Elongation Factor-2 Kinase Regulates Autophagy in Human Glioblastoma Cells

Hao Wu, Jin-Ming Yang, Shengkan Jin, Haiyan Zhang, and William N. Hait

Abstract
Elongation factor-2 kinase (eEF-2 kinase), also known as Ca$^{2+}$/calmodulin–dependent kinase III, regulates protein synthesis by controlling the rate of peptide chain elongation. The activity of eEF-2 kinase is increased in glioblastoma and other malignancies, yet its role in neoplasia is uncertain. Recent evidence suggests that autophagy plays an important role in oncogenesis and that this can be regulated by mammalian target of rapamycin (mTOR). Because eEF-2 kinase lies downstream of mTOR, we studied the role of eEF-2 kinase in autophagy using human glioblastoma cell lines. Knockdown of eEF-2 kinase by RNA interference inhibited autophagy in glioblastoma cell lines, as measured by light chain 3 (LC3)-II formation, acidic vesicular organelle staining, and electron microscopy. In contrast, overexpression of eEF-2 kinase increased autophagy. Furthermore, inhibition of autophagy markedly decreased the viability of glioblastoma cells grown under conditions of nutrient depletion. Nutrient deprivation increased eEF-2 kinase activity and decreased the activity of S6 kinase, suggesting an involvement of mTOR pathway in the eEF-2 kinase regulation of autophagy. These results suggest that eEF-2 kinase plays a regulatory role in the autophagic process in tumor cells; and eEF-2 kinase is a downstream member of the mTOR signaling: eEF-2 kinase may promote cancer cell survival under conditions of nutrient deprivation through regulating autophagy. Therefore, eEF-2 kinase may be a part of a survival mechanism in glioblastoma and targeting this kinase may represent a novel approach to cancer treatment. (Cancer Res 2006; 66(6): 3015-23)

Introduction
Elongation factor-2 kinase (eEF-2 kinase), also known as Ca$^{2+}$/calmodulin–dependent kinase III, was first identified by Nairn et al. (1) and shown to regulate many cellular processes through its essential role in protein translation (2). eEF-2 kinase controls the movement of the elongating peptide chain by specifically phosphorylating eEF-2 at Thr56, which decreases the affinity of the elongation factor and cognate peptide for the ribosome (3, 4). eEF-2 kinase was identified in glioblastoma cell lines as an increased Ca$^{2+}$/calmodulin–dependent enzyme activity (5, 6) and was later shown to be up-regulated in many human cancers (7, 8). Inhibition of eEF-2 kinase by selective (9) and nonselective (10) inhibitors produced cancer cell death in vitro and in vivo. Yet the precise role of this kinase in malignancy is not fully understood.

The activity of eEF-2 kinase is regulated at several levels. These include activation by Ca$^{2+}$/calmodulin (2, 4, 5) and by mitogenic growth factors (11, 12). The kinase is inactivated by phosphorylation of Ser366 by S6 kinase and by autophosphorylation (13–15). S6 kinase is part of the signaling pathway by which the mammalian target of rapamycin (mTOR) senses nutrient deprivation and regulates autophagy (16). Work by Browne et al. (17) showed that eEF2 kinase could also be directly phosphorylated by insulin-dependent mTOR activity at Ser78, a site adjacent to the calmodulin binding domain. Thus, eEF-2 kinase seems to be regulated by mTOR, providing links to several cellular functions including cell transformation and autophagic degradation.

Autophagy is a highly conserved cellular process for large-scale degradation of proteins and organelles (18). It is activated in yeast in response to nutrient deprivation and is believed to protect cell viability by recycling digested proteins for emergency energy utilization (19). In mammals, autophagy may be essential for surviving under conditions of transient nutrient deprivation (20, 21). Furthermore, withdrawal of growth factors from hematopoietic cells induces autophagy, and this seems to protect cell viability (22). In contrast, under circumstances of severe nutrient depletion, this pathway can result in autophagic cell death. Recent studies point to the importance of autophagy in cancer. For example, genetic manipulation of this pathway can increase the appearance of cancer in mice (23); autophagy is regulated by the AKT/phosphatidylinositol 3-kinase and mTOR pathways (24); and treatment with certain cytotoxic drugs (25) or withdrawal of obligate growth factors may induce autophagic cell death (26).

