MECHANISMS OF TRANSCRIPTIONAL REGULATION OF CAT-1 GENE
EXPRESSION BY ENDOPLASMIC RETICULUM (ER) STRESS

by

YI LI

Submitted in partial fulfillment of the requirements
For the degree of Doctor of Philosophy

Dissertation Advisor: Dr. Maria Hatzoglou

Department of Nutrition

CASE WESTERN RESERVE UNIVERSITY

May, 2009
We hereby approve the thesis/dissertation of

Yi Li

______________________________________________________
candidate for the Ph.D. degree *.

Jonathan Whittaker, M.D.
(signed)_______________________________________________
(chair of the committee)

Martin Snider, Ph.D.

Stephen Previs, Ph.D.

Maria Hatzoglou, Ph.D.

March 19, 2009
(date) _______________________

*We also certify that written approval has been obtained for any proprietary material contained therein.
This work is dedicated to my parents and brother,

for their love and support through the years during my study.
TABLE OF CONTENTS

Dedication iii
Table of Contents iv
List of Tables ix
List of Figures x
Acknowledgements xiii
List of Abbreviations xv
Abstract xix

CHAPTER 1: INTRODUCTION

Cationic amino acid transporters 1
Functions of the CAT-1 protein 5
The functions of amino acids in mammalian cells 6
Gene expression in eukaryotic cells 8
Transcriptional regulation of gene expression by amino acid starvation 11
Regulation of cat-1 gene expression by amino acid starvation 13
Transcriptional regulation of cat-1 gene expression by amino acid starvation 15
Endoplasmic reticulum (ER) stress and unfolded protein response (UPR) 17
Transcriptional regulation of gene expression by ER stress 26
ER stress and human diseases 29
Rationale of the thesis research 31

CHAPTER 2: EIF2α PHOSPHORYLATION IS REQUIRED FOR INDUCTION OF CAT-1 GENE TRANSCRIPTION MEDIATED BY ATF4 AND XBP1S
DURING ER STRESS

INTRODUCTION 33

MATERIALS AND METHODS 35

Plasmid constructs 35

Cell culture and DNA transfections 35

Chromatin immunoprecipitation (ChIP) assay 36

Northern blot analysis 37

Western blot analysis 37

Reverse transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR) 38

RESULTS 41

Cat-1 mRNA levels are induced by ER stress

Induction of cat-1 gene transcription during ER stress is mediated by ATF4 and XBP1s 44

ATF4 and XBP1s act via independent cis-acting DNA elements within the cat-1 gene promoter/regulatory region to induce cat-1 gene transcription during ER stress 48

Phosphorylation of the translation initiation factor eIF2α is required for induction of cat-1 gene transcription by ER stress 52

Induction of XBP1s is largely dependent on eIF2α-P but not ATF4 during ER stress 53

Proteasome-mediated degradation is involved in XBP1s turnover during ER stress 58

eIF2α phosphorylation is partially required for XBP1 mRNA splicing
during ER stress

XBP1 expression during ER stress may help cells to survive during ER stress

DISCUSSION

CHAPTER 3: C/EBPβ LAP ISOFORM ACTIVATES AND LIP ISOFORM INHIBITS CAT-1 GENE TRANSCRIPTION VIA THE CIS-ACTING ELEMENT AARE DURING ER STRESS

INTRODUCTION

MATERIALS AND METHODS

Plasmid constructs

Cell culture and DNA transfections

Western blot analysis

Cell viability assay

Electrophoretic mobility shift assay (EMSA)

RESULTS

C/EBPβ LIP is up-regulated at late stage of ER stress and amino acid starvation

C/EBPβ LAP activates cat-1 promoter activity and LIP inhibits ER stress-induced cat-1 promoter activity

C/EBPβ LIP inhibits ATF4-induced and the LAP activates expression of a reporter containing the cat-1 AARE in the enhancer site

C/EBPβ isoforms bind to the cat-1 AARE element in vitro

C/EBPβ LIP mediates better survival of cells during ER stress

DISCUSSION

CHAPTER 4: DIFFERENTIAL CONTROL OF THE C/EBPβ PRODUCTS LAP
AND LIP AND THE REGULATION OF GENE EXPRESSION DURING THE
RESPONSE TO ER STRESS

