AMP-activated Protein Kinase Phosphorylates Golgi-specific Brefeldin A Resistance Factor 1 at Thr\textsuperscript{1337} to Induce Disassembly of Golgi Apparatus\textsuperscript{*5}

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Sufficiency and depletion of nutrients regulate the cellular activities through the protein phosphorylation reaction; however, many protein substrates remain to be clarified. GBF1 (Golgi-specific brefeldin A resistance factor 1), a guanine nucleotide exchange factor for the ADP-ribosylation factor family associated with the Golgi apparatus, was isolated as a phosphoprotein from the glucose-depleted cells by using the phospho-Akt-substrate antibody, which recognizes the substrate proteins of several protein kinases. The phosphorylation of GBF1 was induced by 2-deoxyglucose (2-DG), which blocks glucose utilization and increases the intracellular AMP concentration, and by AICAR, an AMP-activated protein kinase (AMPK) activator. This phosphorylation was observed in the cells expressing the constitutively active AMPK. The 2-DG-induced phosphorylation of GBF1 was suppressed by Compound C, an AMPK inhibitor, and by the overexpression of the kinase-negative AMPK. Analysis using the deletion and point mutants identified Thr\textsuperscript{1337} as the 2-DG-induced phosphorylation site in GBF1, which is phosphorylated by AMPK \textit{in vitro}. ATP depletion is known to provoke the Golgi apparatus disassembly. Immunofluorescent microscopic analysis with the Golgi markers indicated that GBF1 associates with the fragmented Golgi apparatus in the cells treated with 2-DG and AICAR. The expression of the kinase-negative AMPK and the GBF1 mutant replacing Thr\textsuperscript{1337} by Ala prevented the 2-DG-induced Golgi disassembly. These results indicate that GBF1 is a novel AMPK substrate and that the AMPK-mediated phosphorylation of GBF1 at Thr\textsuperscript{1337} has a critical role, presumably by attenuating the function of GBF1, in the disassembly of the Golgi apparatus induced under stress conditions that lower the intracellular ATP concentration.

Recent evidence has indicated that nutrients such as glucose and amino acids play important roles in the control of the cellular activities through the protein phosphorylation reaction, as hormones and growth factors regulate the biological functions by the membrane receptor stimulation and the following protein phosphorylation cascades (1–3). Namely, the mammalian target of rapamycin (mTOR),\textsuperscript{3} a giant serine/threonine protein kinase, is activated in response to the amino acid supplementation and phosphorylates the translational regulators of eIF-4B and p70 S6 kinase (1) and PRAS40 (proline-rich Akt substrate of 40 kDa) (4). A small GTPase, Rheb, locates in the upstream of mTOR as a positive regulator, and the tuberous sclerosis complex composed of TSC1 and TSC2 negatively regulates mTOR by functioning as a GTPase-activating protein toward Rheb. On the other hand, AMP-activated protein kinase (AMPK), a heterotrimeric serine/threonine protein kinase, is regarded to be a major intracellular energy sensor and contributes to the regulation of energy homeostasis in the eukaryotic cells (5, 6). AMPK is activated by the increase in AMP under the ATP-depleted conditions through the binding of AMP to the regulatory \textgamma subunit and the phosphorylation of the catalytic \textalpha subunit at Thr\textsuperscript{172} by AMPK kinases, such as the Peutz-Jeghers syndrome gene product LKB1 and \textgamma\textsuperscript{2} /calmodulin-dependent protein kinase. In practice, the AMPK activity is elevated in the cells treated with 2-deoxyglucose (2-DG), a non-metabolizable glucose analog, that increases the intracellular AMP/ATP ratio. Interestingly, AMPK phosphorylates TSC2 at Thr\textsuperscript{1227} and Ser\textsuperscript{1345} to enhance its ability, resulting in the suppression of the mTOR pathway (7). It seems, therefore, that the signaling pathways through mTOR and AMPK are not independent, but these two nutrient-dependent signaling mechanisms are closely related each other. Moreover, these two protein kinases have been reported to take a part not only in the normal signal transduction mechanisms but also in the pathological states, including obesity, diabetes, and cancer (1, 2). It is thus important to analyze the substrate proteins of these two protein kinases in the nutrient-signaling pathways for better understanding of the regulation of the cellular activities under normal and disease conditions. Only a limited number of the

\textsuperscript{3} The abbreviations used are: mTOR, mammalian target of rapamycin; AMPK, AMP-activated protein kinase; 2-DG, 2-deoxyglucose; PAS, phospho-Akt-substrate; GEF, guanine nucleotide exchange factor; ARF, ADP-ribosylation factor; GST, glutathione S-transferase; DMEM, Dulbecco’s modified Eagle’ medium; FBS, fetal bovine serum; D-PBS, Dulbecco’s phosphate-buffered saline; FCCP, carbonyl cyanide 3-(3-chloroamidopropyl)dimethylammonio)-1-propanesulfonic acid; MS, mass spectrometry; WT, wild type.

