Serum Starvation Activates NF-κB Through G Protein β2 Subunit-Mediated Signal

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Several cell stresses induce nuclear factor-kappaB (NF-κB) activation, which include irradiation, oxidation, and UV. Interestingly, serum-starving stress-induced NF-κB activation in COS cells, but not in COS-A717 cells. COS-A717 is a mutant cell line of COS cells that is defective of the NF-κB signaling pathway. We isolated genes with compensating activity for the NF-κB pathway and one gene encoded the G protein β2 (Gβ2). Gβ2 is one of the G protein-coupled receptor signaling effectors. In COS-A717 cells, Gβ2 expression is significantly reduced. In Gβ2 cDNA-transfected COS-A717 cells, the NF-κB activity was increased along with the recovery of Gβ2 expression. Furthermore, serum-starving stress induced the NF-κB activity in Gβ2-transfected COS-A717 cells. Consistently, the serum-starved COS cells with siRNA-reduced Gβ2 protein expression showed decreased NF-κB activity. These results indicate that Gβ2 is required for starvation-induced NF-κB activation and constitutive NF-κB activity. We propose that serum contains some molecule(s) that strongly inhibits NF-κB activation mediated through Gβ2 signaling.

Introduction

NUCLEAR FACTOR-KAPPAB (NF-κB) is a ubiquitously expressed transcription factor with critical roles in cell survival, proliferation, apoptosis, immune response, and inflammation. NF-κB usually exists as a heterodimer of p50 and p65 (Rel A), and is kept in the cytoplasm through an association with inhibitor of kappaB (IκB) inhibitory proteins. After various stimulations, the serine residues at positions 32 (S32) and 36 (S36) in the IκB proteinare phosphorylated (Brown *et al.*, 1995) by the IκB kinase (IKK) complex (Zandi *et al.*, 1997), and the IκB protein is degraded by the ubiquitin–proteasome pathway (Chen *et al.*, 1995). The IKK complex consists of two catalytic subunits, IKK1 and IKK2 (also referred to as IKKα and IKKβ), and a regulatory subunit, NEMO (Yamaoka *et al.*, 1998). Cytokines and various cell stresses, including irradiation (Criswell *et al.*, 2003), oxidation (Marshall *et al.*, 2000), and UV (Kato *et al.*, 2003), induce NF-κB activation. Serum starvation also activates NF-κB in various cell lines (Ryter and Gomer, 1993; Grimm *et al.*, 1996), indicating that serum contains unknown inhibitor(s) of NF-κB.

On the other hand, constitutively active NF-κB exists in certain normal cells (Pagliari *et al.*, 2000; Lilienbaum and Israel, 2003) and several tumor cells without stimulation (Mori *et al.*, 1999; Lind *et al.*, 2001). However, the mechanism by which NF-κB is constitutively activated in these cells is not known. COS cells have a relatively high level of basal NF-κB activity. We established a mutant cell line, COS-A717, with a defective NF-κB signaling pathway (Kohno *et al.*, 2008). The basal level of NF-κB activity in the COS-A717 cells was reduced by as much as sevenfold, as compared with that in the parental COS cells. Serum starvation induced NF-κB activation in the parental COS cell line, but not in the COS-A717 cell clone. Since the COS-A717 cell clone was constructed by the treatment of COS cells with a frameshift-inducing agent, it is most likely that the NF-κB activating factor(s) expressed in the parental COS cells is not functional in the COS-A717 cells. We previously isolated the B cell activating factor of the TNF family (BAFF) receptor as an NF-κB activator in COS-A717 cells (Kohno *et al.*, 2008). However, the original COS cells do not express BAFF-R, indicating that BAFF-R is not responsible for the defective NF-κB signaling in the COS-A717 cells, and activates NF-κB through a salvage pathway.

In this study, we isolated the guanine nucleotide-binding protein β2 subunit (Gβ2) cDNA as another NF-κB activator by screening a human spleen cDNA expression library. The guanine nucleotide-binding proteins (G proteins) are signal transducers required for various G protein coupled receptor (GPCR)-effector networks (Xie *et al.*, 2000; Wu *et al.*, 2001; Albert and Robillard, 2002). GPCRs 'transduce signals through heteromeric G proteins, and several of them activate NF-κB (Xie *et al.*, 2000; Grabiner *et al.*, 2007; Sun *et al.*, 2009). The heteromeric G proteins consist of α, β, and γ subunits,

and the α subunit has GTPase activity. When GPCRs interact with their ligands, the active GTP-bound α subunit is released from the heteromeric G protein complex, and Gα and Gβγ induce downstream signaling (Stephens *et al.*, 1994). The Gβ2 expression level in the parental COS cells is much higher than that in the mutant COS-A717 cells. Transfection of a Gβ2 expression plasmid activated NF-κB in COS-A717 cells. The knockdown of Gβ2 expression by siRNA in COS and HT1080 cells reduced the basal NF-κB activity. These results indicate that the activation of the GPCR signal pathway by Gβ2 results in constitutive NF-κB activation in the transformed cells, and the defect of Gβ2 expression is one of the determinants for reduced NF-κB activity in the COS-A717 mutant cells.

Serum starvation activates NF-κB in COS cells, but not in COS-A717 cells. Transfection of COS-A717 cells with Gβ2 restored the starvation-induced NF-κB activation. These results show that NF-κB activation by serum starvation occurs through the Gβ2 signaling pathway, and the inhibitor(s) present in serum sppress the Gβ2 signal. Taken together, our findings suggest that the constitutive NF-κB activation in transformed cells is induced by the GPCR signaling pathway through Gβ2, and that serum contains factor(s) reducing NF-κB activity by suppressing the GPCR signaling.

