Published in final edited form as:

Biochem Biophys Res Commun. 2008 December 12; 377(2): 407–412. doi:10.1016/j.bbrc.2008.09.133.

Overlapping signal sequences control nuclear localization and endoplasmic reticulum retention of GRP58

Anbu Karani Adikesavan¹, Emmanuel Unni², and Anil K. Jaiswal^{2,*}

¹ Department of Pharmacology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030

² Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, 655 West Baltimore Street, Baltimore, MD 21201

Abstract

Glucose-regulated GRP58 has shown clinical applications to endoplasmic reticulum (ER) stress and cancer. GRP58 is localized in the cytosol, endoplasmic reticulum (ER) and nucleus. Twenty-four amino acids at the N-terminal hydrophobic region are known to target GRP58 to ER for synthesis at the ER membrane and translocation into the ER lumen. In addition, GRP58 contains putative nuclear localization (494KPKKKKK500) and ER retention (502QEDL505) signals. However, the role of these signals in nuclear import and ER retention of GRP58 remains unknown. Present studies investigated the signals that control nuclear localization and ER retention of GRP58. Deletion/mutation of nuclear localization signal (NLS) abrogated nuclear import of GRP58. NLS attached to EGFP localized EGFP in the nucleus. However, deletion/mutation of putative ER retention signal alone did not alter ER retention of GRP58. Interestingly, a combined deletion/mutation of NLS and ER retention signals blocked the GRP58 retention in the ER. These results concluded that overlapping NLS and ER retention signal sequences regulate nuclear localization and ER retention of GRP58.

Keywords

GRP58; Thioredoxin-like domains; Nuclear import; Endoplasmic reticulum retention; Signals regulating subcellular localization

Glucose regulatory protein (GRP58), a 58-kDa protein with significant homology to protein disulfide isomerase has two thioredoxin-like domains and is suspected to function as thiol-dependent oxidoreductase [1]. Glucose deprivation is known to activate GRP58 [2]. GRP58 has shown clinical applications to endoplasmic reticulum stress associated diseases and cancer [2]. Disruption of GRP58 in mouse is lethal; however B cell specific deletion of GRP58 leads to impaired assembly of the major histocompatibility complex [3]. Besides, GRP58 is shown to play a role in gamete fusion [4], regulation of transcription factors like Ref1 [5], entry of SV40 in host cells [6] and in mitomycin C-induced DNA cross-linking [7–8].

GRP58 is localized in cytosol, nuclear and endoplasmic compartments [9–10]. In the cytosol, GRP58 has been identified as a chaperone for the signal transducer and activator of

Corresponding author: Dr. Anil K. Jaiswal, Professor, Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, 655 West Baltimore Street, Baltimore, MD 21201, Tel. # 410 706-2285; Fax # 410 706-0032, Email: ajaiswal@som.umaryland.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

K499A-5'
 GAGGTCCTCTTGTGCCTTCGCCTTCTTCTTAGGTTTTCTTCTTGAATTATAGG3',
 and K500A-5'
 GAGGTCCTCTTGTGCCGCTTCTTCTTCTTAGGTTTTCTTCTTGAATTATAGG3'.
 All plasmids were verified by DNA sequencing.

The plasmid for GRP58 without QEDL sequence (pcDNA-GRP58 Δ ER-V5) was constructed using forward primer same as above and reverse primer targeting the region just upstream to the QEDL sequence (5'-TGCCTTCTTCTTCTTCTTAGG-3'). Construction of plasmids, pcDNA-GRP58 NLS/ER DD-V5 (deletion of both NLS and ER signals) and pcDNA-GRP58 NLS/ER DM-V5 (alanine substitution of all amino acids in NLS and ER signals) were made by PCR amplifications using same forward primer and reverse primers 5' TTCTTCTTGAATTATAGGGGGGTTGTAGC3' and 5' TGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTTCTTCTTGAATTATAGGGG3' respectively. The PCR amplified products were T/A cloned into pcDNA3.1/V5-His TOPO vector (Invitrogen) as per the protocol suggested by the manufacturer. All the cloned plasmid DNA constructs were sequence verified as well as all of them expressed a V5 peptide tag at their C-terminus. All plasmids were verified by DNA sequencing.