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proteins, however, have been found as the targets of mTOR and AMPK.

The antibodies against phosphorylation sites have been employed to study the roles of the phosphoproteins (8). For example, the phosphotyrosine-specific antibodies are widely used for identification of the target proteins for the receptor-type and non-receptor-type tyrosine protein kinases as well as the tyrosine protein kinase themselves. For the analysis of the substrate proteins for the serine/threonine protein kinases, the phosphorylation motif site-specific antibodies have been employed, including the phospho-Akt-substrate (PAS) antibody (9). Akt is activated downstream of phosphatidylinositol 3-kinase following the receptor stimulation (10), and the Akt consensus recognition motif sites are phosphorylated in the proteins involved in the nutrient signaling, such as mTOR, PRAS40, TSC2, and ribosomal S6 protein (11–15). The PAS antibody was originally designed against the Akt consensus recognition motif, Arg-X-Arg-X-Ser/Thr, where Ser or Thr is phosphorylated by Akt (10). This antibody, however, reacts with shorter phosphopeptides having the Arg-X-X-Ser/Thr sequence (16), which fits with the phosphorylation motif sequence of other protein kinases (17, 18). The PAS antibody is, in fact, shown to react with the substrate proteins recognized by other protein kinases, including the ribosomal S6 protein, which is phosphorylated by p70 S6 kinase (9). We here applied a combination of the immunoprecipitation by the PAS antibody and subsequent mass fingerprinting using mass spectrometry for screening of the proteins phosphorylated by the changes of the nutritional conditions. GBF1 (Golgi-specific brefeldin A resistance factor 1), a guanine nucleotide exchange factor (GEF) for the ADP-ribosylation factor (ARF) family, was isolated as a phosphoprotein from the glucose-depleted cells, and thus further analysis was carried out to identify the protein kinase responsible for the phosphorylation of GBF1 as well as to examine the effect of the phosphorylation of GBF1 on the Golgi apparatus.

**EXPERIMENTAL PROCEDURES**

cDNAs—The human GBF1 clone obtained from Kazusa DNA Research Institute (KIAA0248; accession number D87435) was amplified by PCR, which encodes an open reading frame of 1,859 amino acids. The product was cloned into pcDNA3-FLAG (Invitrogen), and the resulting vector was designated as FLAG-GBF1. The deletion mutants of GBF1 having amino acids 1–621, 1–1,243, 295–1,859, and 1,244–1,859 were generated by PCR, and the resulting expression vectors were designated as FLAG-GBF1-(1–621), FLAG-GBF1-(1–1,243), FLAG-GBF1-(295–1,859), and FLAG-GBF1-(1,244–1,859), respectively. The point mutants of GBF1 replacing each of Ser1335, Thr1337, Ser1395, and Ser1659 by Ala were generated using the QuikChange site-directed mutagenesis kit (Stratagene), and the resulting expression vectors were designated as FLAG-GBF1 (S1335A), FLAG-GBF1 (T1337A), FLAG-GBF1 (S1395A), and FLAG-GBF1 (S1659A), respectively. The pEBG vector of the glutathione S-transferase (GST)-fused constitutively active form of AMPK α1 (GST-AMPK (K45R)) (20) were kindly provided by Dr. Lee A. Witters (Dartmouth Medical School). The DNA sequence of the constructs was verified by using ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems).