INTRODUCTION 102

MATERIALS AND METHODS 106

Plasmid constructs 106

Cell culture and DNA transfections 106

Animal studies 107

Western blot analysis 107

Polysome profiles and RNA isolation 108

Metabolic labeling and immunoprecipitation 108

DNA affinity pulldown 109

Quantitative real time RT-PCR (qRT-PCR) 110

RESULTS 113

Differential regulation of the transcriptional activator LAP and transcriptional
repressor LIP during ER stress 113

LAP and LIP are degraded by the proteasome during ER stress 118

LIP levels increase during amino acid starvation but the LIP/LAP
ratio shows a small change 128

LIP attenuates transcription mediated by ATF4 but not XBP1 or ATF6 128

C/EBPβ attenuates the induction of gene expression mediated by the
PERK-ATF4 pathway but not the XBP1/ATF6 pathway during ER stress 134

C/EBPβ−/− MEF cells have lower levels of GADD34 and CHOP proteins
and reduced eIF2 dephosphorylation 138
LIST OF TABLES

Table 1-1. Epithelial amino acid transport systems and their mediators 3
Table 2-1. DNA primers used in polymerase chain reaction (PCR) reverse transcription PCR (RT-PCR) and quantitative RT-PCR (qRT-PCR) 40
Table 4-1. Primers used for qRT-PCR 112
LIST OF FIGURES

Figure 1-1. Distinct components of the unfolded protein response (UPR) mediated by each of the sensors resident in the endoplasmic reticulum (ER) 19

Figure 1-2. Principal architecture of the ER stress sensors IRE1, PERK, and ATF6 21

Figure 2-1. Cat-1 mRNA levels are induced by ER stress 43

Figure 2-2. Induction of cat-1 gene transcription during ER stress is mediated by ATF4 and XBP1s 47

Figure 2-3. ATF4 and XBP1s act via independent cis-acting DNA elements within the cat-1 gene promoter region to induce cat-1 gene transcription during ER stress 51

Figure 2-4. Phosphorylation of the translation initiation factor eIF2α is required for induction of cat-1 gene transcription by ER stress 55

Figure 2-5. Induction of XBP1s is largely dependent on eIF2α-P but not ATF4 during ER stress 57

Figure 2-6. Proteasome-mediated degradation is involved in XBP1s turn over during ER stress 60

Figure 2-7. eIF2α phosphorylation is partially required for XBP1 mRNA splicing during ER stress 63

Figure 2-8. XBP1 gene expression during ER stress may help cells to survive during ER stress 66

Figure 3-1. Amino acid sequence of the full length C/EBPβ and the predicted translation start sites 76

Figure 3-2. C/EBPβ LIP is up-regulated at late stage of ER stress and
amino acid starvation  

Figure 3-3. C/EBPβ LAP activates cat-1 promoter activity and LIP inhibits ER stress induced cat-1 promoter activity  

Figure 3-4. Both C/EBPβ LAP activates the heterogeneous SV40 promoter and LIP inhibits ATF4 induced SV40 promoter via the cat-1 AARE  

Figure 3-5. C/EBPβ isoforms bind to the cat-1 AARE element in vitro  

Figure 3-6. C/EBPβ LIP functions to help cells to survive during ER stress  

Figure 3-7. Predicted interaction between the leucine zipper domain of C/EBPβ isoforms and the leucine zipper domain of another ER stress involved bZIP transcription factor ATF4, XBP1s, or ATF6  

Figure 4-1. Differential regulation of LIP and LAP levels during ER stress  

Figure 4-2. Differential regulation of LIP and LAP levels during ER stress by inhibitors of the proteasome and protein synthesis  

Figure 4-3. Decreased LIP levels during early ER stress require eIF2 phosphorylation and an active proteasome  

Figure 4-4. The C/EBPβ mRNA associates with heavy polysomes during prolonged ER stress  

Figure 4-5. Amino acid starvation induces LIP and LAP levels but has a small effect on the LIP/LAP ratio  

Figure 4-6. LIP attenuates induction of transcription by ATF4 via the cat-1 AARE enhancer element  

