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Nuclear translocation of 2-amino-3-ketobutyrate coenzyme A ligase by cold and osmotic stress

Akemi Hoshino and Hodaka Fujii

Department of Pathology, New York University School of Medicine, 550 First Avenue, New York, NY 10016, USA

Abstract Cells are continuously exposed to environmental stresses and respond to them to maintain cellular homeostasis. Failure to respond to these stresses may cause pathological states such as renal failure, complications of diabetes, and autoimmune diseases. Signal transduction induced by osmotic and cold stresses is not fully understood. In addition, mechanisms of these stress responses are yet to be elucidated. Activation of many signaling pathways induces translocation of proteins into the nucleus to transduce signals and regulate nuclear functions. By using inducible translocation trap (ITT), a reporter gene–based screening technique, nuclear translocation of 2-amino-3-ketobutyrate coenzyme A ligase (KBL) was detected in response to cold and osmotic stresses. Rapid nuclear translocation of KBL was confirmed by biochemical analysis and fluorescent microscopy. A large region of KBL was required for stress-induced nuclear translocation. The KBL reporter system will be a useful tool for the investigation of cold and osmotic stress responses.

INTRODUCTION

Cells are continuously exposed to environmental stresses such as osmotic and cold stresses, and they respond to these stresses to maintain cellular homeostasis. Cells must preserve a balanced osmolality to prevent dehydration and maintain viability. To respond to hypertonicity, eukaryotic cells have machinery for acute compensatory changes in cell volume. In addition, osmotic stress induces changes in expression of genes including those functioning to increasing intracellular organic osmolytes (Burg et al 1997). In mammalian cells, hypertonicity is detected by unknown mechanisms, leading to activation of stress-activated protein kinase (SAPK)/JNK, p38, and ERK5 (de Nadal et al 2002). Osmotic stress–induced activation of Syk protein tyrosine kinase also has been reported (Roig et al 2000; Takano et al 2002). Then, nuclear factor of activated T cells 5 (NFAT5)/tonicity enhancer binding protein (TonEBP) is activated (Miyakawa et al 1999; Macian et al 2001). Except these small number of proteins, information on signaling/effector molecules involved in mammalian osmotic stress response is limited.

Analysis of signal transduction of cold stress has been limited in contrast to extensive analyses of other stress responses, especially its counterpart, heat shock response. On sensory neurons, the TRPM8 and TRPA1 ion channels of the transient receptor potential (TRP) family plays a critical role in cold perception (McKemy 2005). In other cell types, mechanisms of cold perception are yet to be clarified. Phosphorylation of p38 has been reported (Gon et al 1998), and translocation of β-crystallin from the nucleus to the cytoplasm was detected (Coop et al 1998). Moderate cold stress (25–33°C) has been shown to induce gene expression in the nucleus (Sonna et al 2002). Exposure to cold stress inhibits cell proliferation (Shimura et al 1997; Shimura et al 1998) or leads to expression of inflammatory cytokines (Sonna et al 2002). Mechanisms of these cellular responses are yet to be clarified. Defective cold responses are known to be involved in pathogenesis of several important diseases including cold urticaria and familial cold urticaria ([FCU], Zip et al 1993). Preventive hypothermia is widely used in brain and cardiac surgery to prevent tissue damage. However, this procedure can cause complications such as edema. Understanding of signal transduction of cold stress is essential for treatment of cold stress–induced diseases and reduction of complications by hypothermia and decrease in mortality rate during and after surgery.

Activation of many signaling pathways induces translocation of proteins into the nucleus to transduce signals and regulate nuclear functions. Identification and char-

Correspondence to: Hodaka Fujii, Tel: 212 263-8514; Fax: 212 263-8179; E-mail: hodaka@med.nyu.edu.