Antibodies—The following antibodies were purchased from the commercial sources: anti-FLAG and anti-α-tubulin antibodies from Sigma; anti-phospho-Akt substrate antibody (110B7), anti-phospho-p70 S6 kinase (T389) antibody (1A5), and anti-GST antibody (for immunofluorescence microscopy) from Cell Signaling Technology; anti-phospho-acetyl-CoA carboxylase (S79) antibody and anti-GST antibody (for immunoblot analysis) from Upstate Biotechnology; anti-p70 S6 kinase antibody and normal rabbit immunoglobulin from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-β-actin antibody from Abcam; anti-GM130 antibody from BD Transduction Laboratories; anti-mannosidase II antibody from Covance; horseradish peroxidase-labeled goat anti-mouse and anti-rabbit IgG antibodies from Jackson ImmunoResearch Laboratories and Bio-Rad, respectively; Alexa Fluor 488-labeled goat anti-rabbit IgG antibody and Alexa Fluor 546-labeled goat anti-mouse IgG antibody from Invitrogen. The antibodies against GBF1 (G1844) and phosphorylated GBF1 at Thr1337 (pT1337) were produced by the antibody service of Immunobiological Laboratories against the peptides LATPRPTDPIPTSEVN (amino acids 1,844–1,859) and KIHRSA-pTDAEV (amino acids 1,331–1,341, where pT indicates phospho-Thr), respectively.

Cell Culture, Transfection, and Treatment—CHO-IR, HEK293, and NRK cells were maintained in Ham’s F-12 medium (Sigma), Dulbecco’s modified Eagle’s medium (DMEM; Sigma), and α-MEM (Nacalai Tesque), respectively, containing 10% fetal bovine serum (FBS; Invitrogen) at 37 °C in a 5% CO2 incubator. For nutritional starvation, CHO-IR cells were deprived of FBS for 16 h and were further cultured in Dulbecco’s phosphate-buffered saline (D-PBS; Invitrogen) for 90 min. Then the cells were incubated for 30 min with either D-PBS, DMEM, DMEM lacking glucose, or DMEM lacking amino acids. For the treatment with the reagents, the cells cultured in the presence of FBS were replaced in DMEM lacking glucose supplemented with 5.5 mM 2-DG (Nacalai Tesque) or 10 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) (Sigma) and incubated for the indicated time. When AICAR (Sigma) was employed, the cells were incubated with the reagent at 2 mM in DMEM. Where indicated, the cells were preincubated with 20 μM Compound C (Merck) in DMEM before the treatment. The transfection with the expression vectors was carried out by the lipofection method using Lipofectamine Reagent (Invitrogen) according to the manufacturer’s protocol, and the cells were cultured for 24 h and then employed for the experiments.

Protein Purification—Immunoprecipitation was carried out at 0–4 °C essentially as described (21). Briefly, cells were lysed in buffer A (20 mM Tri-HCl (pH 7.4), 120 mM NaCl, 1 mM EDTA, 5 mM EGTA, 50 mM β-glycerophosphate, 5 mM NaF, 0.3% CHAPS, 1 mM dithiothreitol, 4 μg/ml leupeptin, 4 μg/ml aprotinin), and the lysate was incubated with each antibody and protein G-Sepharose (GE Healthcare Bio-Sciences) for 2 h. As the substrates for the AMPK assay, immunoprecipitated AMPK-mediated Phosphorylation of GBF1 and Golgi Disassembly

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FLAG-GBF1 and FLAG-GBF1 (T1337A) from transfected HEK293 cells were eluted by buffer B (60 mM HEPES (pH 7.0), 120 mM NaCl, 2.5 mM β-glycerophosphate, 1 mM dithiothreitol) containing 200 μg/ml FLAG peptide (Sigma). As the enzymes for the AMPK assay, GST-AMPK (WT) and GST-AMPK (K45R) were expressed in HEK293 cells. After incubation with or without 2-DG for 15 min, the cells were lysed in buffer C (50 mM Tri-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 5 mM sodium diphosphate, 20 mM β-glycerophosphate, 50 mM NaF, 1% Triton X-100, 1 mM dithiothreitol, 4 μg/ml leupeptin, 4 μg/ml aprotinin). After centrifugation, the supernatants were subjected to GST pull-down using glutathione-Sepharose (GE Healthcare Bio-Sciences). The pulled down proteins were washed twice with buffer B and eluted by buffer B containing 20 mM glutathione.

**Mass Fingerprint**—Mass fingerprinting analysis was carried out as described (4). The immunoprecipitates by the PAS antibody from CHO-IR cells were separated by SDS-PAGE and visualized by silver staining. The protein bands were cut out and subjected to in-gel digestion with trypsin. The resulting peptides were analyzed by the liquid chromatography electrospray ionization MS/MS using a LCQ Advantage ion trap mass spectrometer (Thermo Finnigan). Protein identification according to product ion mass lists was performed by the product ion mass fingerprinting using Mascot “MS/MS ion search.”