Figure 4-7. Induction of stress-response proteins in C/EBPβ+/+, C/EBPβ−/−, and C/EBPβ−/+ (LIP) MEFs
Figure 4-8. C/EBPβ differentially regulates expression of genes in the PERK/ATF4 pathway during ER stress 140

Figure 4-9. Attenuation of cat-1 gene transcriptional activation in C/EBPβ−/− (LIP) cells 143

Figure 4-10. LIP modulates the PERK/ATF4-mediated gene expression program during ER stress 152
ACKNOWLEDGEMENTS

First, I am very grateful to my advisor, Dr. Maria Hatzoglou, for her great mentorship and outstanding scientific training guidance during the time of my study. I appreciate her patience to instruct my studies and her support to provide me a wonderful study and research environment. Her scientific enthusiasm has been always inspired me and it will be in the future. I will always remember her wonderful and critical lab meeting discussion, which helped me to develop my critical thinking and scientific ideas. Her kindness and support extended beyond scientific training, I still remember that she spent so much time to help me on my writing and oral presentation skills.

I appreciate Dr. Martin Snider for his useful discussions and suggestions on my projects, and I appreciate his intellectual contributions and help in preparation of our manuscripts.

I appreciate Dr. Colleen Croniger for her help with animal studies.

I would like to express my gratitude to my advisory committee members Drs. Martin Snider, Stephen Previs, and Jonathan Whittaker for their valuable time and suggestions.

I am thankful to my friends and colleagues in the Hatzoglou’s Lab Elena Bevilacqua, Calin-Bogdan Chiribau, Mithu Majumder, Francesca Gaccioli, Chuanping Wang, Charlie Huang, Manas Maity, Celvie Yuan, Dawid Krokowski, and Zachary Wilkerson. I am
grateful to their commitment, help, and their friendship. Without the research environment they have contributed, it would not be possible to finish the studies presented in this thesis. I would especially like to thank Elena Bevilacqua, Calin-Bogdan Chiribau, Mithu Majumder, and Chuanping Wang for their experimental contributions to some parts of the projects presented in this thesis.

Finally, I am especially grateful to my parents and brother. Their love, understanding, and support during the years have made it possible for me to finish the studies presented in this thesis.