Materials and Methods

Plasmids and reagents

The human spleen cDNA library was purchased from Life Technologies. The five-tandem κB luciferase reporter vector (5×κB luciferase) was purchased from Stratagene. The IκBα superrepressor (IκBα-SR) expression plasmid was described previously (Sugita *et al.*, 2002). The expression vectors for the dominant negative forms of IKK1 (IKK1.DN), IKK2 (IKK2.DN), and NEMO (NEMO.DN) were kind gifts from Dr. Yamaoka (Tokyo Medical and Dental University, Tokyo, Japan) (Hironaka *et al.*, 2004). The expression vector for the dominant negative mutant of Akt (Akt.DN) was generously provided by Dr. V. Stambolic (Ontario Cancer Institute, Toronto, Canada). Wortmannin was purchased from Sigma.

Cells

The mutant cell lines COS-A717 and COS-A717-GS were described previously (Kohno *et al.*, 2008). The mouse spleen cells were obtained from a C57BL/6 mouse. COS (Kohno *et al.*, 2008), HT1080 (Jones *et al.*, 1975), and HEK293T cells were maintained in the Dulbecco's modified Eagle's medium, and ST1 cells (Yamada, 1996) were maintained in the RPMI1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified 5% CO_2 atmosphere.

Expression cloning of Gβ2

Isolation of genes with compensating activity for the NF-κB activation pathway was performed according to the previously described method (Kohno *et al.*, 2008). Briefly, COS-A717-GS cells were transfected with a human spleen cDNA library (Life Technologies) using the FuGene 6 reagent. After 48 h of transfection, the top 0.5% fraction of fluorescent cells was collected using a FACStar Plus (Becton, Dickinson and Co.). Plasmids were extracted from sorted

cells (Hirt, 1967), amplified in bacteria, and used in three subsequent rounds of flow cytometry-based enrichment. Individual bacterial colonies obtained from the third sorting were grouped into pools of 50 colonies. Positive pools were subdivided further into subpools with half the number of colonies, and were subjected to repeated screening. This process finally yielded independent clones that conferred compensation for the NF-κB activation pathway in COS-A717 cells.

Transfection and luciferase assay

Cells were transfected with a 5×κB-luciferase reporter and a Gβ2 expression plasmid, as indicated in the text and figure legends. Transient transfections were performed using the FuGene 6 reagent (Roche). When necessary, additional DNA (pcDNA3.1) was added to equalize the amount of transfected DNA in each sample. At 48 h post-transfection, the κB-directed expression of firefly luciferase was determined, using luciferase assay reagents (Promega), and the luciferase activities were measured with a BioOrbit 1254 luminometer. The relative transfection efficiency in each sample was determined by measuring the Renilla luciferase activity. The data were normalized per transfection efficiency. Data shown are averages and SD from three independent experiments.

Western blot analysis

Cell extracts were prepared from the cells transfected for the luciferase assay. Cell lysates were resolved by 12.5% SDS PAGE, transferred onto an Immobilon-P membrane (Millipore), and blocked with 5% nonfat dry milk in TBS with 0.5% Tween 20. The blots were incubated with anti-Gβ2, anti-IKK1, anti-IKK2, anti-NEMO, anti-Akt1, anti- IκBα, and anti-phosho S32, and S36-containing peptide of IκBα antibodies (Santa Cruz Biotechnology; refer to Tables 1 and 2), or an anti-β-actin antibody (Chemicon), followed by an incubation with a horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit Ig (Amersham Pharmacia Biotech). The blots were visualized with the ECL detection system (Amersham Pharmacia Biotech).

Electrophoretic mobility shift assay

Preparation of nuclear extracts for electrophoretic mobility shift assays (EMSAs) was performed as described previously (Sugita *et al.*, 2002). The consensus κB site 5′-AGTTGAGG GGACTTTCCCAGGC-3′ and mutant 5′-AGTTGAGGCGAC TTTCCCAGGC-3′ oligonucleotides were obtained from Santa Cruz Biotechnology. The double stranded oligonucleotides were end-labeled with [γ-^{32}P] ATP, using T4 polynucleotide kinase (Takara). The reaction was conducted in a total volume of 10 μL, using 10 μg of nuclear extract, 1 μg of poly(dI-dC), 20 mM HEPES-NaOH (pH 7.6), 100 mM NaCl, 1 mM DTT, 1 mM PMSF, and 2% glycerol. The binding reaction mixture was incubated with 10,000 cpm of radiolabeled probe for 15 min. For the competition and supershift assays, a 20-fold excess of unlabeled or mutant oligonucleotide, and the antibodies to p65 or p50 (Santa Cruz Biotechnology) were added to the reaction, respectively. The samples were loaded onto a 5% nondenaturing polyacrylamide gel, which was run in a 0.5×TBE buffer. After