**Immunoblot**—The lysates and immunoprecipitates were separated by SDS-PAGE, and the proteins were transferred onto a Hybond-P membrane (GE Healthcare Bio-Sciences) and subjected to immunoblot using each primary antibody. Where indicated, the anti-GBF1 PT1337 antibody preincubated with the antigen phosphopeptide for 16 h at 4 °C was employed. After incubation with the horseradish peroxidase-labeled secondary antibody, the chemiluminescence reaction was carried out. When the same sample was analyzed with different antibodies, the membrane was stripped and employed for the subsequent immunoblot analysis. The results shown are representative of three independent experiments.

**AMPK Assay**—The procedure was carried out essentially as described (19). Purified GBF1 proteins were incubated with either GST-AMPK (WT) or GST-AMPK (K45R) obtained from the 2-DG treated and control HEK293 cells in the AMPK assay mixture (60 mM HEPES (pH 7.0), 120 mM NaCl, 1 mM dithiothreitol, 2.5 mM β-glycerophosphate, 5 mM MgCl2, 20 μM ATP, 0.2 mM AMP, 5 μCi of [γ-32P]ATP) for 20 min at 30 °C. The reaction was stopped by adding SDS-sample buffer, and the proteins were separated by SDS-PAGE, transferred onto a Hybond-P membrane, and analyzed using Bioimage Analyzer BAS2500 (Fujix). Then the membrane was immunoblotted with appropriate antibodies and visualized as described above. The result shown is representative of three independent experiments.

**Immunofluorescence and Quantitative Analysis**—The cells with or without transfection were grown on 35-mm glass bottom dishes and treated with 2-DG for 60 min. The cells were then fixed with methanol for 15 min at −20 °C, permeabilized with D-PBS containing 0.2% Triton X-100 for 15 min at 25 °C, blocked in D-PBS containing 1% bovine serum albumin for 30 min at 25 °C, double-stained with primary antibodies for 16 h at 4 °C, and incubated with both Alexa Fluor 488-labeled anti-rabbit IgG and Alexa Fluor 546-labeled anti-mouse IgG antibodies for 2 h at 25 °C. Nuclei were stained with Hoechst (Sigma). The dishes were washed three times with D-PBS after each step except for blocking, and image data were obtained with fluorescence microscope model BZ-8000 (Keyence). For the examination of microtubules, the cells were observed under fluorescence microscope model IX71 (Olympus). The quantitative analysis of the Golgi elements was performed by NIH Image software (22). Briefly, a fixed threshold was applied to all images, and the number of objects was measured using the Analyze Particle function.

**Sequence Analysis**—The amino acid sequence identity and similarity were analyzed by using the Genetyx program, version 8 (Genetyx).

**RESULTS**

Identification of GBF1 as a Nutrient-regulated Phosphoprotein.—The screening of the phosphoproteins in the CHO-IR cell lysates by immunoprecipitation using the PAS antibody isolated two distinct proteins designated as p500 and p200 from the cells cultured in the absence of FBS and then in D-PBS were incubated with DMEM (Nutrients-+), while D-PBS (Nutrients-−) for 30 min. The proteins immunoprecipitated (IP) from the cell lysates by the PAS antibody were applied to SDS-PAGE and were visualized by silver staining (top) or subjected to immunoblot with the anti-GBF1 antibody (bottom). The positions of p200 and p500 proteins and the molecular markers are indicated by arrows and in kDa, respectively. B, human GBF1 and its deletion mutants. The schematic structure of human GBF1 is shown with those of the deletion mutants employed. The numbers of amino acid residues are indicated on the left. C, analysis of p200 using specific antibodies. The cell lysates prepared as in A were subjected to immunoprecipitation with the anti-GBF1 antibody, and the immunoprecipitated proteins were applied to immunoblot with the antibodies as indicated.