NOTE: Chapter 4 of this thesis has been published previously and presented in its entirety (Li et al., 2008). Experimental contributions by the collaborators are cited in the figure legends.
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4E-BP1</td>
<td>eIF4E binding protein 1</td>
</tr>
<tr>
<td>AARE</td>
<td>amino acid response element</td>
</tr>
<tr>
<td>ARE</td>
<td>AU-rich element</td>
</tr>
<tr>
<td>AS</td>
<td>asparagine synthase</td>
</tr>
<tr>
<td>ATF</td>
<td>activating transcription factor</td>
</tr>
<tr>
<td>BCL2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>BiP</td>
<td>immunoglobulin heavy chain-binding protein</td>
</tr>
<tr>
<td>bZIP</td>
<td>basic leucine zipper transcription factor</td>
</tr>
<tr>
<td>CAT</td>
<td>chloramphenicol acetyl transferase</td>
</tr>
<tr>
<td>CAT-1</td>
<td>cationic amino acid transporter-1</td>
</tr>
<tr>
<td>cat-1</td>
<td>cat-1 gene</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
</tr>
<tr>
<td>CDKs</td>
<td>cyclin-dependent kinases</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT/enhancer binding protein</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CHOP</td>
<td>CEBP homologous protein</td>
</tr>
<tr>
<td>CHX</td>
<td>cycloheximide</td>
</tr>
<tr>
<td>CS</td>
<td>calf serum</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ecoR</td>
<td>receptor for the ecotropic murine leukemia virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>EDEM</td>
<td>ER degradation enhancing alpha mannosidase-like</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>eIF2α</td>
<td>eukaryotic translation initiation factor 2α</td>
</tr>
<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERSE</td>
<td>endoplasmic reticulum stress responsive element</td>
</tr>
<tr>
<td>ERAD</td>
<td>endoplasmic reticulum associated degradation</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GADD34</td>
<td>growth arrest and DNA damage protein 34</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GCN2</td>
<td>general control non-derepressable 2 protein</td>
</tr>
<tr>
<td>GCN4</td>
<td>general control non-derepressable 4 protein</td>
</tr>
<tr>
<td>GLS</td>
<td>Golgi localization sequence</td>
</tr>
<tr>
<td>GRP78</td>
<td>glucose regulated protein 78</td>
</tr>
<tr>
<td>HATs</td>
<td>heteromeric amino acid transporters</td>
</tr>
<tr>
<td>HDAC1</td>
<td>histone deacetylase 1</td>
</tr>
<tr>
<td>hnRNP</td>
<td>heterogeneous nuclear ribonuclear protein</td>
</tr>
<tr>
<td>HRI</td>
<td>heme-regulated inhibitor</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>insulin-like growth factor binding protein-1</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-d-thiogalactopyranoside</td>
</tr>
<tr>
<td>IRE1</td>
<td>inositol-requiring enzyme 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IRES</td>
<td>internal ribosome entry site</td>
</tr>
<tr>
<td>LAP</td>
<td>liver-enriched transcriptional activating protein</td>
</tr>
<tr>
<td>LIP</td>
<td>liver-enriched transcriptional inhibitory protein</td>
</tr>
<tr>
<td>LUC</td>
<td>luciferase</td>
</tr>
<tr>
<td>MEFs</td>
<td>mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NSRE</td>
<td>nutrient sense responsive element</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PERK</td>
<td>PKR-like endoplasmic reticulum kinase</td>
</tr>
<tr>
<td>PI3 Kinase</td>
<td>phosphoinositide 3 kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PKR</td>
<td>RNA-activated protein kinase</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative PCR</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative RT-PCR</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>uORF</td>
<td>upstream ORF</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>UPR</td>
<td>unfolded protein response</td>
</tr>
<tr>
<td>UPRE</td>
<td>unfolded protein responsive element</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>------</td>
<td>-------------</td>
</tr>
<tr>
<td>SLC7</td>
<td>solute carrier family 7</td>
</tr>
<tr>
<td>SNAT2</td>
<td>sodium-coupled neutral amino acid transporter</td>
</tr>
<tr>
<td>Tg</td>
<td>thapsigargin</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane</td>
</tr>
<tr>
<td>TRB3</td>
<td>pseudokinase tribble 3</td>
</tr>
<tr>
<td>Tu</td>
<td>tunicamycin</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>XBP1</td>
<td>x-box binding protein 1</td>
</tr>
</tbody>
</table>
Mechanisms of Transcriptional Regulation of Cat-1 Gene Expression
by Endoplasmic Reticulum (ER) Stress

Abstract
by
YI LI

As a member of cationic amino acid transporter (CAT) family proteins, CAT-1 mediates the bidirectional transport of essential amino acids arginine and lysine by facilitated diffusion. Cat-1 gene expression is modulated by stimuli including endoplasmic reticulum (ER) stress, availability of nutrients, growth factors, and hormones. In mammalian cells, ER stress triggers 3 distinct signaling pathways initiated by the 3 ER membrane-associated sensor proteins PERK, IRE1, and ATF6. It is shown here that cat-1 mRNA level is induced to about 5 fold at the highest level in C6 cells during ER stress. Transcriptional induction of cat-1 by ER stress is mediated by two transcription factors ATF4 and XBP1s. ATF4 interacts with the cis-acting element AARE and XBP1s interacts with the putative cis-acting element ERSE-II to induce cat-1 gene transcription at early stage of ER stress. The sequential binding of XBP1s after ATF4 to the cat-1 promoter during ER stress may be responsible for sustained induction of cat-1 mRNA levels during ER stress. eIF2α phosphorylation is required for induction of cat-1 transcription by ER stress due to induction of ATF4 entirely and induction of XBP1s largely requires eIF2α phosphorylation. Because eIF2α phosphorylation is required for ATF4 mRNA translation as described in the literature and sufficient XBP1 mRNA
splicing showing here. This indicates that there is a crosstalk between the PERK-eIF2α-ATF4 and the IRE1-XBP1 pathways. At the late stage of ER stress, C/EBPβ LIP isoform down-regulates cat-1 gene transcription by specifically suppressing the induction mediated by AARE via binding of ATF4. Transcriptional induction of other two ATF4 induced genes SNAT2 and AS is also suppressed by C/EBPβ LIP during ER stress. The down-regulation of C/EBPβ LIP level at the early stage of ER stress is mediated by proteasome-mediated degradation and eIF2α phosphorylation. The dramatic increase of C/EBPβ LIP at late staged of ER stress is caused by increased mRNA translation and stability of the protein. Physiologically, LIP induction may help cells to survive during ER stress. In our knowledge, the crosstalk between the PERK-eIF2α-ATF4 and the IRE1-XBP1 pathways of the 3 UPR signaling pathways is being demonstrated here at the first time.
CHAPTER 1
INTRODUCTION