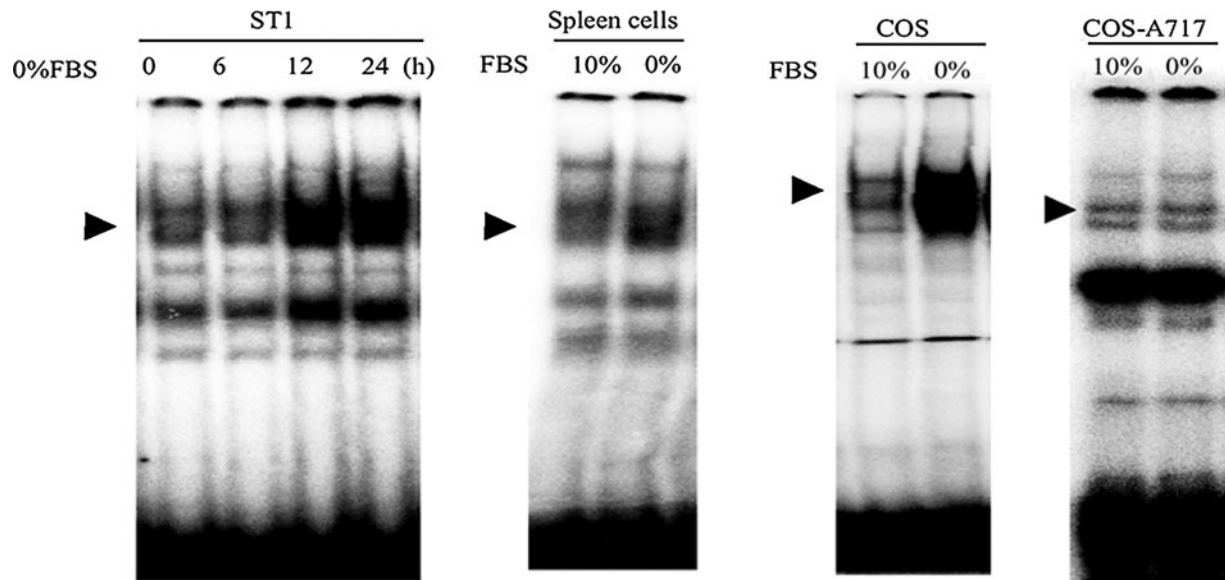**FIG. 1.** Serum-starving stress-induced nuclear factor-kappaB (NF-κB) activation. The nuclear extracts were incubated with a ^{32}P-labeled NF-κB consensus oligonucleotide, and analyzed by an electrophoretic mobility shift assay. Nuclear cell extracts from ST1 cells, which were cultured without fetal bovine serum (FBS) for 0, 6, 12, 24 h (*STI panel*). The mouse spleen cells were cultured with 10% FBS or 0% FBS for 24 h (*Spleen cells panel*). Nuclear cell extracts from COS (*COS panel*) and COS-A717 (*COS-A717 panel*) cells, which were cultured with 10% FBS or 0% FBS for 24 h. The *arrowhead* indicates the NF-κB-containing complex.

electrophoresis, the gel was dried and processed for auto-radiography.

siRNA

The nucleotide sequences of the two siRNAs for Gβ2 are as follows:

#1 sense 5'-CAUCUGCUCCAUCUACAGCdTdT-3', anti-sense 5'-GCUGUAGAUGGAGCAGAUGdTdT-3'; #2 sense 5'-AGACCUUCAUCGGCCAUGAdTdT-3', anti-sense5'-UCAUGGCCGAUGAAGGUCUdGdT;

and sense 5'-GGCUACGUCCAGGAGCGCAdTdT-3', anti-sense 5'-UGCGCUCCUGGACGUAGCCdTdT-3' for GFP. The annealed oligonucleotides were transfected by using Lipofectamine 2000 (Invitrogen). Cells were maintained in the Dulbecco's modified Eagle's medium without FBS at the transfection. Cells were harvested after 24 and 48 h after the transfection for COS and HT1080 cells, respectively. For the luciferase assay, cells were transfected with the 5×κB-luciferase reporter using the FuGene 6 reagent, at 6 h after the siRNA transfection.

Results

#### Serum-starving stress induces NF-κB activation

Cell stresses, such as irradiation, UV, and oxidation (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/dna), induce NF-κB activation. Cells are usually cultured with 10% FBS in medium *in vitro*. Serum includes various factors and nutrients for cell survival and proliferation, and thus serum starvation ceases cell proliferation, and then induces cell death. Serum may include factors affecting NF-κB signaling. To address this issue, we analyzed the effects of serum starvation on NF-κB binding to the target sequence by EMSA of nuclear extracts from several cell lines (ST1,

COS, and COS-A717) and mouse primary spleen cells. The binding of NF-κB to the target sequence was enhanced by serum starvation in all examined cells, except for the mutant COS-A717 cells, which exhibit defective NF-κB signaling (Fig. 1). These results indicate that serum starvation induces NF-κB activation, suggesting that serum contains unknown factor(s) inhibiting NF-κB activity.

Expression cloning of an NF-κB activating molecule using COS-A717 cells

Many transformed cell lines containing the COS cell line have constitutively activated NF-κB signaling. To identify NF-κB activators in the COS cells, a COS-A717 cell derivative containing the GFP gene under the control of the Sp1 site-deleted HIV-1 LTR was constructed, and the cells were designated as COS-A717-GS. The GFP is expressed by NF-κB activation in the COS-A717-GS cells, because the expression from the Sp1 site-deleted HIV-1 LTR is NF-κB dependent. The COS-A717-GS cells were transfected with a human spleen cDNA expression library, and GFP-expressing cells were selected (Fig. 2A). The sequence analysis of the cDNA expressed in the GFP-positive COS-A717-GS cells revealed that it perfectly matched the Gβ2. The expression level of the Gβ2 protein in COS-A717 cells was much lower than that in the parental COS cells (Fig. 2B).

