The glucose-regulated proteins: stress induction and clinical applications

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A protective mechanism used by cells to adapt to stress of the endoplasmic reticulum (ER) is the induction of members of the glucose-regulated protein (Grp) family. The induction of mammalian Grp proteins in response to ER stress involves a complex network of regulators and novel mechanisms. The elucidation of Grp function and regulation opens up new therapeutic approaches to diseases associated with ER stress and cancer.

The mammalian stress response is an evolutionarily conserved mechanism that allows cells to respond to adverse environmental or metabolic conditions. This response is represented at the molecular level by the induced synthesis of specific sets of cellular proteins with protective functions. For example, heat shock and inflammation strongly induce the expression of heat shock proteins (Hsps)\(^1\). This review focuses on the family of so-called glucose-regulated proteins (Grps), a term that encompasses a variety of endoplasmic reticulum (ER) chaperones that can be induced in cell culture under conditions of glucose starvation\(^2,3\). The Grp genes can also be induced by other perturbations of ER function such as agents that affect calcium stores or inhibit glycosylation. As these stress conditions generally lead to the accumulation of misfolded proteins in the ER, induction of Grp gene expression has been used extensively as a marker for the unfolded protein response (UPR). The UPR, an adaptive process conserved from yeast to human that acts in response to the disruption of ER homeostasis, also involves transient attenuation of new protein synthesis, degradation of misfolded proteins and onset of apoptosis\(^4\). Here, I address the characterization of mammalian Grp induction, regulation and function. The role of the Grps (Grp78 and Grp94) in cell survival during stress, and their clinical implications in cancer chemotherapy, adaptive immunity and gene therapy, will be discussed.

Unique inducers for the mammalian Grp genes

The Grps were discovered in 1977 by Ira Pastan through the observation that two proteins of molecular size 78 and 94 kDa were strongly induced in chicken embryo fibroblasts cultured in glucose-free medium\(^5\). These proteins were subsequently identified as Grp78 (also referred to as the immunoglobulin binding protein BiP) and Grp94 (alias gp96). Grp94 is the most abundant glycoprotein in the ER. In addition, whereas Grp78 is evolutionarily conserved from yeast to humans, Grp94 has only been identified in vertebrates, suggesting that Grp94 might serve unique functions that are restricted to higher eukaryotes. Expression of misfolded proteins, underglycosylated proteins and treatment of cells with reducing agents, induce the expression of both yeast and mammalian Grp genes; however, other stress inducers are unique for the Grp genes of higher eukaryotes (Fig. 1). For example, thapsigargin, a non-phorbol ester tumor promoter that specifically inhibits the ER Ca\(^{2+}\)-ATPase, is a highly potent ER stress inducer. This, as well as the Ca\(^{2+}\) ionophore A23187, ionomycin and EGTA, activate Grp transcription through depletion of Ca\(^{2+}\) stores in the ER (Ref. 6). This type of Ca\(^{2+}\) stress is not observed in yeast. In addition, chronic ethanol exposure potentiates inducers of Grp transcription\(^7\), and the mood-stabilizing drug sodium valproate, a branched-chain saturated fatty acid used in the treatment of bipolar disorder and epilepsy, induces Grp78 gene expression in rat brain\(^8\).

Most studies of Grp induction have been conducted with cells in culture; however, there is now new information on Grp expression in vivo. The Grps might be required for tissue-specific development. For example, Grp78 is elevated in embryonic mouse heart and is induced following hypoglycemic stress of the heart\(^9\) and within the rat endometrium at the pre-implantation stage\(^10\). High levels of Grp78 and Grp94 expression were observed in the brains of immature rats compared to the levels found in the brains of adult animals. Their expression was also high in the rat adult brain following cerebral ischemia, head trauma or seizure, induced by the glutamate agonist kainate\(^11\). Pathological conditions such as tumor growth correlate with Grp78 and Grp94 overexpression\(^1\). This could be partly caused by the activation of Grp gene expression through glucose starvation, acidosis and hypoxia, which are hallmark of the microenvironment of poorly vascularized solid tumors\(^12\). Grp78 induction is also associated with injury and dysfunction of human vascular endothelial cells as a result of hyperhomocysteinemia or genetic disorder\(^13\). Indeed,
the majority of pathologically toxic conditions show areas of inflammation, apoptosis and necrosis, which could represent physiological stresses for Grp induction in vivo.