Cationic amino acid transporters

In eukaryotic cells, amino acid transporters have been categorized in systems depending on their biochemical characteristics of transport. One system stands for a group of plasma membrane proteins which transport amino acids with similar characteristics in a variety of cell types (White and Christensen, 1982; Broer, 2008). Amino acid transport has been studied in many different epithelial cells. However more advanced knowledge has been obtained from kidney and intestinal tissues (Broer, 2008). There are many different systems that mediate amino acid transport to these two tissues, such as: System A, System B\(^0\), System B\(^{0+}\), System b\(^{0+}\), System L, System N, System T, System y\(^+\), System y\(^+\)L, etc. (Table 1-1; Broer, 2008). Cationic amino acids are transported via System y\(^+\), y\(^+\)L and b\(^{0+}\) (Broer, 2008). System y\(^+\) amino acid transporters have saturable, sodium ion-independent transport activity with high specificity and affinity for cationic L-amino acids. They mediate transport of the substrates across the membrane in both directions and transport increases by trans-stimulation (White et al., 1982). Trans-stimulation involves increased transporter activity by amino acids at the opposite side of the membrane.

The cationic amino acid transporters (CAT) and the glycoprotein-associated amino acid transporters (gpaAT) mediate transport of cationic amino acids (Hatzoglou et al., 2004; Verrey et al., 2004). These transporters belong to the solute carrier family 7 (SLC7) (Closs et al., 2006).
Table 1-1. Epithelial amino acid transport systems and their mediators. (from Broer, 2008; used with permission)
### TABLE 1-1