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acterization of stress-induced nuclear translocating molecules are essential for understanding molecular mechanisms of stress signaling and effector mechanisms. Inducible translocation trap (ITT) developed in our lab (Hoshino et al 2004) is a novel system for detecting inducible nuclear translocation. In the ITT system, a fusion protein of LexA DNA-binding domain (LexA DB), Gal4 transactivation domain (Gal4 TA), and a test protein (LG-fusion protein) is expressed in a reporter cell line containing green fluorescent protein (GFP) reporter gene fused with LexA-operator sequences (LexA-d1EGFP). Reporter gene expression indicates nuclear translocation. ITT-based screening strategy of cDNA library enables us to identify novel nuclear translocating proteins without prior knowledge about mechanisms of nuclear translocation.

By using ITT, nuclear translocation of 2-amino-3-ketobutyrate coenzyme A ligase (KBL) was detected in response to cold and osmotic stresses. Rapid nuclear translocation of KBL was confirmed by biochemical analysis and fluorescent microscopy. A large region of KBL was required for stress-induced nuclear translocation. The KBL reporter system will be a useful tool for the investigation of cold and osmotic stress responses.

MATERIALS AND METHODS

ITT screening of stress-responsive nuclear translocating proteins

The Ba/F3-derived cDNA library constructed in the pLG vector (Hoshino et al 2004) was transfected into 293T cells with helper plasmid to produce retrovirus particles. pLG expresses a LG-fusion protein consisting of LexA DB, Gal4 TA, and a protein encoded by cDNA insert (Hoshino et al 2004). Following this, BLG cells, which were derived from Ba/F3 cells containing LexA-d1EGFP reporter gene, were infected with the supernatant of the 293T cells containing virus particles. Following nuclear translocation of the LG-fusion proteins by extracellular stimuli, the LexA DB targets the fusion protein to the LexA operator sites of the reporter gene, and then the Gal4 TA activates the expression of GFP. Thus, nuclear translocation of the LG-fusion protein is detected by the expression of GFP. By sorting GFP (+) cells in the presence of stimulation of interest and GFP (−) cells in the absence of the stimulation, cells expressing LG-fusion proteins that translocate into the nucleus in response to stimulation of interest can be enriched (Fig 1; Hoshino et al 2004).

To expose cells to cold stress and high pressure, LG-fusion library-transduced cells were subjected to sorting with the MoFlow superfast cell sorter at room temperature, and GFP (−) cells were sorted. Four hours later, GFP (+) cells that are induced to express GFP by sorting procedure

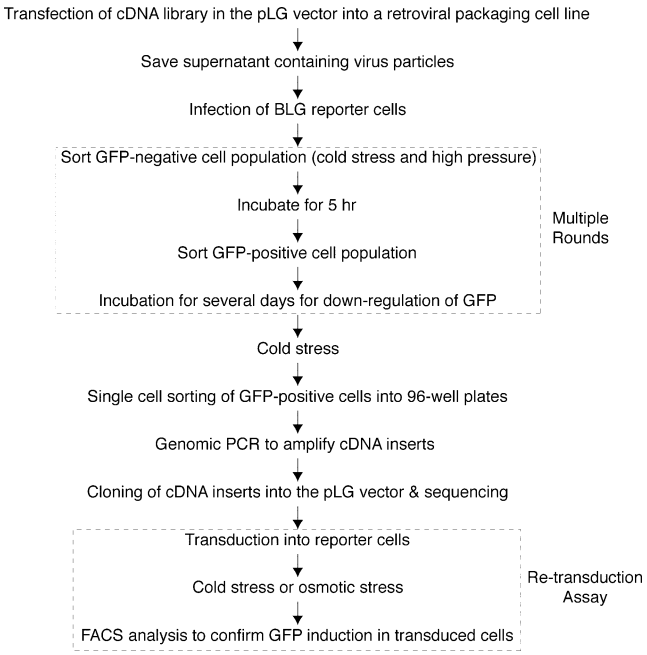

Fig 1. Scheme of ITT screening of cDNA library to identify proteins that translocate into the nucleus in response to stress.