Several lines of evidence suggest that eEF-2 kinase may be involved in autophagy. First, eEF-2 kinase regulates protein synthesis, a major consumer of cellular energy. Second, eEF-2 kinase lies downstream of mTOR, a known negative regulator of autophagy. Third, growth factor deprivation can markedly reduce the activity of eEF2 kinase. Yet, a role for eEF-2 kinase in autophagy has not been identified. Therefore, in the current study, we investigated the effects of eEF-2 kinase on autophagy in human glioblastoma cell lines. We found that this inhibitor of protein translation can induce autophagy and protect cancer cell viability under conditions of nutrient depletion.

Materials and Methods
Reagents and antibodies. pSUPER.retro.neo+green fluorescent protein (GFP) vector was purchased from OligoEngine, Inc. (Seattle, WA). Restriction enzymes, T4 DNA ligase, Oligofectamine, Lipofectamine 2000, and G418 were purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA). Acidine orange, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and anti-α-actin and anti-β-tubulin antibodies were purchased from Sigma Chemical Co. (St. Louis, MO). Anti-eEF-2 kinase, anti-phospho-eEF-2 kinase, anti-eEF-2, and anti-phospho-eEF-2 antibodies...
were purchased from Cell Signaling Technologies (Beverly, MA). Anti-microtubule-associated protein 1 light chain 3 (MAP-LC3) antibody was a gift from Dr. Noboru Mizushima (Department of Bioregulation and Metabolism, The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) or purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All media and cell culture products were purchased from Life Technologies, Inc. (Grand Island, NY). Chemiluminescence Western blot reagents were purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

**Cell lines and culture.** The human glioblastoma cell lines T98G, U138MG, and LN-229 were purchased from American Type Culture Collection (Manassas, VA). TJY-V and TJY2-D cell lines were derived by transfecting T98G cells with empty vector or sense DNA for eEF-2 kinase, respectively (27). These cell lines were maintained in Ham’s F-10/DMEM (10:1) medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO2/95% air. Mouse embryonic fibroblasts from eEF-2 kinase knockout embryos were provided by Dr. Ryazanov (University of Medicine and Dentistry of New Jersey/Robert Wood Johnson Medical School, New Brunswick, NJ).

**Small interfering RNA preparation and transfection.** The small interfering RNA (siRNA) sequence targeting eEF-2 kinase mRNA corresponded to the coding region 144-164 (5’-AAGCTCGAACGAGAATGTC-3’) relative to the start codon. siRNA duplexes were synthesized by Dharmacon Research, Inc. (Lafayette, CO). Cells in exponential phase of growth were plated in six-well plates at 5 x 10^5 cells/well and incubated at 37°C in a humidified atmosphere containing 5% CO2/95% air. Mouse embryonic fibroblasts from eEF-2 kinase knockout embryos were provided by Dr. Ryazanov (University of Medicine and Dentistry of New Jersey/Robert Wood Johnson Medical School, New Brunswick, NJ).

**Plasmid construction and transfection.** pSUPER.retro.neo+GFP vector was linearized with HindIII and BglII, and the annealed 64-nucleotide oligos (synthesized by Qiagen Operon, Alameda, CA), corresponding to the same coding region as the synthesized eEF-2 kinase siRNA, was ligated into the A vector. The concentrations of siRNAs were chosen based on dose-response studies. Silencing effect was examined 72 hours after transfection.