**Epithelial amino acid transport systems and their mediators**

<table>
<thead>
<tr>
<th>System</th>
<th>cDNA</th>
<th>SLC</th>
<th>Amino Acid Substrates</th>
<th>Analogous</th>
<th>Affinity</th>
<th>Mechanisms</th>
<th>Ions</th>
<th>Expression*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASC</td>
<td>SMAT4</td>
<td>SLC38A4</td>
<td>G,A,S,A,C,Q,N,M,AA⁺</td>
<td>MeAID</td>
<td>Medium</td>
<td>S</td>
<td>Na⁺</td>
<td>K</td>
</tr>
<tr>
<td>B⁺</td>
<td>ASCT1</td>
<td>SLC1A4</td>
<td>A,S,C</td>
<td>Cysteic acid</td>
<td>High</td>
<td>A</td>
<td>Na⁺</td>
<td>K</td>
</tr>
<tr>
<td>B⁺⁺</td>
<td>ASCT2</td>
<td>SLC1A5</td>
<td>A,S,C,T,O</td>
<td>Cysteic acid</td>
<td>High</td>
<td>A</td>
<td>Na⁺</td>
<td>K</td>
</tr>
<tr>
<td>L</td>
<td>LAT1</td>
<td>SLC3A2</td>
<td>AA⁺⁺</td>
<td>BCH</td>
<td>Low</td>
<td>A</td>
<td>Na⁺</td>
<td>K</td>
</tr>
<tr>
<td>L</td>
<td>LAT2</td>
<td>SLC3A1</td>
<td>AA⁺⁺</td>
<td>BCH</td>
<td>High</td>
<td>S</td>
<td>Na⁺⁺</td>
<td>I (AM)</td>
</tr>
<tr>
<td>IMINO</td>
<td>LAT3</td>
<td>SLC3A1</td>
<td>AA⁺⁺</td>
<td>BCH</td>
<td>High</td>
<td>S</td>
<td>Na⁺⁺</td>
<td>K (AM)</td>
</tr>
<tr>
<td>Glu</td>
<td>XCT1</td>
<td>SLC3A1</td>
<td>AA⁺⁺</td>
<td>BCH</td>
<td>High</td>
<td>S</td>
<td>Na⁺⁺</td>
<td>K (AM)</td>
</tr>
<tr>
<td>N</td>
<td>LAT5</td>
<td>SLC3A1</td>
<td>AA⁺⁺</td>
<td>BCH</td>
<td>High</td>
<td>A</td>
<td>Na⁺⁺</td>
<td>K (AM)</td>
</tr>
<tr>
<td>PAT1 (trometamol)</td>
<td>LAT1</td>
<td>SLC3A2</td>
<td>AA⁺⁺</td>
<td>BCH</td>
<td>High</td>
<td>A</td>
<td>Na⁺⁺</td>
<td>K (AM)</td>
</tr>
<tr>
<td>PAT2</td>
<td>SLC3A2</td>
<td>P,G,A</td>
<td>MeAIB</td>
<td>Medium</td>
<td>A</td>
<td>K</td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ⁺⁺⁺⁺</td>
<td>LAT1</td>
<td>SLC3A2</td>
<td>P,G,A</td>
<td>MeAIB</td>
<td>Medium</td>
<td>A</td>
<td>K</td>
<td></td>
</tr>
<tr>
<td>LAT2</td>
<td>SLC3A2</td>
<td>P,G,A</td>
<td>MeAIB</td>
<td>Medium</td>
<td>A</td>
<td>K</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*NB: not reported; A, antiport; AA⁺⁺, neutral amino acids; AA⁺⁺, cationic amino acids; U, unport; S, symport; S-A-A⁺⁺, symport together with neutral amino acids; K, kidney; 1, intestine; A5, apical membrane; BM, basolateral membrane; Ub, ubiquitous. Amino acids are given in one-letter codes. O, ornithine; H, histidine. Affinity, high, >100 μM, medium, 100 μM to 1 mM, low, <1 mM. Expression in epithelial cells of kidney and intestine.
SLC7A1-4 are the cationic amino acid transporters. SLC7A1, SLC7A3, and SLC7A4 are CAT-1, CAT-3, and CAT-4, respectively. There are two splicing variants for SLC7A2 or CAT-2, which are CAT-2A and CAT-2B that only differ in a stretch of 42 amino acids (Verrey et al., 2004). CAT-1, CAT-2A, CAT-2B, and CAT-3 are more closely related to each other, with about 60% identity among them and about 40% identity with CAT-4. CAT proteins have 14 putative transmembrane (TM) domains, with some extracellular domains being glycosylated (Verrey et al., 2004). The CAT1-3 members of the CAT family mediate transport of cationic amino acids by facilitated diffusion (exchangers) in a Na\(^+\)-independent manner and compose the y\(^+\) System (Verrey et al., 2004). It seems that CATs play the major role for cationic amino acids to entry most mammalian cells, but cationic amino acids are also transported by other systems (Closs et al., 2006, White and Christensen, 1982). CAT-1 has high affinity for substrates and is subject to trans-stimulation. CAT-2A has a relative low substrate affinity and is not sensitive to trans-stimulation (Closs et al., 2006). CAT-1 is ubiquitously expressed in rat and mouse tissues except the adult liver (Hatzoglou et al., 2004). The other CATs show tissue specific expression (Hatzoglou et al., 2004).