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results suggest that GBF1 is phosphorylated downstream of AMPK, which is activated by an increase in the cellular AMP/ATP ratio caused by metabolic stresses, such as deprivation of glucose, that interfere with ATP production (5, 6). As expected, the compounds that lower the intracellular ATP concentration, such as 2-DG, a nonmetabolizable glucose analog that blocks cellular glucose utilization (6), and FCCP, a mitochondrial uncoupler, induced the phosphorylation of GBF1 (Fig. 2B). Furthermore, AICAR, an AMPK activator (6), caused the phosphorylation of GBF1, and the incubation of the cells with Compound C, an AMPK inhibitor (25), completely prevented the 2-DG-induced phosphorylation of the protein. Therefore, the constitutively active and kinase-negative forms of AMPK were co-expressed with FLAG-GBF1 in HEK293 cells to examine the role of AMPK for the phosphorylation of GBF1 in the cells (Fig. 2C). The phosphorylation of FLAG-GBF1 was induced by 2-DG as the endogenous GBF1 and was observed in the cells expressing the constitutively active form of AMPK (GST-AMPK(1–312)) even in the absence of 2-DG. The phosphorylation of acetyl-CoA carboxylase was, however, not evident in the lysate obtained from the unstimulated cells transfected with GST-AMPK(1–312), presumably because the transfection efficiencies of the active AMPK and FLAG-GBF1 are not high in these cells. Overexpression of the wild-type AMPK slightly enhanced the phosphorylation of FLAG-GBF1, whereas the introduction of the kinase-negative form (GST-AMPK(K45R)) attenuated the 2-DG-induced phosphorylation of the protein. GBF1 was thus concluded to be phosphorylated downstream of AMPK.

Identification of Thr1337 as an AMPK Phosphorylation Site in GBF1—Next, we identified the phosphorylation site of GBF1 in HEK293 cells to examine the role of AMPK for the phosphorylation of GBF1 (Fig. 2B). We hypothesized that the phosphorylation of GBF1 is induced by glucose depletion irrespective of the presence or absence of amino acids, which was attenuated by the addition of glucose (Fig. 2A). These findings indicate that the amino acid supplementation stimulated the phosphorylation of p70 S6 kinase, an efficient substrate of mTOR (Fig. 2A), and plectin was isolated by immunoprecipitation using the phosphorylation motif site-specific antibody from the cells cultured in the medium supplemented with amino acids (Fig. 1A, top), suggesting that plectin is phosphorylated in the downstream of mTOR. On the other hand, the glucose removal provoked the phosphorylation of acetyl-CoA carboxylase, a typical substrate protein of AMPK, and the phosphorylation of GBF1 was induced by glucose depletion irrespective of the presence or absence of amino acids, which was attenuated by the addition of glucose (Fig. 2A). These findings suggest that GBF1 is phosphorylated downstream of AMPK, which is activated by an increase in the cellular AMP/ATP ratio caused by metabolic stresses, such as deprivation of glucose, that interfere with ATP production (5, 6). As expected, the compounds that lower the intracellular ATP concentration, such as 2-DG, a nonmetabolizable glucose analog that blocks cellular glucose utilization (6), and FCCP, a mitochondrial uncoupler, induced the phosphorylation of GBF1 (Fig. 2B). Furthermore, AICAR, an AMPK activator (6), caused the phosphorylation of GBF1, and the incubation of the cells with Compound C, an AMPK inhibitor (25), completely prevented the 2-DG-induced phosphorylation of the protein. Therefore, the constitutively active and kinase-negative forms of AMPK were co-expressed with FLAG-GBF1 in HEK293 cells to examine the role of AMPK for the phosphorylation of GBF1 in the cells (Fig. 2C). The phosphorylation of FLAG-GBF1 was induced by 2-DG as the endogenous GBF1 and was observed in the cells expressing the constitutively active form of AMPK (GST-AMPK(1–312)) even in the absence of 2-DG. The phosphorylation of acetyl-CoA carboxylase was, however, not evident in the lysate obtained from the unstimulated cells transfected with GST-AMPK(1–312), presumably because the transfection efficiencies of the active AMPK and FLAG-GBF1 are not high in these cells. Overexpression of the wild-type AMPK slightly enhanced the phosphorylation of FLAG-GBF1, whereas the introduction of the kinase-negative form (GST-AMPK(K45R)) attenuated the 2-DG-induced phosphorylation of the protein. GBF1 was thus concluded to be phosphorylated downstream of AMPK.
ing of amino acids 1,244–1,859 was recognized by the PAS antibody. The consensus recognition motif for AMPK, $\Phi^{-}[\beta, X]-X-X-Ser/Thr-X-X-$, where $\Phi$ and $\beta$ are hydrophobic and basic, respectively, and the residues in brackets are in any order (6), is compatible with the Arg-X-X-Ser/Thr sequence, which is recognized by the PAS antibody, as mentioned above. Therefore, it is reasonable to assume that the site detected by the PAS antibody in GBF1 is phosphorylated by AMPK. The search for the consensus recognition motif for AMPK in amino acids 1,244–1,859 identified four putative recognition sites for AMPK, Ser1335, Thr1337, Ser1395, and Ser1659, that are conserved among human and rodent GBF1. Each of these Ser and Thr residues in the full-length GBF1 was then replaced by Ala, and these point mutants recovered from the transfected cells were subjected to immunoblot analysis using the PAS antibody (Fig. 3B). The 2-DG-induced phosphorylation was found in the S1335A, S1395A, and S1659A mutants but was not detected in the T1337A mutant. The antibody, pT1337, raised against a phosphopeptide corresponding to amino acids 1,331–1,341 of human GBF1, recognized GBF1 immunoprecipitated from CHO-IR and HEK293 cells treated with 2-DG, as in the case of the PAS antibody (Fig. 3D). The immunoreaction by pT1337 was vanished by adsorbing the antibody with the antigen phosphopeptide. These results indicate that Thr$^{1337}$ is phosphorylated in the 2-DG-treated cells. The data base search showed that human Thr$^{1337}$ in the consensus recognition motif for AMPK is conserved among the GBF1s of vertebrates, including Cricetulus griseus, Mus musculus, and Danio rerio, but is not present in Drosophila melanogaster (Fig. 3C). The phosphorylation at this site, thus, could be a common regulation mechanism for GBF1 among the different species. We then examined whether GBF1 was phosphorylated directly by AMPK at Thr$^{1337}$ in vitro (Fig. 3E). FLAG-GBF1 (WT) and FLAG-GBF1 (T1337A) affinity-purified from the transfected HEK293 cells were incubated with either GST-AMPK (WT) or GST-AMPK (K45R) obtained from the control and 2-DG-treated HEK293 cells, respectively, in the kinase assay buffer. The top panel shows autodigestion, and the lower two panels show immunoblot with the antibodies, as indicated.
AMPK-mediated Phosphorylation of GBF1 and Golgi Disassembly