**Western blot analysis.** Cells were washed twice with PBS containing 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), scraped off the dishes, and pelleted at 500 g for 10 minutes. Cell pellets were then lysed in cold lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na3VO4, 1 μg/mL leupeptin, and 1 mmol/L PMSF] and sonicated for 5 seconds. The lysates were clarified by centrifugation at 12,000 x g for 30 minutes at 4°C. Identical amounts (50 μg of protein) of cell lysates were resolved by 8% or 15% SDS-PAGE. Transfer of proteins to nitrocellulose was carried out by the method of Towbin et al. (28). Membranes were incubated in blocking solution consisting of 5% powered milk in TBST [10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, and 0.1% Tween 20] for 1 hour, then immunoblotted with anti-eEF-2 kinase, anti-phospho-eEF-2 kinase, anti-eEF-2, anti-phospho-eEF-2, anti-LC3, anti-β-actin, or anti-α-tubulin antibodies. Detection by enzyme-linked chemiluminescence was carried out according to the protocol of the manufacturer. Bands were quantified using Scion Image software (Scion Co., Frederick, MD).

**Measurement of acidic vesicular organelles.** Cells were incubated with 0.5 mg/mL of acridine orange for 15 minutes. Acidic vesicular organelle (AVO) staining was examined using a fluorescence microscope (Nikon ECLIPSE TE200 microscope, Nikon, Inc., Melville, NY). To quantify AVOs, the stained cells were detached by trypsinization and the intensity of red fluorescence from 10^5 cells was measured by FACSCalibur using CellQuest software (Becton Dickinson, Bedford, MA).

**Cellular viability.** For MTT assay, cells were plated at 5 x 10^3 per well in 96-well tissue culture plates and incubated at 37°C in a humidified atmosphere. The concentrations of siRNAs were chosen based on dose-response studies. Silencing effect was examined 72 hours after transfection.

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**Electron microscopy.** Cells were harvested by trypsinization, fixed in 2.5% glutaraldehyde/4% paraformaldehyde in 0.1 mol/L cacodylate buffer, then post-fixed in 1% osmium tetroxide buffer. After dehydration in a graded series of acetone, the cells were embedded in epoxy resin. Thin sections (90 nm) were cut on a Reichert Ultracut E microtome. Sectioned grids were stained with saturated solution of uranyl acetate and lead citrate. Sections were examined at 80 kV with a JEOL 1200EX transmission electron microscope.

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atmosphere containing 5% CO₂/95% air. Cell viability was measured by MTT assay after 1 to 7 days of incubation. The formazan product, formed after a 4-hour incubation with MTT, was dissolved in DMSO and read at 570 nm using a VERSAmax microplate reader (Molecular Devices Corp., Sunnyvale, CA).

For trypan blue exclusion assay, cells were plated at 2.5 × 10⁴ per well in 24-well tissue culture plates. Viable cells were counted using a Vi-CELL Cell Viability Analyzer (Beckman Coulter, Inc., Miami, FL).

Results

Generation and characterization of siRNA and shRNA transfectants. We used RNA interference techniques to test the effect of eEF-2 kinase on autophagy. Figure 1A shows that transfection of T98G and U138MG cells with an eEF-2 kinase–targeted siRNA resulted in a significant decrease in expression of the kinase. Seventy-two hours after transfection, the amount of eEF-2 kinase in T98G and U138MG cells decreased by 90% as compared with that of scrambled siRNA–treated controls. Knock-down of eEF-2 kinase was accompanied by a concurrent decrease in enzyme activity as measured by phosphorylation of eEF-2 (Fig. 1A).

We also constructed a vector that expressed a shRNA against eEF-2 kinase and isolated stable transfected clones. As shown in Fig. 1B, T98GeEF2K(-) is a stable clone in which eEF-2 kinase expression was decreased by 90% as compared with that of an empty vector–transfected cell line (T98G). Silencing of eEF-2 kinase expression decreases autophagy. Microtubule-associated protein 1 light chain 3 (LC3) is a human
homologue of the yeast ATG8, an essential autophagy protein. It is processed from a full-length protein (MAP-LC3-I) to a cleaved and lipidated form (LC3-II) during autophagy. The abundance of MAP-LC3-II reflects the level of autophagy and LC3-II has been used as a specific marker for autophagy (29). To determine whether eEF-2 kinase plays a role in autophagic cellular degradation, we compared the formation of LC3-II in glioblastoma cells with or without silencing of eEF-2 kinase expression. As shown in Fig. 2, silencing of eEF-2 kinase decreased the appearance of LC3-II in T98G and U138MG cells grown to high cell density to stimulate autophagy (90% confluence) in comparison with cells treated with scrambled siRNA (Fig. 2A). Similarly, the production of LC3-II was markedly reduced in T98G eEF2K(-/-) cells compared with that of empty vector controls (Fig. 2B). The effect of eEF-2 kinase on autophagy was also observed in the human glioma cell line LN-229, human breast cancer cell line MCF-7, and mouse embryonic fibroblasts from eEF-2 kinase knockout embryos (Fig. 2C and D).