To confirm that Gβ2 activates NF-κB activity, COS and COS-A717 cells were transfected with the Gβ2 expression plasmid, and the NF-κB promoter activity was measured using the 5×κB-luciferase plasmid. Gβ2 activated the NF-κB promoter activity in both COS and COS-A717 cells, in a dose-dependent manner (Fig. 2C). Transfection of the COS-A717 mutant cells with the Gβ2 expression plasmid (0.5 μg) resored the NF-κB activity comparable to the parental COS cells (sevenfold). When 0.5 μg of the Gβ2 expression plasmid was transfected
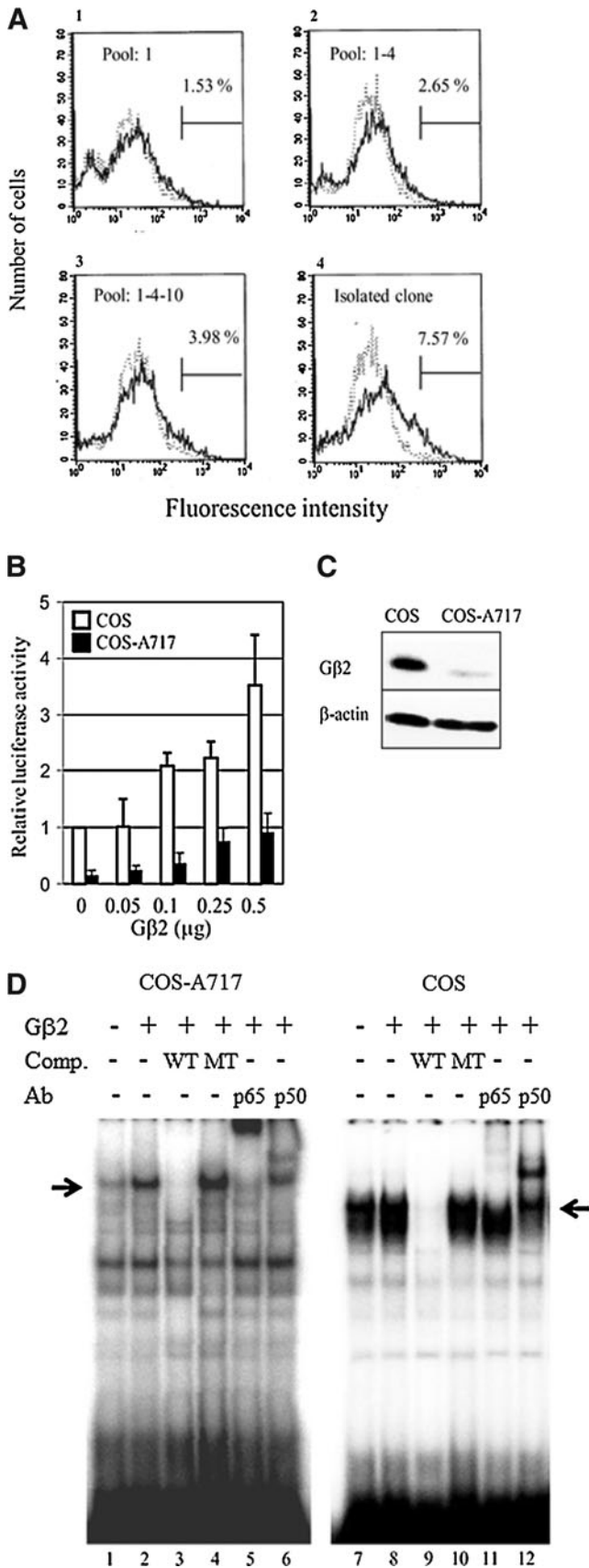
into the COS cells, the NF-κB activity was also increased by threefold. The DNA-binding activity of NF-κB was elevated by Gβ2 by about 1.7- and 6.2-fold in COS and COS-A717 cells, respectively (Fig. 3D). The complex formation was inhibited by a wild-type κB oligonucleotide competitor, but not by a mutant κB oligonucleotide. The complex was supershifted by both anti-p65 and -p50 antibodies, indicating that the complex consisted of p65 and p50 (Fig. 3D). The Gβ2 transfection activated the NF-κB signal more efficiently in the mutant COS-A717 cells than in the COS cells that originally express Gβ2. These results indicate that Gβ2 activates NF-κB signaling and the defect of Gβ2 expression is one of the determinants for the reduced NF-κB activity in the COS-A717 cells.

Gβ2 is required for NF-κB activation induced by serum starvation

Serum starvation activated NF-κB by 10-fold in COS cells, but had no effect in COS-A717 cells (Fig. 3A). Since the level of the Gβ2 protein is much lower in the COS-A717 cells than in the parental COS cells, we examined whether Gβ2 was involved in the NF-κB activation by serum starvation. Serum starvation elevated the NF-κB activity by fourfold in the Gβ2-transfected COS-A717 cells, indicating that Gβ2 is required for the serum starvation-induced NF-κB activation and that the Gβ2-activated signal is inhibited by the unknown factor(s) present in serum. However, because the level of NF-κB activity in the starved Gβ2-expressing COS-A717 cells was lower than that in the starved COS cells, the COS-A717 cells have additional defect(s) in the NF-κB signal activation.