**Conservation and divergence of the induction mechanism of yeast and mammalian Grp genes**

Stress induction of both yeast and mammalian Grp genes is primarily regulated at the level of transcription (Fig. 2). The promoter of KAR2, the yeast homolog of Grp78, contains one copy of the unfolded protein response element (UPRE). This element, CAGCGTG, serves as the binding site for an activator Hac1, which undergoes ER stress-induced mRNA splicing catalyzed by an ER membrane protein, Ire1p, using an unconventional mechanism4. The yeast UPR is negatively regulated by the phosphatase Ptc2p, which directly interacts with and dephosphorylates the phosphorylated form of Ire1p.

In yeast, deletion of the gene encoding Ire1p, IRE1, abolishes the UPR and the associated induction of Grp genes. Some features of the yeast UPR are conserved in mammalian cells14 (Fig. 2). For example, a subset of mammalian Grp promoters contain a UPRE-like sequence15, to which yeast Hac1 can bind and activate transcription14. Mice and humans have two IRE1 alleles. Support of an Ire1p-dependent pathway for mammalian UPR induction is based on the observation that overexpression of Ire1p activates the Grp promoter in a manner that is dependent on the endoribonuclease activity of Ire1p (Refs 4,16,17). This shows that the induction of Grp by Ire1p overexpression is mediated by its enzymatic activity and not merely as a consequence of creating ER stress through overexpressing an ER protein. However, the target for the endoribonuclease activity of Ire1p in mammalian cells has not yet been identified and the mammalian homolog of yeast Hac1 has not been isolated. Surprisingly, genetic disruption of both IRE1 alleles in mice had no effect on the UPR (Ref. 17). Therefore, unless there are as yet undiscovered mammalian IRE1 alleles, it appears that the putative Ire1p homolog is not obligatory for the mammalian UPR.

Evidence is accumulating suggesting that mammalian cells use Ire1p-independent signaling pathways to activate the Grp genes in response to ER stress. Different stress stimuli could also elicit distinct or overlapping pathways. WEHI17.2 lymphoma cells are able to increase Grp78 transcript levels in response to a block in protein glycosylation but not in response to depletion of the ER Ca2+ store; further investigation suggested that the proto-oncogene product c-fos was involved in Grp78 induction following ER Ca2+ release18. Stress induction of Grp78 and Grp94 is suppressed by genistein, an inhibitor of protein tyrosine kinases that works by obstructing the ATP-binding site of the kinases19. ER-stress treatment is also known to induce JNK and p38 MAP kinase signaling17. However, the direct relationship between the targets of these and other stress-activated kinases, and their role in the induction of Grp genes upon ER stress, remain to be established.

**Complex regulatory network for induction of Grp transcription**

An unusual feature shared by mammalian Grp promoters is that they contain multiple copies of the...
CCAAT element flanked by GC-rich sequences. These turn out to be repetitive units of the ER stress response element (ERSE)\(^{20,21}\), which is an evolutionarily conserved tripartite structure CCAAT(N\(_9\))CCACG (N represents a 9-bp region) (Fig. 2). This sequence is notably more complex than the UPRE. Recently, another sequence motif, ATTGG(N\(_9\))CCACG, referred to as ERSE-II, has been identified in the promoter of Grp78, also contains a heat shock element (HSE). In mammalian cells, the Grp promoters contain multiple endoplasmic reticulum stress elements (ERSEs) but no HSE. A subset of mammalian Grp promoters, including Grp78, also contains UPRE-like sequences. ER stress leads to activation of Ire1p; however, its downstream target is not known. ATF6, an ER transmembrane protein, undergoes proteolytic cleavage and other modifications upon ER stress. The nuclear form of ATF6 is a strong activator of the mammalian Grp genes. Other transcription factors such as NF-Y, YY1 and TFII-I, can also bind and activate the ERSE. Both NF-Y and YY1 are known to interact with proteins that modify histone acetylation and with cofactors that bind to the TATA element. Other activating pathways might involve tyrosine kinases and the proto-oncogene product c-fos; however, their downstream targets are not known. Members of the cold shock domain proteins, dbpA and YB-1, which also show affinity for the CCACG motif, suppress Grp78 promoter activity\(^{26}\). In genistein-treated cells, in which Grp78 induction is severely suppressed, interaction of both NF-Y and YY1 with the ERSE is inhibited\(^{19}\). Within a subset of vertebrate Grp promoters, the N\(_9\) sequence is highly conserved and has a strikingly high GC content that binds an ER-stress-inducible complex\(^{15,21,27}\). Indeed, mutation of the GC-rich motif reduces ER stress induction\(^{21,23}\). Through affinity chromatography, the protein that binds to the GC-rich motif of the ERSE has been identified as the transcription factor TFII-I, known to facilitate protein–protein interactions\(^{27}\). Suppression of TFII-I expression by antisense approaches inhibited ER stress induction of the ERSE. Because the stimulatory activity of TFII-I is dependent on tyrosine phosphorylation, it can potentially provide a link between the tyrosine kinase signaling pathway and Grp transcription. The yeast UPR model predicts that chromatin modification by acetylation could be important for induction of ER stress response genes\(^3\). Both NF-Y and YY1 have been shown to interact with cofactors that can modify histone acetylation. Therefore, in addition to enhancing factor cooperativity, they might modify the chromatin structure of Grp genes in response to ER stress\(^{14,15}\). Furthermore, YY1 and TFII-I, through their ability to interact with components of the basal transcription machinery, such as TBP, TFII B and TAFII 55k, can also link the upstream regulatory and the basal transcription complexes.

