biotech). The resulting plasmid GST367 encoding a recombinant protein was transformed into *E. coli* BL21(DE3). GST367 was induced by using 0.1 mM IPTG at 30°C for 3 h and purified using glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). 50 μl hGBP3-$(His)_6$, 50 μl GST367 and 100 μl buffer 1 (50 mM Tris–Cl pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40, 50 μg/ml soybean trypsin inhibitor, 100 μg/ml leupeptin and 40 μg/ml phenylmethylsulfonyl fluoride) were mixed and incubated for 1 h at room temperature with end-on-end shaking. The beads were separated by a brief centrifugation, washed five times with buffer 1, and suspended in 25 μl SDS sample loading buffer (50 mM Tris–Cl pH 6.8, 100 mM DTT, 2% bromophenol blue, 10% glycerol). The bound protein was analyzed by Western blot using anti-His antibody (NeoMmarkers).

For co-immunoprecipitation, hGBP3 was cloned into pcDNA3.1 (−)/Myc-His A and tagged at the C-terminal with a Myc epitope, and the C-terminal 367 amino acids of NIK/HGK (HGK367) were inserted into pcDNA3.0 and tagged at the C-terminal with a FLAG epitope (DYKDDDDK). Meanwhile, as controls *Aequorea victoria* green fluorescent protein (GFP) and NIK/HGK without the last 367 amino acids (HGKΔ367) were also inserted into pcDNA3 and tagged with a FLAG epitope at the C-terminals. The plasmids were transfected into 293T cells cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) using the lipofectamine plus reagent (Gibco) following the manufacturer's instruction. The cells were harvested 48 h after transfection and lysed using M-PER mammalian protein extraction reagent (Pierce) at room temperature for 10 min. Nuclear and cellular debris was removed by centrifugation at $10\,000\times g$ for 15 min at 4°C. Cell extracts were incubated with 2 μg/ml of mouse anti-myc antibody (Calbiochem) overnight at 4°C. Protein A-agarose beads (Amersham Pharmacia Biotech) were then added and incubated for 2 h at room temperature. The immunoprecipitated samples were washed extensively with phosphate-buffered saline (PBS) and then boiled in SDS loading buffer. The supernatant was subjected to 12% SDS–PAGE and protein was transferred to nitrocellulose membrane (Schleincher and Schucell). The membrane was immunoblotted with chicken anti-FLAG antibody and horseradish peroxidase-conjugated goat anti-chicken IgY (Aves labs). The signals were detected using the ECL system (Amersham Pharmacia Biotech).

3. Results

3.1. Brain-specific hGBP3 has a GTP-binding activity

An EST contig encoding a partial protein homolog to hGBP1 and hGBP2 was identified by searching brain-specific ESTs in a human-expressed sequence tag (EST) database (www.ncbi.nlm.nih.gov/). The complete mRNA sequence obtained by 5′-RACE and reverse transcriptase (RT)-PCR was based on the EST sequences. The novel mRNA consisted of a 1675-bp open reading frame (ORF) with the ATG translation initiation code in a Kozak consensus sequence [31], and a putative polyadenylation signal AATAAA in the 3′ untranslated region (GenBank accession number AF444143). The putative protein deduced from the ORF is approximately 63 kDa in molecular mass, contained two of the three guanylate-binding classical motifs: GXXXXGK (S/T) and DXXG, and lacked the third motif (N/T) KXD (Fig. 1), suggesting a structural homology to hGBP1 and hGBP2. It was designated hGBP3.

HGBP3 is conserved during the evolution. Both *Drosophila melanogaster* and *C. elegans* have hGBP3 homologs. HGBP3 displays 49.0 and 51.6% amino acid identities to the homologs from *C. elegans* (GenBank accession number AAK68527) and *D. melanogaster* (GenBank accession number AAF56318), respectively.

It was previously suggested that the GXXXXGKS (T) and DXXG motifs are highly indicative for associated guanine nucleotide-binding activity [3]. Since the hGBP3 contained the motifs, *E. coli*-expressed hGBP3 was incubated with agarose-GTP beads to test its GTP-binding capacity. As expected, agarose-GTP was able to recover hGBP3 protein while agarose-CTP failed to do so (Fig. 2A). Competition experiment showed that the binding of hGBP3 to agarose-GTP could be significantly reduced by free GTP but not affected by ATP and CTP (Fig. 2A), indicating the binding of hGBP3 to GTP was specific.