SLC7A5-11 are the gpaATs or the light chain of the heteromeric amino acid transporters (HATs). These proteins have 12 TM domains and are not glycosylated. In order to be functional, they have to be associated with the glycosylated heavy chains to form HATs (Verrey et al., 2004). HATs have a broad range of selectivity for substrates. They mediate transport of large and small neutral amino acids, negatively charged amino acids, and cationic amino acids (Verrey et al., 2004).
**Functions of the CAT-1 protein**

The cat-1 cDNA was initially identified in searching for the gene encoding a novel cell membrane receptor that mediates murine type ecotropic retrovirus infection, so the protein was initially named ecoR (ecotropic retrovirus receptor; Albritton et al., 1989; Christensen, 1992). The predicted protein of 622 amino acids from the cloned cDNA was known to be hydrophobic, with 14 predicted membrane-spanning domains (Christensen, 1992). Besides its function as a virus receptor, the studies that followed this discovery indicated that the ecoR is the principal cationic amino acid transporter CAT-1 in mammalian cells, that belongs to the System y+ of amino acid transporters (Wang et al., 1991; Weiss, 1984; Handelin and Kabat, 1985; Christensen, 1989; White, 1985). In most non-epithelial cells, the major entry pathway for cationic amino acids is mediated by System y+ activity by members of the CATs family (Closs, 2002). CAT-1 was the first System y+ amino acid transporter cloned and characterized at the molecular level (Hatzoglou et al., 1995; Hatzoglou et al., 2004; White and Christensen, 1982).

CAT-1 may also play a role in cell proliferation. It was observed that expression of the cat-1 gene and its human homolog H13 gene is enhanced in proliferating cells (Yoshimoto et al., 1992). Studies have demonstrated that the levels of CAT-1 affects the amount of intracellular nitric oxide (NO) synthesized by inducible nitric oxide synthase (iNOS), which might in turn affect cell proliferation (Schnorr et al., 2005). In psoriatic skin cells, overexpressed arginase-1 leads to up-regulation of cat-1 gene expression, which is due to lowered intracellular L-arginine levels and limited NO synthesis at physiological L-arginine concentrations (Schnorr et al., 2005). In bovine aortic epithelial cells, CAT-1 directly interacts with eNOS, which enhances NO production with a
mechanism independent of the amino acid transport function of CAT-1 (Li et al., 2005). CAT-1 may also affect intracellular arginine hydrolysis, since transport of arginine into hepatocytes is the rate-limiting step for its hydrolysis by arginase (White and Christensen, 1982). It has also been demonstrated that platelet-derived growth factor induced vascular smooth muscle cell proliferation by stimulating polyamine synthesis is controlled by the levels of CAT-1 and CAT-2B, which provide the cells with the necessary intracellular precursor for polyamine synthesis (Durante et al., 1996). So it is possible that CAT-1 may also function as a signal molecular besides its function as a amino acid transporter and a receptor mediating virus infection.

The functions of amino acids in mammalian cells

In eukaryotic cells, amino acids have many different functions. One important function of amino acids is the role of serving as substrates for protein synthesis. Amino acids are the basic structural units of proteins. They are linked by peptide bonds to form linear chains called polypeptides, which are the components of proteins. Amino acids can be precursors for synthesis of other compounds. For example, tryptophan is a precursor for a neurotransmitter and arginine is a precursor for nitric oxide (Farfournoux et al., 2000). Amino acids can also function as signaling molecules to regulate protein synthesis and turnover (Kilberg et al., 1994).

Among all of these functions of amino acids, the function as substrates for protein synthesis is more closely related with regulation of gene expression. Of all the naturally occurring amino acids, the 20 amino acids normally involved in protein synthesis are: alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine,
tryptophan, tyrosine, and valine, These 20 regular amino acids are encoded by standard genetic codes for protein biosynthesis, so they are called standard amino acids. Nonstandard amino acids are often found as intermediates in metabolic pathways (Curis et al., 2005). Two of the nonstandard amino acids are specified by genetic codes. Selenocysteine is encoded by the stop codon UGA and pyrrolysine is encoded by the stop codon UAG on some occasions (Krzycki, 2005).