were sorted. Sorted cells were incubated for several days for downregulation of GFP expression, then subjected to the next round of GFP (−) sorting. After 5 rounds of GFP (−) and GFP (+) sorting, cells were subjected to single-cell sorting, and GFP expression was examined in the presence and absence of cold and/or osmotic stress. Genomic DNAs extracted from clones that showed induction of GFP expression by stress were used as templates for polymerase chain reaction (PCR) amplification using viral vector primers as described previously (Hoshino et al 2004). The amplified PCR fragments were subcloned into the pLG vector and sequenced. To verify that fusion proteins of LG and proteins or protein fragments encoded by the recovered cDNA inserts confer stress-responsiveness, the LG-fusion constructs were transduced into BLG cells and exposed to cold and osmotic stress. GFP expression was analyzed by flowcytometry.

Plasmid construction and generation of cell lines

For construction of pEGFP-KBL, a vector-expressing mouse KBL fused in-frame with GFP (GFP-KBL), KBL cDNA was inserted into pEGFP-C2 (Clontech, Mountain View, CA, USA). pEGFP-KBL was electroporated into Ba/F3 cells. Subsequently, clones expressing GFP-KBL were selected by G418 selection as described previously (Miyazaki et al 1994). For construction of LGV-fusion proteins with the full-length and mutants of KBL, the full-length or mutants of KBL cDNA was inserted into the pLGV vector (Hoshino et al 2004). Retroviral gene transfer was performed as described previously (Hoshino et al 2004).

ITT assay

BLG cells expressing LG-fusion proteins or BL2 cells expressing LGV-fusion proteins were analyzed for GFP or human CD2 (hCD2) expression with FACS Calibur (BD Biosciences, San Diego, CA, USA) as described previously (Hoshino et al 2004, 2006). GFP and hCD2 expression was quantified by FlowJo software (Tree Star).

Fluorescent microscopy

NIH 3T3 was transfected with pEGFP-KBL with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells also were stained with 4′,6-diamidino-2-phenylindole (DAPI) to indicate the positions of nuclei. Cells were observed with a deconvolution microscope.

Immunoblot analysis

Preparation of nuclear/cytoplasmic extracts and immunoblot analysis were performed as described previously (Hoshino et al 2004). We used anti-LexA (Upstate Biotechnology, Charlottesville, VA, USA), anti-GFP (Clontech), and anti-Sp1 (PEP 2, Santa Cruz Biotechnology, Santa Cruz, CA, USA) Ab.

RESULTS AND DISCUSSION

Identification of KBL as a stress-induced nuclear translocating protein

By using the ITT system, we attempted to isolate cDNAs that encode proteins translocating into the nucleus by extracellular stress. Scheme of screening of cDNA library is shown in Figure 1. The Ba/F3-derived cDNA library constructed in the pLG vector (Hoshino et al 2004) were transfected into 293T cells with helper plasmid to produce retrovirus particles. Then, BLG reporter cells were infected with the supernatant of the 293T cells containing virus particles. To expose cells to cold stress and high pressure, transduced cells were subjected to sorting with the MoFlow superfast cell sorter at room temperature, and GFP (−) cells were sorted. Four hours later, GFP (+) cells that are induced to express GFP by sorting procedure were sorted. Sorted cells were incubated for several days at 37°C for downregulation of GFP expression, and then subjected to the next round of GFP (−) sorting. After 5 rounds of GFP (−) and GFP (+) sorting, we detected marked increase of GFP (+) cells in response to cold stress and osmotic stress (Fig 2A). Subsequently, cells were subjected to single-cell sorting, and GFP expression was examined in the presence and absence of cold and/or osmotic stress. Genomic DNAs extracted from clones that showed induction of GFP expression by stress were used as templates for PCR amplification using viral vector primers. The amplified PCR fragments were subcloned into the pLG vector and sequenced. In this screening, we sequenced cDNA inserts from 13 positive clones to find that they encoded 7 different genes. To verify that fusion proteins of LG and proteins or protein fragments encoded by the recovered cDNA inserts confer stress-responsiveness, the LG-fusion constructs were transduced into BLG cells and exposed to cold and osmotic stress (retransduction assay). One of the 4 cDNA species positive in the retransduction assay encoded the entire coding region of mouse KBL (Edgar and Polak 2000). Results of the retransduction assay using LG-KBL fusion protein are shown in Figure 2B.