FIG. 2. G protein β2 (Gβ2) activates NF-κB. **(A)** Identification of Gβ2 by expression cloning. **(A-1)** COS-A717-GS cells were transfected with plasmids obtained from a positive pool of 50 bacterial transformants (pool 1) following four rounds of FACS enrichment. **(A-2)** COS-A717-GS cells were transfected with plasmids from a positive pool (1-4), containing 20 bacteria colonies. **(A-3)** COS-A717-GS cells were transfected with plasmids from a positive pool (1-4-10), containing 10 bacterial colonies. **(A-4)** COS-A717 GS cells were transfected with a Gβ2-encoding clone. **(B)** Western blot analysis of Gβ2 in COS and COS-A717 cells. Proteins were analyzed by immunoblotting with an anti-Gβ2 Ab (*top*) and an anti-β-actin Ab (*bottom*). **(C)** Gβ2-mediated NF-κB activation in COS-A717 cells. COS and COS-A717 cells were transiently transfected with 0.25 μg of the 5×κB-luciferase reporter and the Gβ2 expression construct (0.05, 0.1, 0.25, and 0.5 μg), and then additional DNA (pcDNA3) was added to make the total amount of DNA 1 μg/well. At 48 h post-transfection, the cells were harvested and the luciferase activity was measured. The relative transfection efficiency in each sample was determined by the measurement of the Renilla luciferase activity. The relative luciferase activity in control COS cells (without Gβ2) was set to 1.0. Data shown are averages +SD from three independent experiments. **(D)** Gβ2 induced the NF-κB-binding ability in COS-A717 cells and COS cells. Nuclear proteins from untransfected (*lanes 1, 7*) or Gβ2 transfected (*lanes 2–6, 8–12*) COS-A717 cells or COS cells were isolated. The unlabeled consensus κB oligonucleotide (*lanes 3, 9*) or the mutant κB oligonucleotide (*lanes 4, 10*) was added as a competitor in a 20-fold molar excess to the binding reaction. Abs against p65 (*lanes 5, 11*) and p50 (*lanes 6, 12*) were added to the reaction for a supershift assay. The *arrow* indicates the NF-κB-containing complex.
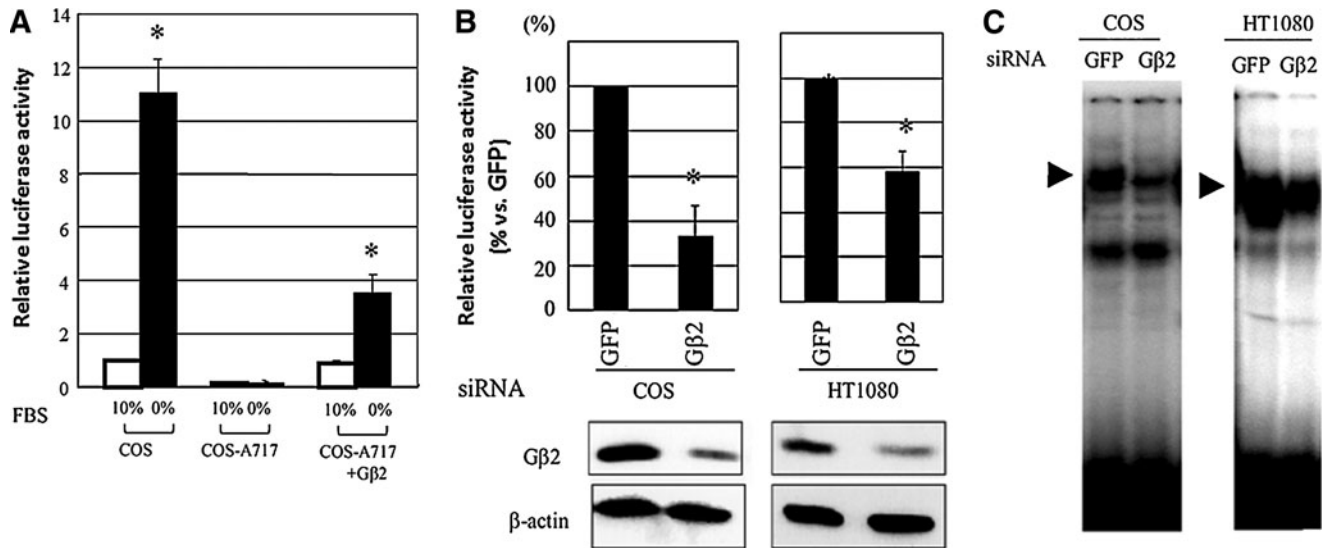
FIG. 3. Gβ2 is required for serum starvation-induced NF-κB activation and for constitutive NF-κB activation in transformed cells. **(A)** The serum-starving stress-induced NF-κB activation was analyzed by a luciferase assay. COS, COS-A717, and Gβ2-transfected COS-A717 cells were transiently transfected with the 5×κB-luciferase reporter. Six hours after transfection, the cells were washed with phosphate buffered saline (PBS) and incubated with (10%) or without FBS (0%) for 36 h for the luciferase assay. The luciferase activities in COS cells incubated with 10% FBS were set as 1.0. The activations were significant (*$p < 0.05$). **(B)** The Gβ2 siRNA reduced the NF-κB activity in COS and HT1080 cells. The NF-κB activity was determined by transfection with the 5×κB-luciferase reporter together with the GFP or Gβ2 siRNA, and shown as the % of that in cells transfected with the GFP siRNA. Western blot analyses of Gβ2 (*top*) and β-actin (*bottom*) in cells transfected with the GFP or Gβ2 siRNA#1 were performed. The inhibitions were significant (*$p < 0.05$). **(C)** Nuclear cell extracts were isolated from COS and HT1080 cells transfected with the GFP or Gβ2 siRNA. The *arrowhead* indicates the NF-κB-containing complex.

Gβ2 is involved in NF-κB in HT1080 human fibrosarcoma cell line

HT1080 cells also have a relatively high level of basal NF-κB activity. We examined whether Gβ2 contributes to the constitutive activation of NF-κB in HT1080 cells. Knockdown of Gβ2 expression by siRNA reduced the basal NF-κB activity not only in COS cells, but also in HT1080 cells (Fig. 3B). The siRNA against Gβ2 indeed reduced the Gβ2 protein level. Consistent with the κB promoter activity, the knockdown of Gβ2 inhibited the NF-κB-binding capability to the target sequence (Fig. 3C). These results indicate that Gβ2 is required for the constitutive activation of NF-κB in COS and HT1080 cells, suggesting that Gβ2-mediated signaling contributes to the constitutive NF-κB activation. The serum-deprived Gβ2-mediated NF-kB activation in COS cell was also confirmed using another siRNA (Supplementary Fig. S2).

Impact of IKKs, NEMO, and IκB in Gβ2-induced NF-κB activation

To determine whether the Gβ2 induced NF-κB activation requires IκB phosphorylation, a IκBα-SR with mutations at the inducible phosphorylation sites, S32G and S36A, was coexpressed with Gβ2 in COS-A717 cells. The IκB-SR abolished the Gβ2-induced NF-κB activation in a dose-dependent fashion (Fig. 4A). This result suggests that the phosphorylation of IκBα at S32 and S36 is necessary for the Gβ2-induced NF-κB activation. An important regulator of phosphorylation in the IκB pathway is the IKK complex, which comprises multiple kinases, including IKK1 (IKKα), IKK2 (IKKβ), and NEMO (IKKγ). We examined whether IKK1, IKK2, and/or