Another measure of autophagic activity is the appearance of AVOs (30). Acridine orange is a lysosomotropic agent, the protonated form of which accumulates in acidic compartments and forms bright red fluorescent aggregates. As shown in Fig. 3B and C, silencing of eEF-2 kinase decreased acridine orange staining of AVOs in T98G eEF2K(-/-) cells as compared with empty vector controls. Quantification of these results by flow cytometry revealed that the mean red fluorescence in T98G eEF2K(-/-) cells (3.7 ± 0.6) was significantly less than that seen in the control cells (27 ± 2.1; P < 0.01). A similar effect was observed in U138MG cells and the eEF-2 kinase knockout mouse embryonic fibroblasts (Fig. 3G and H).

Autophagy is characterized by the formation of double-membrane vacuoles that sequester organelles. Electron microscopy visualized abundant double or multi-membrane vacuoles in the cytoplasm of control T98G cells (Fig. 4A). In contrast, these vacuoles were rarely seen in T98G eEF2K(-/-) cells (Fig. 4B).

To verify the specificity of the siRNA against eEF-2 kinase and the role of the kinase in autophagy, we tested the effects of eEF-2 kinase overexpression on acridine orange staining of AVOs in T98G cells transfected either with eEF-2 kinase sense cDNA (TJY2-D) or control vector (TJY-V). These cell lines were previously developed and characterized by our laboratory (27). As shown in Fig. 3D, TJY2-D cells had a 2.5-fold increase in eEF-2 kinase expression compared with TJY-V cells and a significant increase in AVO formation compared with that of TJY-V cells (Fig. 3E and F; P < 0.01).

Silencing of eEF-2 kinase expression blunts the autophagocytic response to nutrient deprivation. Because nutrient deprivation induces autophagy in several well-described systems (19, 31), we investigated the effects of eEF2 kinase depletion on the induction of autophagy following nutrient removal. Because cell

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**Figure 3.** Formation of AVOs in glioblastoma cells with silencing or overexpression of eEF-2 kinase (A-G) and in mouse embryonic fibroblasts from eEF-2 kinase knockout embryos (H). A and D, cell lysates were prepared from the transfected cells and equal amounts (50 μg proteins) of the lysates were resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membrane and eEF-2 kinase was detected by immunoblotting as described in Fig. 1. B and E, cells were incubated with 0.5 mg/mL of acridine orange for 15 minutes and AVO staining was examined under a fluorescence microscope (Nikon ECLIPSE TE200 microscope, Nikon). C to H, quantification of red fluorescence intensity in acridine orange-stained cells was done by flow cytometry using CellQuest software. Representative of three similar experiments.
Regulation of Autophagy by eEF-2 Kinase

confluence affects the amount of LC3-II formation (Fig. 5A), T98G cells were grown at low cell density (20-30% confluence) in fully supplemented medium for 24 hours, at which time the medium was replaced with PBS, and then incubated for 30 to 60 minutes. Figure 5B shows that nutrient deprivation increased autophagy in parental and empty vector controls as measured by the increase of LC3-II. In contrast, the appearance of LC3-II was markedly reduced in the T98GeEF2K(-) cells. LC3-II levels were 6- and 3-fold lower in T98GeEF2K(-) than in T98GV cells at 30 and 60 minutes after nutrient deprivation, respectively (Fig. 5B). In similar experiments done in LN-229 glioblastoma cells, we found that siRNA to eEF-2 kinase completely blocked the autophagic response to nutrient deprivation (Fig. 5C).