Although not detectable as an ERSE-binding complex in gel shift assays using nuclear extracts, ATF6, a transmembrane protein with an ER and perinuclear location, is a potent activator of the Grp promoters\(^{20}\). There are two forms of ATF6, a 90-kDa form referred to as ATF6 (\(\alpha\)) and a 110-kDa form referred to as CREB-RP or ATF6 (\(\beta\)). Both forms of ATF6 undergo ER-stress-induced cleavage, generating cytoplasmic fragments of 50–60 kDa that enter the nucleus and activate the ERSE through complex formation with NF-Y in a manner dependent on the CCACG motif\(^{24}\). In the presence of a proteasome inhibitor, ER-stress-induced cleavage of exogenous ATF6 expressed at a low level can be visualized and is mediated by the Site-I and Site-2 proteases (S1P and S2P, respectively)\(^{28}\). These novel findings also imply that ATF6 has to undergo ER stress-induced translocation to the Golgi apparatus to be processed by these specific proteases. Interestingly, Brefeldin A, a drug that causes redistribution of the Golgi components into the ER and blocks protein transport out of the mixed ER–Golgi system\(^{29}\), induces Grp78 transcription in hamster fibroblasts; this induction is mediated by the region of the Grp78 promoter containing the ERSE (Ref. 30). Future studies will be required to determine whether the impairment of Grp induction
transmembrane protein that pumps Ca\textsuperscript{2+} into the ER lumen. Blockage of SERCA-2 results in ER Ca\textsuperscript{2+} depletion, leading to cell death and subsequent Grp induction. Oxidoreductin 1-L is an ER transmembrane protein that contains ubiquitin-like domains at its N-terminus, and genes encoding both Grp78 and Herp are induced in homocysteine-exposed human vascular endothelial cells. Oxidoreductin 1-L, Grp94, and adapt78 can protect cells against cell death, as illustrated in Table 1. The majority of the Grps [Grp78, Grp94, Grp170, ERP72, PDI, calreticulin, and GRP58 (alias ERP57)] are ER molecular chaperones that assist in protein folding and assembly. Grp78, Grp94, and adapt78 are other mechanisms for ATF6 activation, but the anti-apoptotic effect of the Grps is not understood, as the mechanism for the anti-apoptotic effect of the Grps is not understood, according to the authors. A Grp78 mutant that is defective in ATP hydrolysis, the level of the 90-kDa uncleaved ATF6(α) actually increases at the peak of Grp78 stress induction concurrent with the appearance of other modified forms that can be recovered in soluble nuclear fractions. It is possible that ATF6 is retained in the ER through interaction with ER chaperones. Following ER stress, a fraction of ATF6 could dissociate from the chaperone complex and, with changes in ER membrane fluidity, assume new cellular locations. Because ATF6 is a substrate of p38 MAP kinase, its activity can also be modulated by phosphorylation.