GFP fusion protein

The putative NLS coding sequence (KPKKKKK) of GRP58 was fused with green fluorescent protein (EGFP) coding sequence to construct the plasmid that expressed the fusion protein EGFP-NLS. For this, the upper and lower strand oligonucleotide primers (5' CGGGGTACCCAAACCTAAGAAGAAGAAGGGATCCGCG3' and 5' CGCGGATCCCTTCTTCTTCTTCTTAGGTTTGGTACCCCG3') were annealed and the resulting double-stranded oligonucleotide contained KpnI and BamHI restriction sites at the 5' and 3' ends respectively. Subsequently, this product was cloned into corresponding sites in pEGFP-C1 vector (Clontech, Palo Alto, CA). The construct was sequence verified and named as pEGFP-NLS.

Cell culture and transfections

HCT116 cells were grown in McCoy's 5A (modified) medium and Hepa-1 cells in Dulbecco's modified Eagle's medium, both supplemented with 10% fetal bovine serum, penicillin (40units/ml) and streptomycin (40 μ g/ml). The cells were grown in monolayer at 37°C in 95% air and 5% CO₂. Transient transfections with different plasmids were done in cells grown overnight to ~50% confluence using Effectene transfection reagent (Qiagen, Valencia, CA) as per manufacturer's protocol.

Immunofluorescence staining and fluorescence microscopy

Hepa-1 cells were grown in monolayer on sterile poly-D-lysine coated coverslips (BD Biosciences, MA) in 6-well plates to ~50% confluence. Cells were transiently transfected with desired plasmids using the protocols described above. 24h after transfection, the cells were fixed in 10% formalin (Polysciences, Inc., PA) and permeabilized with 0.5% Triton X-100 in phosphate buffered saline (PBS). The cells were then incubated with anti-V5-FITC antibody (Invitrogen, CA) for immunostaining V5-tagged proteins (GRP58-WT-V5, GRP58- Δ NLS-V5 and GRP58 NLS K-A mutant-V5). In case of EGFP-NLS fusion protein that fluoresces on its own, the cover slips were directly processed for visualization after mounting. The cover slips were mounted onto glass slides using Vectashield mounting medium (Vector Laboratories, CA). The mounting medium contained DAPI to counter stain DNA for visualizing the nucleus. The cells were examined for FITC/GFP fluorescence localization using Nikon eclipse TE 2000-U fluorescence microscope and images were captured simultaneously using Photometrics Coolsnap CF camera fitted to the microscope.

Subcellular fractionation

HCT116 and Hepa-1 cells transiently transfected with different plasmids were briefly washed with ice-cold PBS. Total cell lysates (TCL) were made by lysing the cells in RIPA buffer (50mM Tris, pH 8.0, 150mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 1mM phenylmethylsulfonyl fluoride and 1mM sodium vanadate) supplemented with protease inhibitor mixture (Roche Applied Science, IN), followed by centrifugation at 21000×g for 10 min at 4°C to collect the supernatant. Cytosolic and nuclear fractionations of the cells were obtained using the nuclear extract kit (Active Motif, CA). ER fractions were made by differential centrifugation after homogenizing the whole cells by douncing under cold condition in homogenization buffer [0.25M sucrose, 0.05M Tris (pH 7.4), 1 mM EDTA, 2 µg/ml antipain and 2 µg/ml leupeptin]. The crude cell homogenates were centrifuged at 500×g for 10 min and then 12,000×g for 15 min to generate nuclear and mitochondrial fractions. The resulting supernatants were centrifuged at 105,000×g for 1h and 20 min at 4°C to get the ER pellet and the supernatant cytosolic fraction. The nuclear and ER pellets were subsequently lysed in RIPA buffer to get the respective fractions.

SDS-PAGE and Western analysis

50 µg of total cell lysates (TCL) or the cellular fractions were resolved in 10% SDS-PAGE, transferred to nitrocellulose membranes and blocked with 5% skim milk powder in TBS-Tween20 buffer followed by incubation with anti-V5 (1:5000, Invitrogen), or anti-βactin (1:5000, Sigma) antibodies. The membranes after incubation with respective secondary antibodies were probed with chemiluminescence detection kit (Amersham Biosciences). The membranes were stripped and re-probed with cytoplasm-specific, anti-lactate dehydrogenase (1:1500, Chemicon International); nucleus-specific, anti-laminB (1:2000, Santa Cruz Biotechnology) and ER specific, anti-calnexin (1:5000, StressGen Bioreagents) and/or anti-cyp1A1 (1:2000) antibodies.