The expression pattern of hGBP3 was investigated in a variety of human tissues by Northern hybridization. Two transcripts approximately 2.4 and 2.0 kb in length in brain

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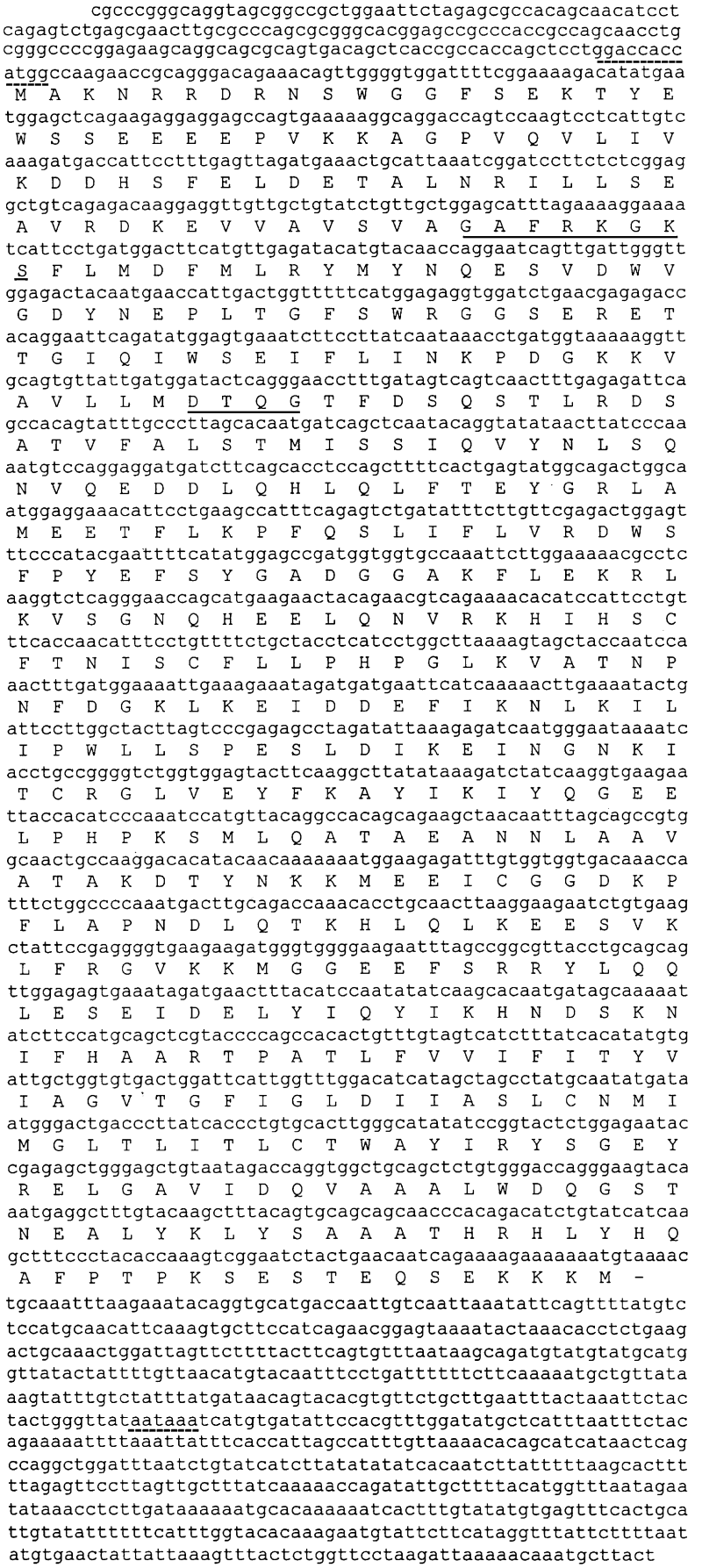
Fig. 1. Sequence analysis of hGBP3. The nucleotide sequence and the deduced amino acid sequence. The Kozak consensus and polyadenylation signal AATAAA are underlined by dashed lines; the GXXXXKS (T) and DXXG motifs are underlined by solid lines. The hGBP3′ GenBank accession number is AF444143.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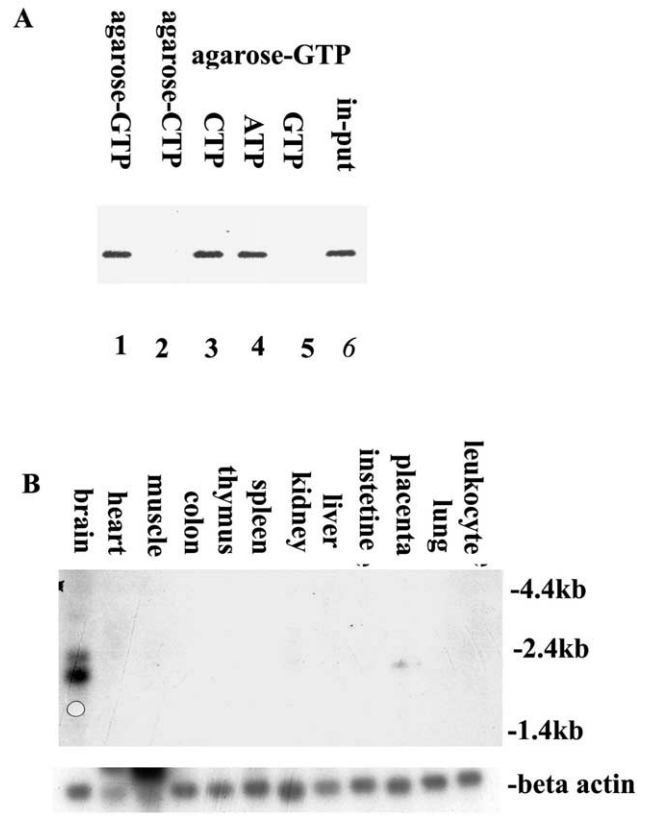