**Multiple functions of the Grps**

A list of proteins encoded by mammalian genes that exhibit induction profiles similar to Grp78 and Grp94 in response to ER stress is summarized in Table 1. The majority of the Grps [Grp78, Grp94, Grp170, ERP72, PDI, calreticulin, and GRP58 (alias ERP57)] are ER molecular chaperones that assist in protein folding and assembly. Grp78, Grp94, ERP72 and calreticulin are also Ca\textsuperscript{2+}-binding proteins. Newly identified ER proteins that are inducible by ER stress include SERCA-2, Herp, oxidoreductin 1-Lβ and adapt78, all with functions related to maintenance of ER homeostasis. SERCA-2 is an ER transmembrane protein that pumps Ca\textsuperscript{2+} from the cytosol into the ER lumen. Blockage of SERCA-2 activity by thapsigargin results in ER Ca\textsuperscript{2+} depletion and subsequent Grp induction. Herp contains ubiquitin-like domains at its N-terminus, and genes encoding both Grp78 and Herp are induced in human vascular endothelial cells exposed to homocysteine. Oxidoreductin 1-Lβ is an N-glycoprotein required for maintaining a suitable redox environment in the ER. Although CHOP does not regulate Grp gene expression, the levels of two activators for the Grp promoters, ATF6(α) and TFII-I, increase upon ER Ca\textsuperscript{2+} release in some cell lines. Autoregulation of these transcription factors in response to ER stress might represent novel feedback mechanisms that sustain the ER stress response.

**Role of Grps in cell survival following ER stress**

Overexpression, antisense and ribozyme approaches in tissue culture systems directly showed that Grp78, Grp94 and adapt78 can protect cells against cell death. The protective function of the Grps suggests that their induction could be beneficial in situations...
Grp induction and protection. ER stress triggers transcriptional activation of the Grp genes mediated by the ERSE and subsequent increase in the levels of Grp proteins. Components of the transcription activating complex include NF-Y, ATF6, YY1, and TFII-I. The stress-inducible properties of the Grp78 promoter can be used for transcriptional targeting in gene therapy. The induction of Grp can be blocked at the level of transcription by drug inhibitors such as genistein. The stability and translation of Grp can also be downregulated by ribozymes and antisense expression vectors. Suppression of Grp induction in ER stressed cells leads to higher cytotoxicity and programmed cell death. The induction of Grp confers a survival advantage. This can be beneficial to organ preservation, for example, by protecting against brain damage or endothelial cell injury. By contrast, induction of Grp in pre-neoplastic and neoplastic cells could lead to escape of the tumor cells from immune surveillance, resulting in tumor progression and malignancy. Induction of Grp in cancer cells can also lead to drug resistance. Grp94 derived from necrotic cancer cells acts to present antigenic peptides when taken up by scavenging antigen-presenting cells.

Grps as targets for cancer chemotherapy
Whereas Grp overexpression could limit damage in organs exposed to ER stress, the anti-apoptotic function of the Grps also predicts that their induction in neoplastic cells could lead to cancer progression and drug resistance (Fig. 3). In a variety of cancer cell lines, solid tumors and human cancer biopsies, the levels of Grp78 and Grp94 are elevated, correlating with malignancy. In addition, induction of Grp78 has been shown to protect cancer cells from immune surveillance, whereas suppressing the stress-mediated induction of Grp78 enhanced apoptosis, inhibited tumor growth and increased the cytotoxicity of chronic hypoxic cells. Therefore, targeted suppression of Grp expression or function in cancer cells could represent a novel approach to cancer chemotherapy. Genistein, which suppresses both the Grp and the heat shock responses, inhibits the growth of carcinogen-induced tumors in rats and in human leukemia cells transplanted into mice. In another example, Grp94 has been shown to associate with and stabilize p185 erbB2 (also referred to HER-2/neu), which is commonly overexpressed in breast carcinomas and is associated with poor prognosis. Treatment of breast cancer cells with geldanamycin, an antiproliferative agent, enables the degradation of p185 in the breast cancer cells by disrupting the Grp94–p185 complex.