KBL is involved in the conversion of L-threonine to glycine (Dale 1978; Boylan and Dekker 1981). L-Threonine is first converted into 2-amino-3-ketobutyrate by L-threonine dehydrogenase. KBL then catalyzes the reaction between 2-amino-3-ketobutyrate and coenzyme A to form glycine and acetyl-CoA. Expression of KBL mRNA was detected in human tissues including heart, brain, liver, and pancreas (Edgar and Polak 2000).

Stress-induced nuclear translocation of KBL

To confirm stress-induced nuclear translocation of KBL, stress-induced expression of GFP was monitored in BLG clones expressing LG-KBL (Fig 3A). Cells were mock-stimulated, exposed to cold stress (25°C) for 30 min or exposed to osmotic stress (0.5 M sorbitol). Reporter expression was monitored 4 hours after stimulation. Cold stress or osmotic stress clearly induced GFP expression (Fig 3A,B).

Next, Ba/F3 cells expressing LG-KBL were exposed to cold stress, and nuclear extracts were prepared. They were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to immunoblot analysis with anti-LexA Ab. As shown in Figure 3C, we observed rapid increase of nuclear amounts of LG-KBL in response to cold stress. To exclude a possibility that the nuclear translocation of KBL is induced by cryptic nuclear localization signal (NLS) caused by fusion with the LG, we constructed a retroviral vector expressing a fusion protein of GFP and KBL (GFP-KBL). Ba/F3 cells were transduced with this vector, and cells expressing GFP-KBL were selected by G418 selection. They were exposed to cold stress, and nuclear extracts were prepared, separated by SDS-PAGE, and subjected to immunoblot analysis with anti-GFP Ab. As shown in Figure 3D, we observed rapid increase of nuclear amounts of GFP-KBL. It should be noted that the size of the band in the nucleus detected by anti-GFP Ab was identical with that detected in the cytoplasm (data not shown), suggesting that the entire KBL protein translocates into the nucleus by stress exposure.