NEMO were involved in the Gβ2-induced NF-κB activation. Dominant negative forms of IKK1 (IKK1.DN), IKK2 (IKK2.DN), and NEMO (NEMO.DN) were each coexpressed in the Gβ2-transfected COS-A717 cells. As shown in Figure 4B–D, IKK1.DN, IKK2.DN, and NEMO.DN were each able to reduce the Gβ2-induced NF-κB activation in COS-A717 cells in a dose-dependent fashion, indicating that IKK1, IKK2, and NEMO are involved in the Gβ2-induced NF-κB activation. Especially, the IKK2.DN more efficiently suppressed the Gβ2-mediated NF-κB activation than IKK1.DN and NEMO.DN. This result suggests that IKK2 plays an important role in the Gβ2-induced NF-κB activation, like the bradykinin-induced NF-κB activation through Gαq and Gβγ (Xie *et al.*, 2000). To confirm the expressions of the dominant negative and endogenous IKK1, IKK2, NEMO, IκBα, and Akt, COS cells were transfected with the mutant expressing plasmids, and the cell lysates were subjected to Western blotting analysis using their specific antibodies. The descriptions of the dominant negative mutants and their specific antibodies used here are summarized in the Tables 1 and 2, respectively. As shown in Figure 5A, we have confirmed that the anti-IKK1, NEMO, and Akt1 antibodies were able to detect the simian endogenous proteins as well as their human and mouse dominant negative mutants. On the other hand, the antibodies against IKK2 and IκBα reacted to their human dominant negative mutants and the human endogenous proteins in HEK293T cells, but not to the simian endogenous proteins. Considering the different affinities of the antibodies between endogenous simian proteins and their human or mouse counterparts, we could not assess precisely the relative amounts of dominant negative mutants to endogenous proteins in COS cells. However, each
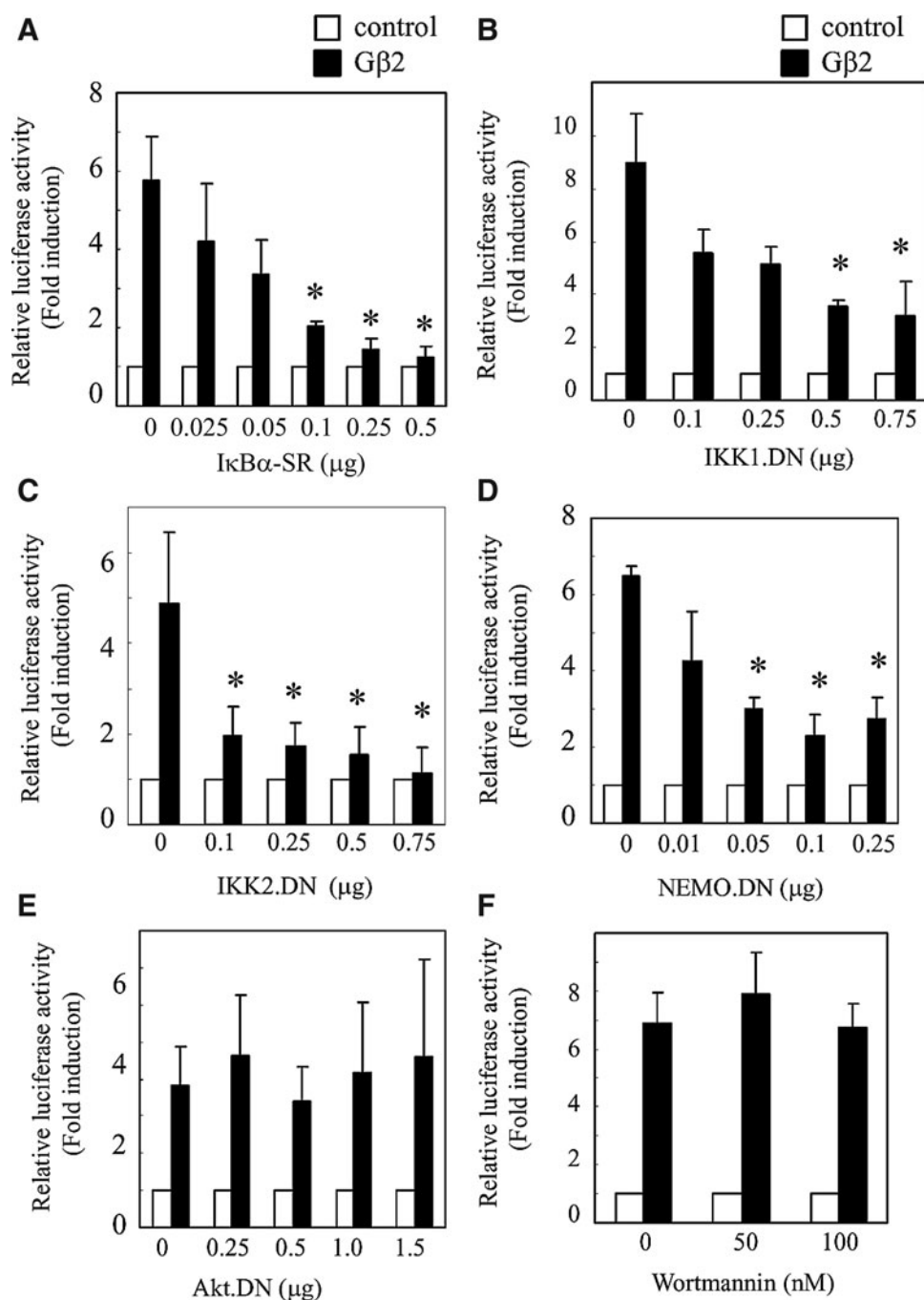
FIG. 4. Involvement of IκB, IκB kinase 1 (IKK1), IKK2, and NEMO in Gβ2-induced NF-κB activation. COS-A717 cells were transfected with 0.25 μg of the 5×κB-luciferase and the mutant expression plasmid of IκBα super-repressor (IκBα-SR) **(A)**, IKK1 (IKK1.DN) **(B)**, IKK2 (IKK2.DN) **(C)**, NEMO (NEMO.DN) **(D)**, or Akt (Akt.DN) **(E)**, together with the Gβ2 construct (0.25 μg) or pcDNA3.1. The pcDNA3.1 plasmid was added to make the total amount of DNA 1 μg/well. COS-A717 cells were cotransfected with 0.5 μg of the 5×κB-luciferase reporter without (control) or with 0.5 μg of the Gβ2 construct. Wortmannin was added 1 h before transfection **(F)**. The relative luciferase activity in the control cells without Gβ2 was set as 1.0. Data shown are averages and SD from three independent experiments. The inhibitions were significant (*$p < 0.05$) .