Fig. 2. The GTP-binding property and expression of hGBP3 in human tissues. A: GTP-binding assay. 5 μg purified recombinant hGBP, $(his)_6$ tagged at the C-terminal was mixed with 30 μl agarose-GTP beads (lane 1), or 30 μl agarose-CTP beads (lane 2). The bound material was analyzed by 10% SDS–PAGE and stained with Coomassie blue. For the competition experiment, the reacting system was suspended with 10 mM GTP (lane 5), CTP (lane 3), and ATP (lane 4). Lane 6, purified hGBP3 protein. B: Northern blot analysis of hGBP3 mRNA in human tissues. 1 μg of $polyA^+$ RNA from human tissue was run on each lane. The same was hybridized with β-actin probe (bottom panel). The molecular size was marked on the right side of the panel.

and a 2.0-kb transcript in placenta were observed, but not in other tissues examined (Fig. 2B), suggested that the hGBP3 expression was brain-specific in adult, consistent to the result from searching the EST database.

3.2. *The N-terminal region of hGBP3 specifically binds to the CHN domain of the HIK/HG*

Biological function of hGBP3 was investigated by screening a human adult brain cDNA library using a yeast two-hybrid assay in order to identify proteins binding to hGBP3 protein. Approximately 2×10^6 yeast transformants were screened. One clone capable of growing in selective media and displaying a strong galactosidase signal was identified. Sequence analysis indicated that the clone encoded a partial regulatory domain (a complete CHN domain) of the NIK/HGK protein, a member of the group I GCK kinases which could activate JNK kinase in 293T cells. The C-terminal regulatory domain of NIK/HGK is necessary for its N-terminal kinase activity.

In order to confirm the interaction of hGBP3 and NIK/HGK, a fusion protein (GST367) containing the regulatory domain (the C-terminal 367 residuals) of NIK/HGK fused to a bacterial GST was employed to pull-down the *E. coli*-expressed hGBP3 in vitro. GST protein was used as a control to test the specificity of the binding. The Western blot results showed that the hGBP3 protein bound to the GST367 fusion protein whereas no interaction between hGBP3 and GST was detected (Fig. 3A), confirming the specificity of the GST367–hGBP3 interaction.