No obvious phosphorylation of the wild-type protein was detected after incubation with the kinase-negative AMPK. FLAG-GBF1 (T1337A) was phosphorylated; however, it was phosphorylated far less significantly than FLAG-GBF1 (WT) by the incubation with the wild-type AMPK obtained from the incubation with the wild-type AMPK (WT) in the 2-DG-treated cells. These results indicate that GBF1 is phosphorylated directly by AMPK at Thr1337, although GBF1 still has other minor phosphorylation site(s) recognized by AMPK at least in vitro. Taken together, it was concluded that GBF1 is phosphorylated at Thr1337 by AMPK in the cells under the stress conditions that decrease the intracellular ATP concentration and concomitantly increase that of AMP.

Phosphorylation of GBF1 at Thr1337 and Disassembly of the Golgi Apparatus—To study the role of the AMPK-mediated phosphorylation of GBF1, the intracellular localization of GBF1 was first observed, comparing it with that of the Golgi marker proteins by immunofluorescent microscopy in CHO-IR cells (Fig. 4A). We examined the effect of 2-DG at 5.5 mM on the Golgi apparatus, because 2-DG at this concentration efficiently induced the AMPK activation, as previously reported (7, 19) and shown in Fig. 2B. In the control cells, GM130, a cis-Golgi marker protein (26), localized primarily in the juxtanuclear region and appeared in the small punctuate structures surrounding the cell nucleus after the 2-DG treatment. The treatment increased significantly the number of the punctuate structures per cell, but the total fluorescence of GM130 did not change evidently, as indicated by quantitative image analysis. Similar results were obtained by staining another cis-Golgi marker protein, βCOP (data not shown). Mannosidase II, a medial Golgi marker as an integral Golgi membrane protein (27), was also localized in the small elements as GM130 in the 2-DG-treated NRK cells (Supplemental Fig. S1). AICAR, the AMPK activator, showed an effect similar to that of 2-DG on the Golgi apparatus (Fig. 4B). These results indicate that the small punctuate structures observed in the cells treated with 2-DG and AICAR are the fragmented Golgi rather than a nonspecific scattering of the Golgi proteins. It has been reported that the disruption of the microtubules causes structural change of the Golgi apparatus and its redistribution to the endoplasmic reticulum export sites (28). The immunostaining of CHO-IR cells with the anti-α-tubulin antibody, however, showed that the 2-DG treatment does not change the organization of microtubules in the cells (Supplemental Fig. S2), indicating that the 2-DG-induced Golgi disassembly is independent of the change of the microtubule structure. It is worth noting that the endogenous GBF1 co-localized with the Golgi marker proteins, such as GM130, βCOP, and mannosidase II, both in the control cells and in the cells treated with 2-DG and AICAR, which induce the AMPK activation.