TABLE 1. CHARACTERISTICS OF THE DOMINANT NEGATIVE MUTANTS

Molecule	Species	Structure	MW (kDa)	Tag
IκBα	Human	S32G, S36A mutant	40	—
IKK1	Human	N145D mutant	85	VSV
IKK2	Human	N145D mutant	87	VSV
NEMO	Mouse	97–412 C-terminal fragment	37	VSV
Akt	Mouse	K179A, T308A, S473A mutant	69	–

MW, molecular weight.

TABLE 2. CHARACTERISTICS OF THE ANTIBODIES

Antigen	Cross-reactivity
Human IκBα C-terminal peptide	Human/mouse/rat
Human IKK1 full-length	Human/mouse/rat
Human IKK2 C-terminal peptide	Human/canine
Human NEMO full-length	Human/mouse/rat
Human Akt1 345–480 peptide	Human/mouse/rat
Human phospho-S32 and S36-containing peptide	Human/mouse

A

FIG. 5. **(A)** Expressions of the dominant negative and endogenous IKK1, IKK2, NEMO, IκBα, and Akt. COS cells were transfected with 1 μg of the mutant expressing plasmids of IKK1.DN, IKK2.DN, NEMO.DN, superrepressor of IκB (IκB-SR), and Akt.DN (refer to Table 1) in a six-well plate, and the cell lysates were prepared using 100 μL Glo lysis buffer (Promega) after 48h incubation. HEK293T cells were also transfected with the same plasmids, and prepared for Western blot. The description of antibodies is shown in Table 1. The *arrows* indicate the described proteins. N.D: not done. **(B)** COS, COS-A717, and COS-A717-Gβ2 cells were washed with PBS and incubated with (10%) without FBS (0%) for 24 h, and lysed with Glo lysis buffer. The positions of total IκBα, and phospho-IκBα were indicated with *arrowhead* and *arrow*, respectively.
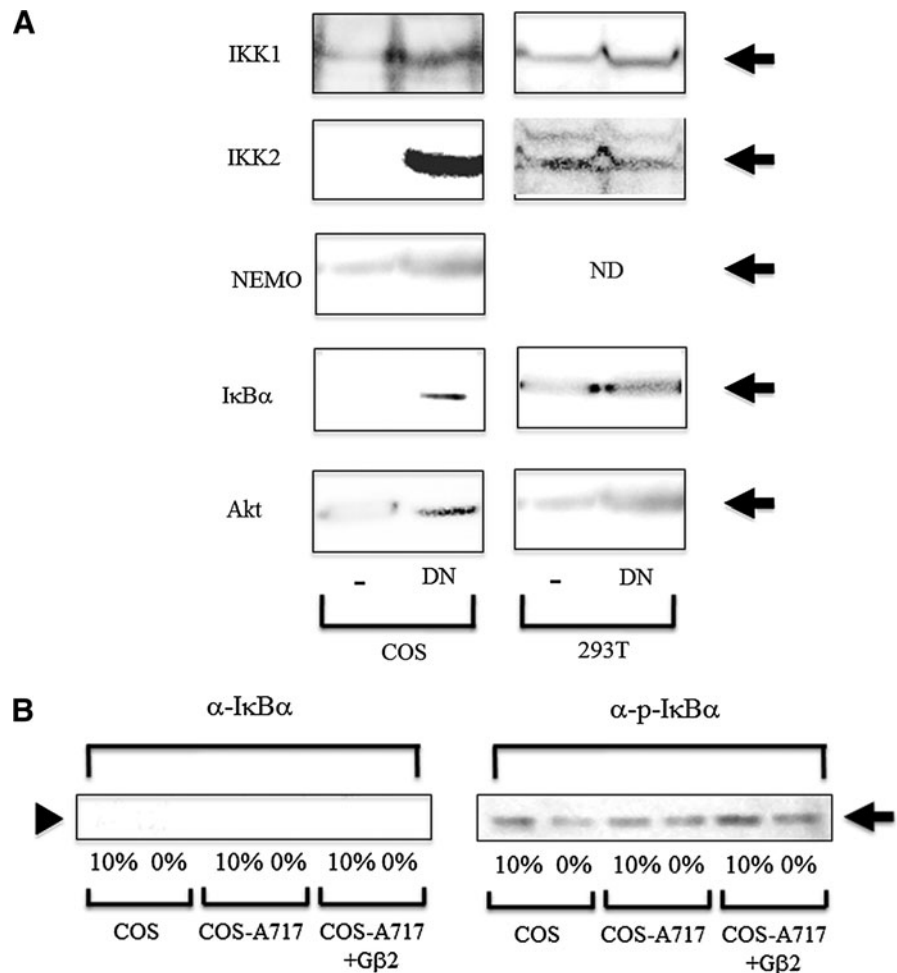
dominant negative mutant is likely to be expressed enough to suppress its endogenous protein. To evaluate the phosphorylation and degradation of IkBα in COS, COSA717, and COSA717-Gβ2 cells by serum deprivation, we carried out Western blotting analysis using their specific antibodies. There were no significant changes in the phosphorylation of IκBα of COS, COSA717, and COSA717-Gβ2 cells by serum deprivation, using a phosphorylation-specific antibody (right panel of Fig. 5B). We could not assess the degradation of IκBα, because the anti-IκBα antibody was actually able to detect the human IκBα protein, but not simian COS IκBα, or because the IκBα expression in COS cell is too low to be detected by this antibody (Fig. 5A and left panel of Fig. 5B). The NF-κB activation by serum deprivation was dependent on IKK1, IKK2, NEMO, and IκBα, and this unique characteristic was not related to the IκBα phosphorylations at S32 and S36.