It is noteworthy that all the experiments were repeated three to five times. Only the representative results are shown.

Results

Prosit analysis of mouse GRP58 revealed the presence of putative nuclear localization (494KPKKKKK500) and ER retention (502QEDL505) signals in the C-terminus of the protein that were conserved among other species (Fig. 1A–B). We generated a series of progressive C-terminus deletions (M1–M5) in mouse GRP58 (Fig. 1A). Wild type and deletion mutants were transfected in HCT116 cells, subcellular fractionated into cytosolic, nuclear and ER fractions and analyzed by immunoblotting (Fig. 1C). The results are shown only for wild type, M1 and M5 constructs (Fig. 1C). However, M3 and M4 constructs showed results same as M1 and M5 (data not shown). The results as expected demonstrated the presence of wild type GRP58 in all the three compartments. The subcellular distribution of transfected GRP58 was similar as observed with endogenous protein (data not shown). However, all of the C-terminal mutants showed exclusive localization in the cytosolic compartment of the cell (Fig. 1C). The deletion mutants were absent in nucleus and ER compartments. Deletion mutant M5 contained shortest deletion of only 12 amino acids (aa 494 to 505) from the C-terminus and yet it was only present in cytosolic compartment. We also performed experiment if C-terminal deletion mutant proteins are getting degraded through ER-associated degradation pathway to remove damaged/misfolded proteins. HCT116 cells were transfected with mutant GRP58-M5-V5, treated with either DMSO or MG132, lysed and subcellular fractionated in cytosolic and nuclear fractions. Total cell lysate (TCL), cytosol (C) and nuclear (N) proteins were separated on SDS PAGE, Western blotted and probed with anti-V5 to detect GRP58-M5-V5, anti-LDH (cytosolic marker), anti-Lamin B (nuclear marker) and anti-calnexin (ER marker). The results are shown

in right most panel of Fig. 1C. The results revealed that GRP58-M5 mutant protein was exclusively localized in the cytosol and absent in nuclear and ER compartments in both DMSO and MG132 treated cells. In other words, the mutant GRP58-M5 protein is not degraded through ER-associated degradation system. However, treatment with MG132 led to stabilization of mutant protein in the cytosol. These results combined demonstrated that 12 amino acids at the C-terminus of the GRP58 protein contains nuclear localization and ER retention signals. Antibodies against lactate dehydrogenase (LDH), laminB, and calnexin were used as markers of cytosolic, nuclear, and endoplasmic reticulum fractions respectively.

The NLS region was deleted from the mouse GRP58-V5 to generate V5-tagged GRP58 lacking in NLS. Plasmids encoding GRP58 wild type (GRP58 WT-V5) and NLS deficient GRP58 Δ NLS-V5 were transfected in HCT116 and Hepa-1 cells. The transfected cells were subcellular fractionated to obtain cytosolic and nuclear fractions. Western analysis showed that the deletion of NLS region from mouse GRP58 abrogated nuclear localization of GRP58 in both cell types (Fig. 1D). In similar experiments, six lysine residues in NLS region were mutated to alanine and analyzed for subcellular distribution in HCT116 and Hepa-1 cells (Fig. 1E). The NLS mutant failed to localize in the nucleus. In related experiments, plasmids encoding GRP58 WT-V5, GRP58 Δ NLS-V5 and GRP58 NLS K-A Mutant-V5 were individually transfected in Hepa-1 cells. The transfected cells were analyzed for subcellular localization of wild type and mutated GRP58 protein by immunohistochemistry with FITC conjugated anti-V5 antibody (Fig. 2A). The results supported observations from Western analysis in Figure 1D and 1E. Wild type GRP58 localized in cytosol and nucleus. However, both GRP58 lacking in NLS and GRP58 with mutated NLS were absent in the nucleus. In further experiments, the GRP58 NLS domain attached to green fluorescence protein (EGFP) localized in the nucleus as compared to EGFP alone localization in cytosol and nucleus (Fig. 2B). The immunohistochemistry results as shown in figure 2A and 2B in Hepa-1 cells were also observed with HCT116 cells (data not shown). The amino acid residues in NLS (494KPKKKKK500) region were mutated one by one to alanine to generate seven GRP58 mutants. These mutants along with wild type GRP58 were transfected in HCT116 cells in separate experiments, subcellular fractionated and analyzed localization of wild type and mutant GRP58 by Western blotting and probing with anti-V5 antibody (Fig. 3). Interestingly, all of the mutant GRP58 proteins localized to cytosolic and nuclear compartments, and also retained in ER compartment, as observed with wild type GRP58 protein (Fig. 3). In other words, mutation of a single residue in NLS region had more or less no effect on nuclear localization of GRP58. Interestingly, all the seven mutants carrying mutation of a single residue in NLS region showed significantly reduced retention of GRP58 in ER compartment, as compared to wild type GRP58 (Fig. 3). Similar results were observed in Hepa-1 cells (data not shown).