The role of AMPK in the Golgi disassembly was next examined by introducing the wild-type and kinase-negative AMPK in CHO-IR cells (Fig. 5). The GST fusion proteins of AMPK were observed mostly in the cytosol. The expression of the wild-type AMPK (GST-AMPK (WT)) did not affect the localization of the Golgi apparatus in the unstimulated cells or its disassembly in the 2-DG-stimulated cells, as observed by the staining of GM130. The introduction of the kinase-negative AMPK (GST-AMPK (K45R)) did not affect the juxtanuclear localization of the Golgi apparatus in the unstimulated cells or its disassembly in the 2-DG-stimulated cells, as observed by the staining of GM130. The introduction of the kinase-negative AMPK (GST-AMPK (K45R)) did not affect the juxtanuclear localization of the Golgi apparatus in the unstimulated cells or its disassembly in the 2-DG-stimulated cells, as observed by the staining of GM130. The introduction of the kinase-negative AMPK (GST-AMPK (K45R)) did not affect the juxtanuclear localization of the Golgi apparatus in the unstimulated cells or its disassembly in the 2-DG-stimulated cells, as observed by the staining of GM130. The introduction of the kinase-negative AMPK (GST-AMPK (K45R)) did not affect the juxtanuclear localization of the Golgi apparatus in the unstimulated cells or its disassembly in the 2-DG-stimulated cells, as observed by the staining of GM130.
The effect of the phosphorylation of GBF1 on the Golgi disassembly was then studied by introducing the FLAG epitope-tagged GBF1 and its T1337A mutant in CHO-IR cells (Fig. 6). FLAG-GBF1 (WT) was not only stained in the cytosol but also merged with the endogenous GM130 in the typical structure of the Golgi apparatus, and the 2-DG treatment induced disassembly of the Golgi apparatus as in the untransfected cells and disrupted the juxtanuclear localization of GM130 with FLAG-GBF1 (WT). Quantitative image analysis confirmed the increase in the number of the perinuclear punctuate structures after the 2-DG treatment in the cells expressing FLAG-GBF1 (WT). FLAG-GBF1 (T1337A) was also detected in the cytosol and the Golgi apparatus as the wild-type protein in the unstimulated cells, but the structural change of the Golgi apparatus was not evident after the 2-DG treatment in the cells overexpressing FLAG-GBF1 (T1337A). This mutant protein was found with GM130 in the juxtanuclear region as observed under a microscope, consistent with the results of quantitative image analysis. Namely, the expression of this phosphorylation site mutant prevented the 2-DG-induced disassembly of the Golgi apparatus. It is less possible that an excess amount of the T1337A mutant protein expressed in the cells might have an effect on the Golgi structure and susceptibility to stress, because the expression of the wild-type GBF1 did not induce a significant change, and the introduction of the kinase-negative AMPK also prevented the 2-DG-induced disassembly of the Golgi apparatus. These results indicate that the phosphorylation of GBF1 at Thr\textsuperscript{1337} mediated by AMPK has a critical role in the disassembly of the Golgi apparatus induced by stress conditions that lower the intracellular ATP concentration.

**DISCUSSION**

The PAS antibody has been employed to identify the Akt sub-
strates, which was originally designed using phosphopeptide sequences according to the Akt consensus recognition motif, Arg-X-Arg-X-Ser/Thr (9). This antibody, however, practically reacts with shorter phosphopeptides having the Arg-X-X-Ser/Thr sequence, which corresponds with the phosphorylation motif sequences of several other protein kinases (17, 18). In this study, we employed the PAS antibody for the screening of the phosphoproteins produced by the nutrient signals, and GBF1 was isolated, which was then revealed to be a substrate of AMPK. It is reasonable that GBF1 was identified by the PAS antibody as the AMPK substrate, because the Arg-X-X-Ser/Thr sequence is contained in the consensus recognition motif for AMPK, $\phi-[\beta,\chi]-X-X-Ser/Thr-X-X-X-\phi$ (6). The PAS antibody does not cover all of the AMPK substrates, because the consensus recognition motif for AMPK contains not only Arg-X-X-Ser/Thr but also Arg-X-X-X-Ser/Thr; however, this antibody will be useful for the detection of the substrates of AMPK, which regulate the metabolism and cell proliferation through the phosphorylation reaction (5, 6).