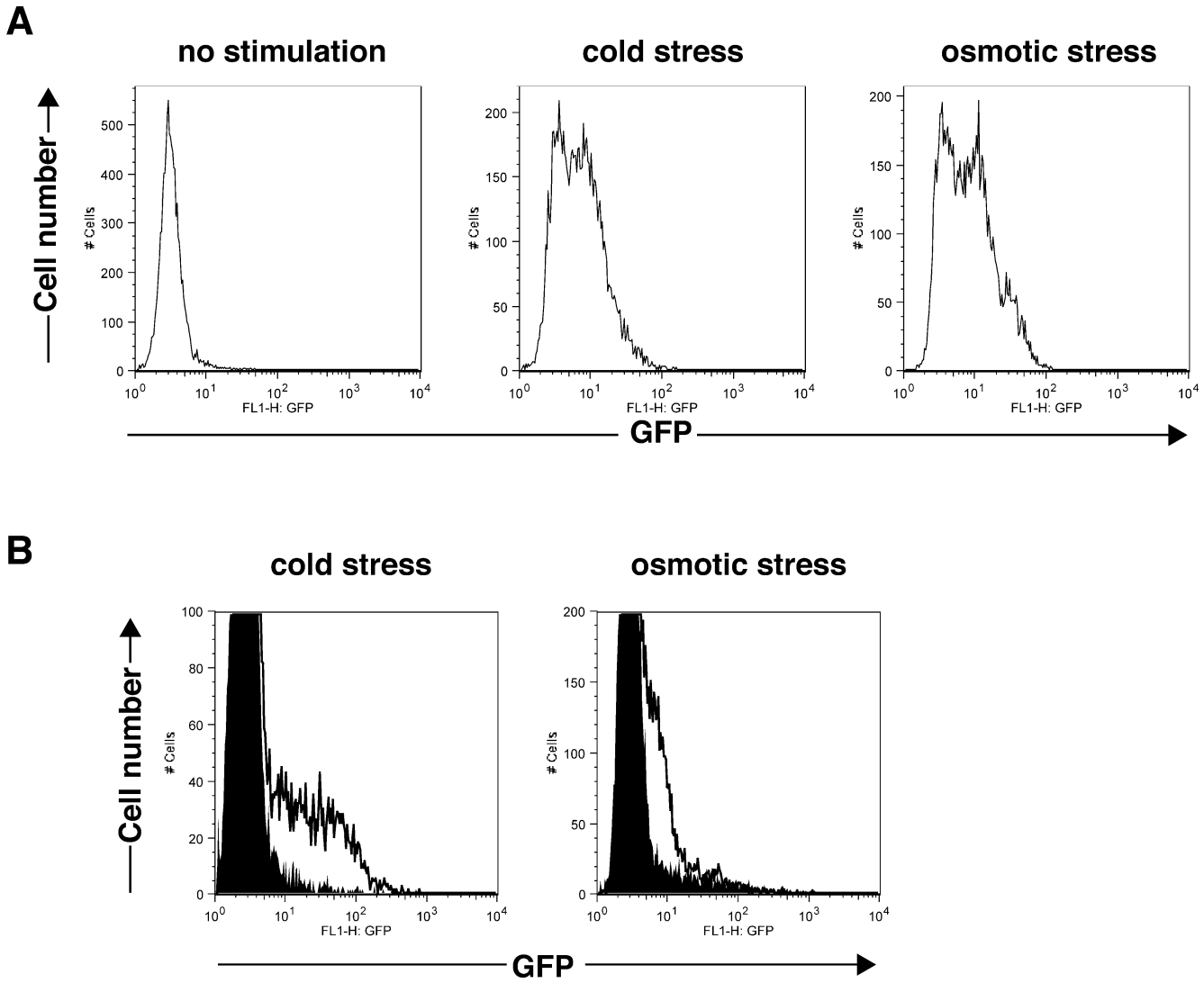

Fig 2. Identification of proteins that translocate into the nucleus in response to cold and osmotic stress. (A) cDNA library from Ba/F3 cells in the pLG vector was transfected into 293T cells with amphotropic helper plasmid to produce retrovirus particles. Then, BLG cells were infected with the supernatant of the 293T cells containing virus particles. To expose cells to cold stress and high pressure, transduced cells were subjected to sorting with the MoFlow superfast cell sorter at room temperature, and GFP (−) cells were sorted. Four hours later, GFP (+) cells in the sorted cells were sorted. Sorted cells were incubated for several days for downregulation of GFP expression, then subjected to the next round of sorting. After 5 rounds of GFP (−) and GFP (+) sorting, cells were mock-stimulated and exposed to cold stress (25°C) or osmotic stress (0.5 M sorbitol). GFP expression was analyzed by flow cytometry. (B) Re-transduction assay. cDNA inserts retrieved by genomic PCR were subcloned in pLG and transduced into BLG cells. Transduced cells were exposed to cold stress (25°C for 30 min) or osmotic stress (0.5 M sorbitol), and GFP expression was examined 4 hours later by flow cytometry. Please note that not all the cells respond to stresses to express GFP because the transduction efficiency was 20–30%. Closed: mock-stimulation; open: cold stress or osmotic stress.