We deleted putative ER retention domain (502QEDL505) from GRP58 to determine the role of ER retention signal in retention of GRP58 in ER compartment. The plasmids encoding wild type and ER deleted GRP58 were transfected in HCT116 and Hepa-1 cells and analyzed for subcellular localization of wild type and deletion mutant protein (Fig. 4A). Surprisingly, the deletion of ER retention signal alone had no effect on ER retention of GRP58. Both wild type and ER mutant proteins were retained in ER compartment. In related experiments, we generated NLS and ER combined deletion mutant (NLS/ER DD) and amino acid mutant (NLS/ER DM). We transfected these two NLS/ER DD and NLS/ER DM mutant plasmids along with wild type and deletion ER mutant in HCT116 cells in separate experiments, subcellular fractionated and analyzed localization of wild type and mutant proteins by Western blotting and probing with anti-V5 antibody (Fig. 4B). Interestingly, combined deletion or mutation of NLS and ER regions resulted in complete loss of retention of double deletion and mutant proteins in ER compartment. In other words, both NLS and ER regions were required for ER retention of GRP58 protein. The NLS/ER deleted and mutated GRP58 proteins were also absent in nuclear

fraction as expected because of absence of NLS (Fig. 4B). Similar results were also observed with another cell line Hepa-1 (data not shown).

Discussion

In this report, we identified and characterized a lysine rich NLS (494KPKKKKK500), which is required for the nuclear localization of GRP58. Deletion and mutation of NLS resulted in the complete loss of GRP58 protein in the nucleus. This was clearly evident from several observations. GRP58 mutant lacking the NLS or containing mutated NLS failed to enter the nucleus. In similar experiments, the wild type GRP58 was localized in the nuclear compartment. The GRP58 NLS sequence fused to green fluorescence protein (GFP), resulted in the nuclear accumulation of GFP indicating that this signal sequence was sufficient to direct nuclear localization of GRP58. It appears that GRP58 contains a single NLS since no other NLS was found by sequence analysis and deletion or mutation of NLS led to complete loss of entry of mutant GRP58 in the nucleus. Interestingly, mutation of amino acid residues one at a time in NLS region had no effect on nuclear localization of GRP58. This indicated that mutation in the NLS region could be tolerated without loss of NLS function.

It is known that GRP58 contains twenty-four amino acids N-terminal hydrophobic region that serves as ER targeting sequence. The N-terminal ER targeting sequence directs GRP58 for synthesis at the ER membrane and translocation into the ER lumen. The present studies also revealed that C-terminal NLS/ER both regions are required for ER retention of GRP58 after hydrophobic N-terminal targeting sequence targeted GRP58 to the ER. Deletion or mutation of both NLS and ER and not individual region abrogated ER retention of GRP58. These results demonstrated that an overlapping NLS/ER retention signal is required for nuclear localization and ER retention of GRP58. Mutations in NLS region and not in ER retention region resulted in significant reduction of retention of GRP58 in the ER compartment. Several studies have identified a carboxyl-terminal retrieval signal (KKXX or KKKXX; X is any amino acid) for targeting of proteins from Golgi to ER retention [12–13]. This is especially true for proteins that undergo post-translational modifications in Golgi before retention in ER. Therefore, it is possible that de novo synthesized GRP58 is modified in Golgi. The lysine rich NLS (493KPKKKKK499), targets GRP58 from Golgi to its ER destination and ER retention signal (502QEDL505) contributes to retention of GRP58 in ER. However, these predictions have to be confirmed by experiments.