The Golgi apparatus is a complex and dynamic organelle that functions in protein sorting and modification, and numerous structural and regulatory proteins are involved in the budding, docking, and fusion of the Golgi-directed vesicles (29). GBF1 is a high molecular weight GEF associated with the Golgi apparatus and has a Sec7 domain and a Pro-rich region in the middle portion and the carboxyl-terminal end, respectively (24), as shown in Fig. 1B. The SEC7 domain is sufficient to catalyze the exchange of GDP for GTP on ARFs, and GBF1 is known to act on class I and class II ARFs in the cis-Golgi elements (30). The Pro-rich region is essential for the interaction with the p115 protein, which binds with a variety of proteins involved in the membrane traffic from the endoplasmic reticulum to and through the Golgi apparatus (31). In addition, GBF1 is one of the molecular targets for brefeldin A, a fungal heterocyclic lactone, which causes reversible inhibition of vesicle trafficking by trapping sensitive ARF-GEFs on the membrane to block the GEF activity (32). Importantly, the membrane transport between intracellular compartments is an energy-dependent process (33), and it has been reported that the treatment of the cells with 2-DG, which induces ATP depletion, inhibits the membrane traffic and induces vesiculation and disassembly of the Golgi apparatus (34–36). The molecular mechanism for the 2-DG-induced disassembly of the Golgi apparatus, however, has not been clarified yet. In this study, it was revealed that GBF1 is phosphorylated by AMPK at Thr$^{1337}$ in the cells treated with 2-DG, where the AMP/ATP ratio is increased to activate the protein kinase, and that the mutant replacing this Thr residue by Ala blocks the 2-DG-induced disassembly of the Golgi apparatus. It has been shown that the GEF activity of GBF1 is important for the maintenance of the Golgi structure, because the expression of the GBF1 mutant causes fragmentation of the Golgi apparatus, in which Glu$^{79}$, the essential residue for the GEF activity in the Sec7 domain, is replaced by Lys (37). It is, thus, attractive to assume that AMPK inhibits the membrane traffic processes, resulting in the disassembly of the Golgi apparatus, through the phosphorylation of GBF1 at Thr$^{1337}$ to suppress its GEF activity. Concerning the effect of the phosphorylation on GBF1, the 2-DG treatment of the cells decreased in the amount of activated ARFs, as judged by the pull-down assay by using a GST-GGA-GAT domain construct (38), suggesting that the GEF activity of GBF1 is attenuated by the phosphorylation reaction (data not shown). The overexpression of the T1337A mutant, however, did not prevent significantly the 2-DG-induced inactivation of ARF as monitored by this assay method (data not shown). On the other hand, the punctate structure in the cells treated with 2-DG and AICAR, which was recognized by the antibodies against the Golgi markers, such as GM130 and mannosidase II, was stained with the anti-GBF1 and anti-βCOP antibodies. The GEF activity of GBF1 seems to remain at least in part in the punctuate structure, because βCOP, a component of the COP1 as the effector of ARFs, is recruited in the structure. Thus, it is plausible that the function of GBF1 is attenuated in 2-DG-treated cells by the multiple mechanisms dependent on and independent of the inhibition of its GEF activity. The regulation mechanism for the GBF1 still needs to be clarified.

The Golgi apparatus repeats disassembly and reassembly according to cell cycle (39, 40). The fragmentation begins in prophase, and the Golgi apparatus disperses into cytosol before mitosis and is rebuilt in daughter cells during cytokinesis. The Golgi-related proteins, such as GRASP55 and GM130, are phosphorylated in M phase. GRASP55, a Golgi-localized protein that participates in the docking of transport vesicles, is phosphorylated in the MEK/ERK pathway to unstack Golgi cisternae; GM130 is phosphorylated by Cdc2/cyclin B to be released from the p115 protein, resulting in the inhibition of the transport and fusion of the transport vesicles. It has been reported that AMPK is involved in the regulation for the cell cycle progression in relation to the ATP consumption during mitosis (41, 42). It is thus interesting to assume that AMPK phosphorylates GBF1 not only under the nutrient depletion to reduce ATP consumption but also in mitosis as a part for the regulation of the Golgi apparatus during the cell cycle progression.

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