With respect to drug resistance, preinduction of Grp in a variety of human cancer cell lines confers resistance to inhibitors of topoisomerase II (e.g., etoposide) but increases sensitivity to DNA cross-linking agents such as cisplatin. Direct suppression of Grp94 levels by antisense knockdown strategies results in enhanced sensitivity to etoposide-induced cell death. Interestingly, etoposide-induced cell death triggers proteolytic cleavage of Grp94 by calpain, which also degrades involving tissue or organ damage. The higher levels of Grp94 and Grp78 in the brains of immature rats when compared to those of adult animals might account for the higher resistance of immature rats to seizure. In addition, specific induction of these Grps in the dentate gyrus region of the adult rat brain following seizure might contribute to a neuroprotective effect in that particular region of the brain. For early-onset familial Alzheimer’s disease (FAD), overexpression of Grp78 in neuroblastoma cells expressing a mutant presenilin-1 (PS1) protein was reported to restore resistance to ER stress. However, another study showed that the induction of Grp78 and CHOP by the UPR is independent of PS1 expression and that UPR is intact in individuals with sporadic Alzheimer’s disease or presenilin-mediated FAD (Ref. 46). Thus, whether clinical manipulation of Grp level will be therapeutic for FAD requires further resolution. Recently, expression of Grp78 was reported to prevent the aggregation and facilitate the proteasomal degradation of mutant prion proteins, which are implicated in neurodegenerative disorders such as prion diseases and transmissible spongiform encephalopathies. However, when the proteasomal function is impaired, Grp78 might render the mutant prion protein even more pathogenic by promoting its refolding. Induction of Grp78 has also been observed in endothelial cells damaged by reductive stress that was caused by hyperhomocysteinemia which, with both genetic and environmental components, is a common risk factor for thrombotic vascular events such as premature arteriosclerosis, stroke, myocardial infarction and thrombosis. Therefore, the induction of Grp could be an adaptive response evolved in mammals to protect endothelial cells against stress-induced cell death.
Bcl-xL during apoptosis, therefore turning an anti-apoptotic protein into a pro-apoptotic molecule\(^{12,44}\). Within the microenvironment of a solid tumor, unique stress conditions can lead to induction of Grp. Because the chaperone function of Grp is essential for its protective function, it is possible that this function requires retention of growth factor receptors, among other proteins, in the E.R., leading to G1 arrest and, therefore, resistance to cytotoxic drugs targeting S-phase cells\(^5\). Other mechanisms for Grp-mediated protection could involve Grp interaction with effectors of apoptosis, leading to the blockade of cell death induced by drug treatment.

**Role of Grp in immunotherapy**

Grp94 has been shown to chaperone a broad array of peptides, including those derived from normal proteins as well as from foreign and altered proteins present in cancer or virus-infected cells\(^2\). Thus, tumor-derived Grp94 carries tumor antigenic peptides, and Grp94 preparations from virus-infected cell's carry viral epitopes. Although Grp94 is normally intracellular, necrotic cells release Grp94-peptide complexes, which are taken up by scavenging antigen-presenting cells. Presentation of the peptides on the surface of these cells leads to stimulation of T lymphocytes and a pro-inflammatory response. Complexes of Grp94 with peptides, whether isolated from cells or reconstituted in vitro, have served as immunogens to elicit immune responses specific to the Grp94-chaperoned antigenic peptides\(^3\). Immunization using low concentrations of Grp94–peptide preparations in mice inhibited tumor growth. Autoimmunity has not been observed, suggesting that the immune response is targeted against the peptides and not against Grp94. In extrapolating the animal studies to human applications, cancer patients were immunized with autologous cancer-derived Grp94 preparations as a test for the feasibility of a patient-specific chaperone-based vaccine for immunotherapy of human cancer\(^3\). Recently, another peptide-binding Grp, referred to as Grp170, has also been shown to be effective as a cancer vaccine\(^4\). If successful, this approach could also be applied as therapy for infectious diseases.

**Use of the stress-inducible Grp78 promoter in cancer gene therapy**

Aggressive tumors often suffer from insufficient blood supply resulting in areas of acidosis, nutrient deprivation and hypoxia. Such adverse conditions specifically activate the Grp78 promoter. The use of the stress-inducible Grp78 promoter to drive therapeutic gene expression offers a novel approach for gene therapy of poorly perfused tumors and ischemic diseases. The Grp78 promoter confers higher efficacy than viral promoters in eradication of sizable tumors in mouse models bearing fibrosarcoma and breast tumors\(^12,50\). An added advantage associated with use of the Grp78 promoter is that it is also inducible by a form of laser therapy (photodynamic therapy), which promotes oxidative stress and ischemia in vivo\(^5\), enabling the expression of the therapeutic gene to be further controlled temporally and spatially.

**Conclusion**

Since the discovery of Grp78 and Grp94 20 years ago, there has been an explosion of new knowledge regarding the function and regulation of Grp in cell culture. Future experiments addressing the physiological roles played by Grp in vivo will further enhance our understanding of the importance of these proteins in human development and in diseases associated with the disturbance of E R homeostasis.

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