An interesting question is what regulates localization of GRP58 in cytosolic and nuclear compartments, and retention of GRP58 in ER compartment of the cell? It has been shown earlier that mutations in thioredoxin like domains have no effect on subcellular localization of GRP58 [8]. There is very little information available on phosphorylation of GRP58 protein. Cytosolic GRP58 is phosphorylated at serine 150 [14]. It raises questions if the subcellular localization of GRP58 is regulated by phosphorylation at tyrosine and serine/threonine residues and remains to be determined.

In conclusion, our results indicate that GRP58 is localized in cytosolic, nuclear and ER compartments. The results also characterized NLS sequence that directs nuclear localization of GRP58. Results further characterized and showed that NLS in combination with ER signal directs GRP58 for retention in ER.

Acknowledgements

This work was supported by NIH grant RO1 CA81057.

References

1. Hirano N, Shibasaki F, Sakai R, Tanaka T, Nishida J, Yazaki Y, Takenawa T, Hirai H. Molecular cloning of the human glucose-regulated protein ERp57/GRP58, a thiol-dependent reductase: identification of its secretory form and inducible expression by the oncogenic transformation. *Eur J Biochem* 1995;234:336–342. [PubMed: 8529662]
2. Lee AS. The glucose-regulated proteins: stress induction and clinical applications. *Trends in Biochem Sci* 2001;26:504–510. [PubMed: 11504627]
3. Garbi N, Tanaka S, Momburg F, Hammerling GJ. Impaired assembly of the major histocompatibility complex class I peptide-loading complex in mice deficient in the oxidoreductase ERp57. *Nat Immunol* 2006;7:93–102. [PubMed: 16311600]
4. Ellerman DA, Myles DG, Primakoff P. A role of sperm surface protein disulfide isomerase activity in gamete fusion: evidence for the participation of ERp57. *Dev Cell* 2006;10:831–837. [PubMed: 16740484]
5. Grillo C, D'Ambrosio C, Scaloni A, Maceroni M, Merluzzi S, Turano C, Altieri F. Cooperative activity of REF-1/APE and ERp57 in reductive activation of transcription factors. *Free Radic Biol Med* 2006;41:1113–1123. [PubMed: 16962936]
6. Schelhaas M, Malmstrom J, Pelkmans L, Haugstetter J, Ellgaard L, Grunewald K, Helenius A. Simian virus 40 depends on ER protein folding and quality control factors for entry into host cells. *Cell* 2007;131:516–529. [PubMed: 17981119]
7. Celli M, Jaiswal AK. Role of GRP58 in mitomycin C-induced DNA cross-linking. *Cancer Res* 2003;63:6016–6025. [PubMed: 14522930]
8. Adikesavan AK, Jaiswal AK. Thioredoxin-like domains required for glucose regulatory protein 58 mediated reductive activation of mitomycin C leading to DNA cross-linking. *Mol Cancer Ther* 2007;6:2719–2127. [PubMed: 17938265]
9. Coppari S, Altieri F, Ferraro A, Chichiarelli S, Eufemi M, Turano C. Nuclear localization and DNA interaction of protein disulfide isomerase ERp57 in mammalian cells. *J Cell Biochem* 2002;85:325–333. [PubMed: 11948688]
10. Ndubuisi MI, Guo GG, Fried VA, Etlinger JD, Sehgal PB. Cellular physiology of STA3: where's the cytoplasmic monomer? *J Biol Chem* 1999;274:25499–25509. [PubMed: 10464281]
11. Jessop CE, Chakravarthi S, Garbi N, Hammerling GJ, Lovell S, Bulleid NJ. ERp57 is essential for efficient folding of glycoproteins sharing common structural domains. *EMBO J* 2007;26:28–40. [PubMed: 17170699]
12. Jackson MR, Nilsson T, Peterson PA. Retrieval of transmembrane proteins to the endoplasmic reticulum. *J Cell Biol* 1993;121:317–333. [PubMed: 8468349]
13. Pelham HRB. Sorting and retrieval between the endoplasmic reticulum and golgi apparatus. *Current Opinion in Cell Biology* 1995;7:530–535. [PubMed: 7495573]
14. Kita K, Okumura N, Takao T, Watanabe M, Matsubara T, Nishimura O, Nagai K. Evidence for phosphorylation of rat liver glucose-regulated protein 58, GRP58/ERp57/ER-60, induced by fasting and leptin. *FEBS Lett* 2006;580:199–205. [PubMed: 16375900]

Fig. 1.