HGBP3-myc was subsequently used to co-immunoprecipitate the FLAG-tagged CHN domain of NIK/HGK by transient transfection of pcDNA3.1-hGBP3-myc and pcDNA3-NIK/HGK-FLAG plasmids in 293T cells, using anti-myc antibody and protein A-agarose. Truncated NIK/HGK protein lacking the CHN domain was not precipitated by hGBP3, consistent with the in vitro GST pull-down experiment (Fig. 3B).

To further map the region binding to NIK/HGK, the C- and N-terminal truncated hGBP3 were tested for their interaction with the CHN domain of NIK/HGK in the yeast two-hybrid system (Fig. 4). The N-terminal 129 amino acid

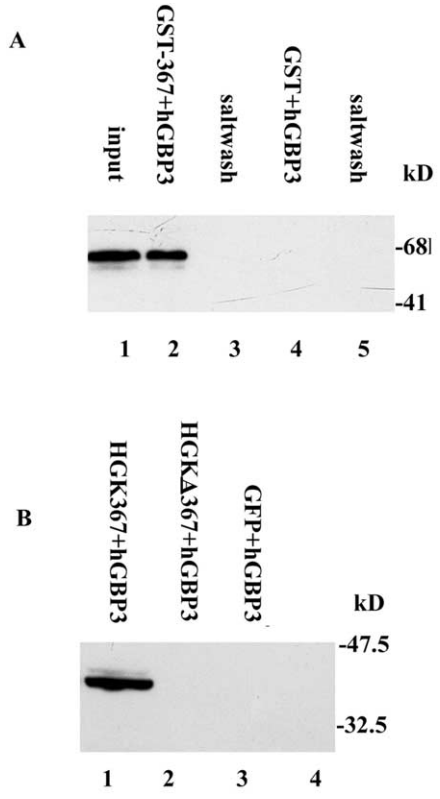

Fig. 3. Confirmation of hGBP3–NIK/HGK interaction. A: GST pull-down assay of hGBP3 and NIK/HGK. Proteins bound to the GST fusion proteins were eluted and analyzed by Western blot using an anti-His antibody. Bacterially expressed hGBP-His (lane 1) was mixed with bacterially expressed GST fused to the C-terminal of NJK/HGK (lane 2) or GST (lane 4), and incubated with glutathione-Sepharose 4B beads. lanes 2 and 5, wash buffer from fifth washing. B: Co-immunoprecipitation of hGBP3 and NIK/HGK. Plasmid encoding hGBP3-myc was co-transfected into 293T cells with the plasmid encoding the CHN domain of NIK/HGK-FLAG (HGK367) (lane 2), the plasmid encoding NIK/HGK deleting the CHN domain also tagged with FLAG (HGKΔ376) (lane 3), or the plasmid encoding GFP-FLAG (lane 4). 48 h after transfection, the cells were lysed and co-immunoprecipitation was performed using anti-myc antibody and protein A. The co-immunoprecipitation complex was washed extensively and run on a SDS–PAGE. The proteins were transferred to membrane and blotted with anti-FLAG antibody.