Finally, NIH 3T3 cells were transfected with the expression vector of GFP-KBL, kept at 37°C, or exposed to cold stress or osmotic stress. Subcellular localization of GFP-KBL was examined by deconvolution microscopy. As shown in Figure 3E (upper panel), GFP-KBL was localized in the cytoplasm when cultured at 37°C. In contrast, accumulation of GFP-KBL was induced by cold stress (middle panel) or osmotic stress (lower panel).

These data consisting of ITT reporter assay, biochemistry, and fluorescent microscopy collectively showed rapid nuclear translocation of KBL in response to cold and osmotic stresses.

A large region of KBL is required for cold stress–induced nuclear translocation

Next, we attempted to delineate a critical region(s) of KBL for stress-induced nuclear translocation. The full-length or deleted forms of KBL were cloned in the pLGV vector and transduced into BL2 cells that have integrated LexA-

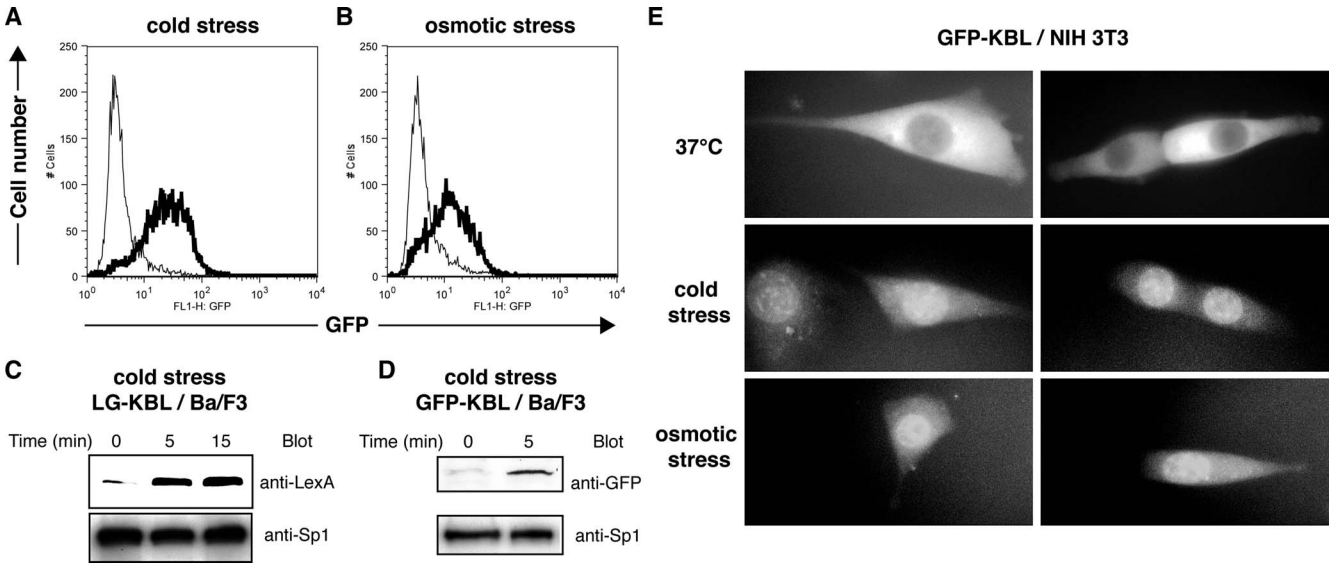

Fig 3. Stress-induced nuclear translocation of KBL. (A,B) Stress-induced GFP expression in BLG cells expressing LG-KBL. Cells were mock-stimulated (thin line) or exposed to cold stress (25°C) for 30 min (A, thick line) or osmotic stress (0.5 M sorbitol) (B, thick line). GFP expression was examined 4 hours after the initiation of stress exposure. (C,D) Cold stress–induced nuclear translocation of LG-KBL (C) or GFP-KBL (D). Ba/F3 cells expressing LG-KBL or GFP-KBL were exposed to cold stress (25°C), and nuclear extracts were subjected to immunoblot analysis with anti-LexA (C), anti-GFP (D), or anti-Sp1 Ab. (E) NIH 3T3 cells transfected with GFP-KBL were kept at 37°C (upper panel), exposed to cold stress (middle panel), or exposed to osmotic stress (lower panel) for 30 min. GFP fluorescence was observed by deconvolution microscopy.