Because PI3K and Akt are upstream factors of IKKs in the NF-κB activation pathway (Ozes *et al.*, 1999; Romashkova and Makarov, 1999; Xie *et al.*, 2000), we examined whether the Gβ2-induced NF-κB activation occurs through PI3K and Akt activation. However, the PI3K inhibitor, Wortmannin, and a dominant negative mutant of Akt did not affect the Gβ2-induced NF-κB activation in COS-A717 cells (Fig. 4E, F). This result suggests that PI3K and Akt is not involved in the Gβ2-induced NF-κB activation.

Discussion

Many cell stresses activate NF-κB. We have shown here that serum starvation activates NF-κB signal, indicating that serum contains unknown inhibitor(s) of NF-κB signal. Cell stresses, such as radiation (Criswell *et al.*, 2003), oxidation (Marshall *et al.*, 2000), and UV (Kato *et al.*, 2003) positively control the NF-κB signaling. Interestingly, serum negatively regulates the NF-κB signaling, and starvation stress induces NF-κB activation by exclusion of the negative factor of serum.

Serum starvation activated NF-κB signaling in COS cells, but not in COS-A717 cells. The transfection of COS-A717 cells with Gβ2 partially restored the serum starvation-induced NF-κB activation. This result indicates that Gβ2 is required for the starvation-induced NF-κB activation, and the serum inhibitor suppresses the Gβ2-induced signaling pathway (Fig. 6).

Serum starvation of cells is frequently used in many biological experiments, including cell cycle synchronization and induction of apoptosis and autophagy. These biological events induced by starvation unexpectedly include the activation of Gβ2 and NF-κB signals. Therefore, these signaling might affect the synchronization of the cell cycle and the induction of apoptosis and authophagy by starvation, and scientists should consider the effects of the Gβ2 and NF-κB signals in the biological experiments using serum starvation.
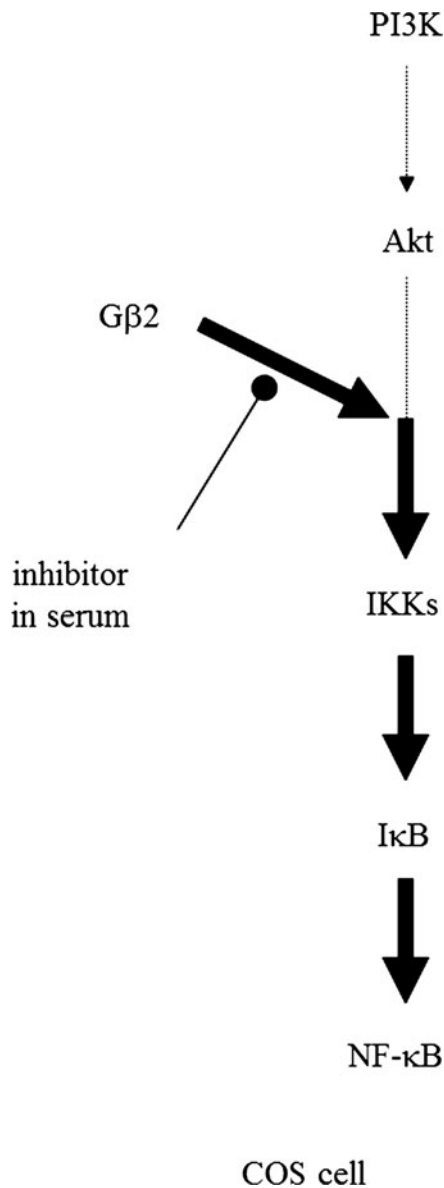
PI3K

Akt

Gβ2

inhibitor
in serum

IKKs

IκB

NF-κB

COS cell

FIG. 6. Signaling pathway of NF-κB activation by Gβ2 or serum starvation.

We are trying to identify the serum inhibitor, and it will provide great impacts into many biological research fields.

NF-κB is constitutively activated in several transformed cell lines, suggesting that NF-κB signaling is involved in cellular transformation. However, the mechanism has not been elucidated yet. COS-A717 cells are mutant cells in which the basal NF-κB activity is much lower compared with the parental COS cells. Here, we showed that COS-A717 cells expressed a lower level of Gβ2 than COS cells, and the transfection of COS-A717 cells with Gβ2 restored the basal NF-κB activity, suggesting that the reduced expression level of Gβ2 is responsible for the defective NF-κB signaling in COS-A717 cells. Furthermore, the knockdown of Gβ2 expression by siRNA reduced the basal NF-κB activity not only in the COS cells, but also in the HT1080 cells, another transformed cell line with constitutively activated NF-κB signaling. These results indicate that Gβ2 is required for the constitutive activa-

tion of NF-κB in these transformed cells. This conclusion is strongly supported by previous reports showing that certain GPCR signals or the Gβ1γ2 complex activate NF-κB signaling (Xie *et al.*, 2000; Grabiner *et al.*, 2007; Sun *et al.*, 2009). Furthermore, the Tax oncoprotein of HTLV-1 activates NF-κB (Mori *et al.*, 1999; Gohda *et al.*, 2007) as well as the signals of CXCR4, a GPCR, by binding to the Gβ subunit (Twizere *et al.*, 2007), consistent with our conclusion. Although the Gβγ complex activates NF-κB through PI3K (Stephens *et al.*, 1994; Xie *et al.*, 2000), a PI3K inhibitor did not affect the Gβ2-induced NF-κB activation, suggesting that Gβ2 activates independently of PI3K in the NF-κB activation pathway (Fig. 6).

In summary, this study found that Gβ2-induced signaling activates NF-κB independently of PI3K and Akt in COS cells (Fig. 6). Unknown factor(s) present in serum inhibit the Gβ2-induced signaling. Therefore, serum starvation activates NF-κB by removing the serum inhibitor(s). The Gβ2-induced signaling is the target of the serum inhibitor, because exclusion of the serum inhibitor by starvation elevates NF-κB activity in Gβ2-expressing COS cells, but does not affect in Gβ2-defective COS-A717 cells.

Disclosure Statement

No competing financial interests exist.

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