If eEF-2 kinase was causally involved in the response to nutrient depletion, we reasoned that these conditions would increase the expression or activity of the kinase. Therefore, we measured the expression and activity of eEF-2 kinase following nutrient deprivation. Figure 5D shows that nutrient depletion increased the expression of the kinase in parental and vector control T98G cell lines and that this was associated with an increased phosphorylation of eEF-2. In contrast, eEF-2 kinase expression did not increase in the T98GeEF2K(-) cells (Fig. 5D). The phosphorylation of eEF-2 in T98GeEF2K(-) was lower than that in T98GV cells and also increased but to a lesser extent than controls following nutrient deprivation.

The activity of eEF-2 kinase can be regulated by phosphorylation on Ser366 by S6 kinase (13). In response to nutrient deprivation, the activity of S6 kinase is decreased by inactivation of mTOR (32). Therefore, we asked whether the increased eEF-2 kinase activity seen with nutrient deprivation was due to inactivation of S6 kinase. As shown in Fig. 5E, nutrient removal led to the rapid loss of phosphorylated S6 kinase from all the three cell lines without a change in the expression of S6 kinase. A similar observation was obtained in the LN-229 cell line (Fig. 5F).

eEF-2 kinase protects cell viability under conditions of nutrient deprivation. Autophagy can protect cells from nutrient and growth factor deprivation (18, 22). Therefore, we determined the effects of eEF-2 kinase on cancer cell viability under conditions shown to activate autophagy. T98GeEF2K(-) and T98GV cells were incubated in medium with or without serum and cell viability measured by the MTT (Fig. 6A and B) and trypan blue exclusion (Fig. 6C and D) assays. Figure 6A and C shows that the viability of T98GeEF2K(-) cells in serum-free medium was significantly lower than that observed in T98GV cells (P < 0.01). In contrast, the viability of T98GeEF2K(-) cells maintained in serum-containing medium was similar to that of controls (Fig. 6B and D).

Discussion

These studies show a role for eEF-2 kinase in the altered cell death pathways commonly seen in cancer cells. Autophagy, or type II programmed cell death, can protect cells from transient nutrient depletion (22, 26, 33) but, if left unchecked, will ultimately lead to cell death by self digestion. Eukaryotic cells have developed a sophisticated sensing mechanism for environmental nutrients. For example, when adequate nutrients are present, mTOR is active and promotes the synthesis of new proteins and the consumption of energy. This insulin-regulated activity is mediated through the assembly of ribosomes and the protein translation apparatus (34). eEF-2 kinase controls the rate of protein elongation through its phosphorylation of eEF-2, which in the nonphosphorylated state mediates the movement of the elongating peptide chain from the A site to the P site on the ribosome. In contrast, when the activity of eEF-2 kinase is increased, eEF2 is phosphorylated and its affinity for the ribosome decreases, leading to transient inhibition of protein synthesis (2, 3). In the presence of nutrients, insulin leads to the phosphorylation of Ser78 of eEF-2 kinase and blocks the activation of the kinase by Ca2+/calmodulin (17). Because protein synthesis accounts for the majority of cellular energy consumption, it is not surprising that eEF-2 kinase lies within the mTOR pathway and can be regulated at several levels (13, 17).

We identified eEF-2 kinase in glioblastoma as a Ca2+/calmodulin–dependent protein kinase (5, 6). Subsequently, we (35) and others (1, 36) reported the unique features of this enzyme, including the presence of an unusual catalytic domain and its total dependence on calmodulin for activity. Several laboratories, including our own, showed that the activity of the kinase was
exquisitely sensitive to growth factor stimulation (8, 11, 12) and nutrient deprivation (6).