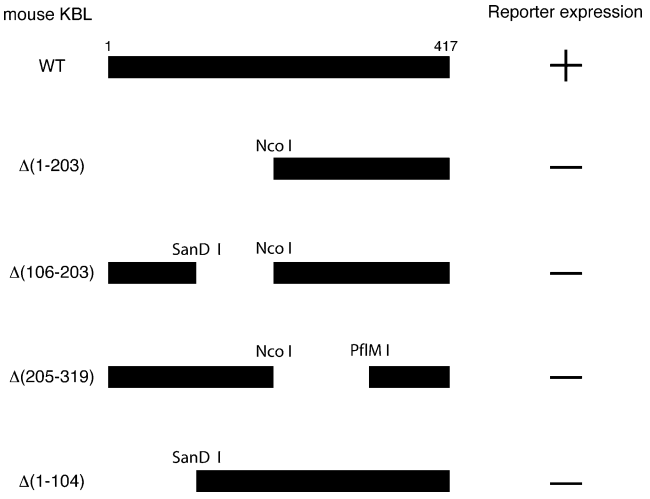

Fig 4. A large region of KBL is required for cold stress–induced nuclear translocation. The full-length or deleted forms of KBL was cloned in the pLGV vector and transduced into BL2 cells that have integrated LexA-hCD2 reporter gene (Hoshino et al 2004). Cells were exposed to cold stress for 30 min and analyzed for hCD2 reporter expression 7 hours after exposure. hCD2 expression was analyzed on GFP (+) populations.

hCD2 reporter gene (Hoshino et al 2004). Cells were exposed to cold stress and analyzed for hCD2 reporter expression 7 hours after exposure. As summarized in Figure 4, none of the deletion mutants expressed hCD2 in response to cold stress, suggesting that a large region of KBL is required for cold stress–induced nuclear translocation. Online motif analysis with PSORT II (http://psort.ims.u-tokyo.ac.jp) did not detect the classical and bipartite NLS rich in Arg and Lys (Hicks and Raikhel 1995) in KBL. This result is consistent with the notion that a large region of KBL is required for stress-induced nuclear translocation (Fig 4).

It has been shown that cellular stresses, including osmotic stress and cold stress, activate p38 serine/threonine kinases. To examine potential involvement of p38 in stress-induced nuclear translocation of KBL, we investigated the effects of SB203580, an inhibitor of p38, on nuclear translocation of KBL with the ITT reporter assay. SB203580 only partially inhibited cold and osmotic stress-induced GFP reporter expression in BLG cells expressing LG-KBL even at 10 μM (data not shown). This result suggests that p38 plays a marginal role in cold and osmotic stress–induced nuclear translocation of KBL. It is an interesting future issue how nuclear translocation of KBL is regulated.

In conclusion, we showed that osmotic and cold stresses induce nuclear translocation of KBL. A large region of KBL was required for stress-induced nuclear translocation. Physiological significance of stress-induced nuclear translocation of KBL will be an interesting future issue. In response to hypertonicity, cells accumulate large amounts of certain osmotically active organic solutes (compatible organic osmolytes; Burg et al 1997). Because KBL is involved in conversion of threonine to glycine, it is possible that osmotic stress–induced nuclear translocation of KBL might affect this metabolic pathway, lead-

ing to increase of compatible organic osmolytes by unknown mechanisms. Alternatively, KBL might function as a signaling molecule to transmit signals into the nucleus. The KBL reporter system will be a useful tool for the investigation of cold and osmotic stress responses.

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