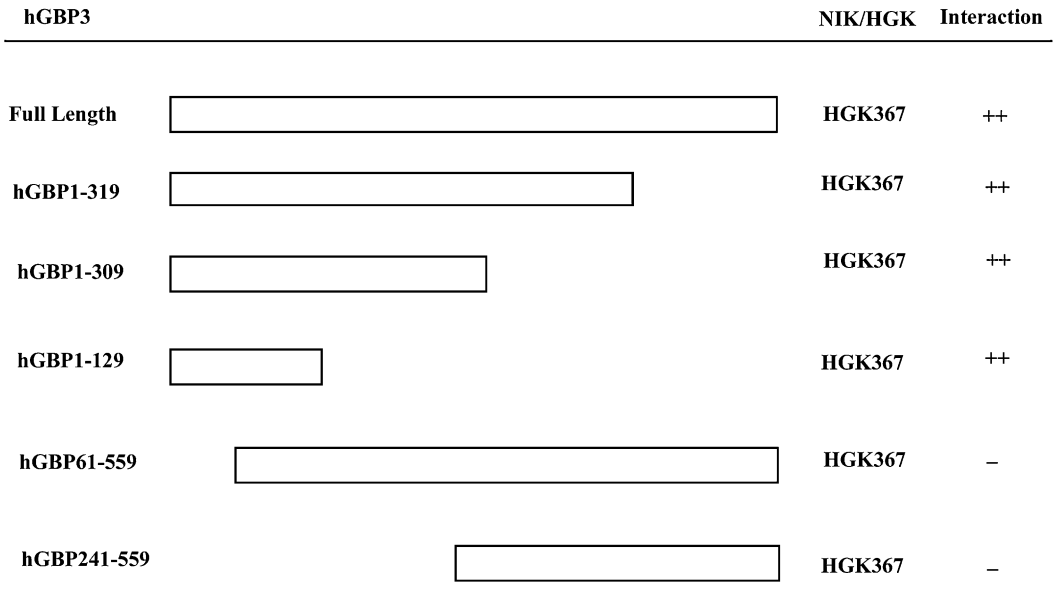

Fig. 4. Mapping of the NIK/HGK-binding site of hGBP3 by a two-hybrid system. The cDNAs encoding hGBP were inserted into pLexA vector and the cDNAs encoding CHN domain of NIK/HGK (HGK367) were inserted into pB42AD vector. The protein–protein interactions were analyzed by yeast two-hybrid assays as described in Section 2. ++: Growth of blue colonies on the leucine-deficient selection medium containing 80 μg/ml X-gal was detected within 1 day; −: no blue colony was detected after 5 days.

residuals of hGBP3 were sufficient to bind to NIK/HGK, whereas a deletion of the first 60 amino acids completely eliminated the interaction between the two proteins. Thus the N-terminal of hGBP3 is essential for mediating its interaction with NIK/HGK.

4. Discussion

GBPs are synthesized upon interferon activation [16,17] and can be accumulated to high levels in interferon-treated cells, thus qualify as potential intracellular mediators of the interferon-induced antiviral and antiproliferation effect. However, the mechanism leading to the effect was unknown [25]. Our discovery that hGBP3 directly interacts with NIK/HGK suggested a possible involvement of JNK signal transduction in interferon-induced antivirus processes.

JNK, also known as stress-activated MAP kinase (SAPK), plays crucial roles in stress response, cell proliferation, apoptosis and ontogenesis. JNK kinase activity can be activated by treatment of cells by cytokines (e.g. TNF and IL-1) and by exposure of cells to various environmental stresses including osmotic stress, redox stress, and radiation [28]. Previous studies have demonstrated that double strand RNA and encephalomyocarditis infection can activate JNK [31,33], and overexpression of human GBP proteins blocks encephalomyocarditis replication in HeLa cells [25]. These reports were consistent with our findings that hGBP3 could regulate the phosphorylation of JNK through direct interaction with NIK/HGK, and might possibly provide a link between the JNK signaling and the antiviral effect of interferons.

The C-terminal regulatory domain of NIK/HGK is composed of a CHN domain, two PEST motifs and two polyproline consensus-binding sites for proteins containing Src homology (SH3) domains [4]. Nck binds to the SH3-binding motif of NIK/HGK and activates the latter [28]. The C-terminal of NIK/HGK also binds MEKK1; the latter is subsequently phosphorylated, and leads to JNK activation [28]. Our observation suggested that the CHN domain of NIK/HGK bound to hGBP3 and thus assigns a new biological function to the important region of the MAP4K.

Mammals have two protein kinase families related to *Saccharomyces cerevisiae* STE20 kinase; both activate JNK. One is the PAK kinase family, which has an N-terminal regulatory domain and a C-terminal kinase domain. The N-terminal of PAKs contains a CDC42/Rac interaction and binding (CRIB) domain [4], or PAKs are activated by GTP-bound Cdc42 and Rac [4]. The other family is the GCK family including GCKs and MST1, containing an N-terminal kinase domain and a C-terminal regulatory region, and lacking a Cdc42- and Rac-binding domain [4,19]. Our studies showed that although not regulated by small GTP-binding proteins Cdc42 and Rac, the GCK family member NIK/HGK could interact with another GTP-binding protein, hGBP3. Since GBP proteins might also bind GDP [18], it will be very interesting to find if the hGBP3 regulation of NIK/HGK is also affected by the kind of cofactors it binds, i.e. GTP or GDP. Further, it will be extremely interesting to investigate the possible involvement of hGBP1 and hGBP2 in the regulation of NIK/HGK, and even other group I GCKs including GLK, GCK, GCKR, and HPK1.

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