To begin to understand the role of eEF2 kinase in autophagy, we used RNA interference to silence eEF-2 kinase expression in human glioblastoma cells, derived stable eEF-2 kinase–depleted clones, and measured the effects on several variables of autophagy. In support of the hypothesis that eEF-2 kinase is involved in autophagy, we found that silencing eEF-2 kinase activity in glioblastoma cell lines reduces autophagy as measured by three methods: LC3-II production (Fig. 2), AVO formation (Fig. 3), and appearance of multilamellar autophagosomes (Fig. 4). In contrast, overexpression of the kinase increased autophagy in isogenic glioblastoma cell lines (Fig. 3D–F). The level of LC3-II (expressed as a ratio to actin), not the ratio to LC3-I, is the standard method for measuring autophagy. Nonetheless, we did not view this single measurement as sufficient and, therefore, also measured autophagy by AVO formation (Fig. 3) and by electron microscopy (Fig. 4). Thus, it is possible that eEF-2 kinase depletion may also affect LC3-I levels; however, the use of complementary methods to assay autophagy, which yielded the same results, indicates that any effect that eEF-2 kinase had on LC3-I would be in addition to the effect of eEF-2 kinase on autophagy.

To determine the effect of nutrient deprivation on the induction of autophagy, we chose conditions of low cell density (20–30% confluence)/low basal autophagy. Nutrient deprivation increases the expression and activity of eEF-2 kinase (Fig. 5D and F) and suppresses the activity of S6 kinase (Fig. 5E and F). In the absence of inducible eEF-2 kinase, S6 kinase remains repressed but the autophagocytic response to nutrient deprivation is blunted (Fig. 5B and C). These data confirm the location of eEF-2 kinase downstream of S6 kinase (13) and show the essential role of eEF-2 kinase in the autophagocytic response. The differences in the baseline degree of autophagy in T98G cells seen in Figs. 2 and 5 are explained by cell density. We found that the degree of autophagy...
Regulation of Autophagy by eEF-2 Kinase

Figure 5. Continued. D to F, eEF-2 kinase, phospho-eEF-2 kinase, eEF-2, phospho-eEF-2, S6 kinase, phospho-S6 kinase, β-actin, and α-tubulin were determined as described in Materials and Methods. Representative of three similar experiments.
increases with cell density (Fig. 5A). In Fig. 2, to study the effect of eEF-2K depletion on cells undergoing autophagy, we grew cells to high density (90% confluence)/high basal autophagy. In Fig. 5, to study the effect of nutrient deprivation on the induction of autophagy, we chose conditions of low cell density (20-30% confluence)/low basal autophagy. Therefore, the differences in autophagy based on cell density readily explain the baseline differences between the T98G controls in these figures and were not a consequence of transfection or infection.

Consistent with the concept that cells can use autophagy to protect against transient nutrient deprivation (22, 26, 33), we found that blunting autophagy through depletion of eEF-2 kinase has deleterious effects on cell viability. The data in Fig. 6 show that cells with an incapacitated autophagic response, due to depletion of eEF-2 kinase, cannot survive even transiently without nutrients whereas the control cell line can actually proliferate and remain viable for up to 5 days. Others have reported similar dependence on autophagy for cell survival under nutrient-depleted conditions. For example, Lum et al. (22) found that removal of an obligate growth factor (interleukin-3) from hematopoietic precursors triggered autophagy and promoted cell survival through reutilization of cellular components to generate energy.

These results allow a more detailed understanding of how nutrient deprivation may link inhibition of protein synthesis to autophagy. In this model, the presence of nutrients activates mTOR, mTOR phosphorylates and activates S6 kinase, and both mTOR and S6 kinase phosphorylate and inhibit eEF2 kinase, thereby promoting protein synthesis. In contrast, in the absence of adequate nutrients, inhibition of mTOR results in the dephosphorylation and inhibition of S6 kinase, thereby relieving the inhibition of eEF-2 kinase, which in turn inhibits protein synthesis and conserves cellular energy. Under conditions of nutrient stress, the cell has acquired a mechanism to link conservation of energy (i.e., via inhibition of protein synthesis) to replenishment of energy supplies by digesting cellular organelles through activation of autophagy.

In summary, the results of our experiments show for the first time that eEF-2 kinase can play a role in autophagy in cancer cells and that increased expression of this kinase may provide a mechanism for survival of glioblastoma cells under conditions of cellular stress and, thus, may help explain why the activity of this kinase is increased in most human malignancies.

**References**


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