Nuclear localization (NLS) and ER retention signals. (A). Schematic representation of mouse GRP58 wild type and GRP58 C-terminal truncated mutant (M1 to -M5) proteins. (B). Amino acid sequence alignment of mouse, rat and human GRP58 showing putative NLS and ER retention signals conserved at the C-termini. (C). Subcellular distribution of GRP58 wild type and GRP58 mutants M1–M5 in HCT116 cells. Cells were transfected with plasmids for V5-tagged GRP58-WT or mutants, indicated in Fig. 1A. In a related experiment, HCT116 cells transfected with GRP58 mutant M5 was treated with either DMSO or MG132 (20 μ M) for 8 hours. 24h after transfection, cells were harvested, total cell lysates (TCL), cytosolic (C), nuclear (N) and endoplasmic reticulum (ER) fractions were made and analyzed by Western blotting. The immunoblots were stripped and re-probed with anti-lactate dehydrogenase (LDH, cytosolic), anti-lamin B (nuclear) and anti-calnexin (microsomal) antibodies to confirm purity of fractionation. Same experiment with similar results was also done in Hepa-1 cells (data not shown). (D/E). Effect of deletion/mutation of the putative NLS sequence on nuclear localization of GRP58. HCT116 and Hepa-1 cells were transfected with pcDNA-V5 plasmids for GRP58-WT or GRP58- Δ NLS mutant (D) or GRP58-NLS K-A mutant (E) in separate experiments. The cells were harvested 24h post-transfection, cytosolic and nuclear fractions were made and analyzed by Western blotting with anti-V5 antibody. The same blots were also re-probed with anti-LDH and anti-laminB antibodies to show purity of fractionation.

Fig. 2.

Immunohistochemical analysis. (A). Immunofluorescence localization of GRP58-WT, GRP58-NLS mutant proteins in Hepa-1 cells. Cells were transfected with pcDNA-V5 plasmids encoding GRP58-WT or GRP58- Δ NLS mutant or GRP58-NLS K-A mutant proteins. After 24h, cells were fixed, permeabilized and indirect immunostaining was performed with anti-V5-FITC antibody to visualize localization of GRP58 wild type and mutant proteins under fluorescence microscope and then counterstained with DAPI to show the nucleus. (B). Nuclear localization activity/potential of GRP58-NLS sequence. Hepa-1 cells were transfected with plasmids encoding green fluorescent protein (GFP) alone or GFP fused to GRP58-NLS. GFP fluorescence was visualized in the cells at 24h post-transfection by fluorescence microscopy. DAPI staining was also done to visualize the nucleus.

Fig. 3. Effect of individual amino acid mutation in GRP58 NLS sequence on subcellular localization of the protein. GRP58 wild type and alanine substitution mutations of individual amino acids in GRP58 NLS sequence were transfected and total cell lysate (TCL) or cytosolic, nuclear and ER fractions were generated. 50µg of cellular fractions were used for SDS-PAGE, immunoblotted with anti-V5 antibody. The blots were stripped and re-probed with sucellular specific antibodies.

Fig. 4.

ER retention of GRP58. (A). Effect of deletion of QEDL sequence on ER localization of GRP58. HCT116 and Hepa-1 cells in separate experiments were transfected with pcDNA-GRP58-WT-V5 or pcDNA-GRP58- Δ ER-V5. Cytosolic, nuclear and ER fractions were made and analyzed by Western blotting and probing with anti-V5 antibody. (B). Requirement of both NLS and ER retention (QEDL) sequences for ER retention of GRP58. NLS and putative ER retention (QEDL) sequences of GRP58 were both deleted (GRP58 NLS/ER DD) or alanine substituted (GRP58 NLS/ER DM) and cloned in pcDNA-V5 expression plasmid. HCT116 cells were transfected with GRP58-WT, GRP58 NLS/ER DD, GRP58 NLS/ER DM and GRP58- Δ ER plasmids. After 24h, cells harvested and subcellular fractions (cytosol, nuclear and ER) were made and analyzed by immunoblotting with anti-V5, anti-LDH, anti-laminB, anti-calnexin and anti- β -actin antibodies. Similar results were observed in Hepa-1 cells (data not shown).