From a nutritional perspective, amino acids are traditionally classified as indispensable (essential) and dispensable (nonessential) amino acids (Reeds, 2000). For humans, there are 8 essential amino acids (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine). These amino acids can’t be synthesized from other compounds in the human body at the necessary levels to support normal growth (Young, 1994). The essential amino acids have to be taken from diet. The rest of the amino acids are called nonessential amino acids (Reeds, 2000). The classification for essential and nonessential is not absolute. Some nonessential amino acids can become essential amino acids and are called conditionally essential amino acids. For example, proline is essential for burned patients (Jaksic et al., 1991). Normally, cysteine, tyrosine, histidine, and arginine are nonessential amino acids for adults, but are essential amino acids for children (Reeds, 2000), since the metabolic pathways for synthesis of these amino acids are not fully developed in children (Imura and Okada, 1998). Evidence has suggested that newly synthesized conditional amino acids may be only used within the cells synthesized (Miller et al., 1996). However from a metabolic view, there are only 3 essential amino acids (lysine, threonine, and tryptophan) and 2 nonessential amino acids (glutamate and serine). From a functional view, every amino acid is essential for its function (Reeds,
According to the chemical and physical properties of their side chains (R chain), amino acids are classified in four groups: neutral-nonpolar, neutral-polar, acidic, and basic. These properties are important for transport and solubility. Polar amino acids are more hydrophilic and nonpolar amino acids are more hydrophobic. At the protein level, the properties of amino acids determine solubility of proteins, and affect protein structure and protein-protein interactions (Lodish et al., 2007). The cationic amino acids are lysine, arginine, and histidine. Lysine and arginine are the main substrates for CAT-1 (Hatzoglou et al., 2004).

**Gene expression in eukaryotic cells**

Gene expression is the process of synthesis of molecules of RNA and protein that are necessary for cellular functions based on inherited genetic information stored in DNA sequences. For the protein-encoding genes or class II genes, gene expression includes a two-step process, transcription and translation. The first step is the synthesis of RNA from a DNA template that is called transcription, and the second step is the synthesis of protein from an mRNA template that is called translation (Lodish et al., 2007).

Transcription occurs in the nucleus in eukaryotic cells. RNA polymerase, transcription factors, and promoter are the main players in transcription of a gene. RNA polymerase is a multi-subunit protein complex. Three different kind of RNA polymerase has been found in eukaryotic cells. RNA polymerase I (Pol I) transcribes rRNA genes to produce the precursor RNAs for 28S, 18S, and 5.8S ribosomal RNAs (rRNA). RNA polymerase II (Pol II) transcribe protein-coding and snRNA genes. RNA polymerase III (Pol III) transcribes all transfer RNA (tRNA) genes and 5S rRNA genes (Spencer and Groudine,
Transcription factors other than the RNA polymerase normally include general and specific transcription factors. General transcription factors are the transcription factors that directly interact with the RNA polymerase, and form the basal transcription apparatus that is required to initiate transcription. Specific transcription factors are the factors that interact directly or indirectly through coactivators with the basal transcription apparatus (Lodish et al., 2007). Specific transcription factors are usually subject to regulation and have positive or negative effects on transcription by binding specific DNA elements within the promoter region or other regions of the relevant genes (Lodish et al., 2007). The promoter is the transcriptional regulatory sequences of a gene. It contains binding sites for the RNA polymerase and the transcription factors required for basal transcription, normally located upstream within 200 base pair (bp) of the transcription start site. For a class II gene, the basal promoter is the region within 40 bp from the transcription start site that contains an initiator (Inr) \[5'-(C/T)(C/T)AN(A/T)(C/T)(C/T)-3']\ and the TATA box \[5'-TATA(A/T)A(A/T)-3']\ for a promoter with a TATA box, the Inr is normally located between -3 and +5, and the TATA box is normally located at -27 of the transcription start site. The basal promoter is bound by general transcription factors (Lodish et al., 2007).

To initiate transcription, the transcription factors have to sequentially bind to the promoter region to recruit the polymerase to the Inr. TFIID is responsible for recognition of a promoter to initiate binding of other transcription factors and the polymerase. TFIID contains about 15 subunits, in which one subunit is the TATA box binding protein (TBP) that recognizes and binds the TATA box, and other subunits are all TBP associated factors (TAFs). The TAFs are recruited to the promoter after binding of TBP. The
formation of a transcription complex starts by binding of TFIID to the TATA box, TFIIA then binds to TFIID, and the next step is the binding of TFIIB downstream of the TATA box. The binding of TFIIB may provide the surface for polymerase binding and initiation of transcription (Lodish et al., 2007).

Gene expression can be regulated at the levels of transcription. Inducible factors and transcription factors binding to enhancers and silencers can either positively or negatively regulate gene expression. Enhancers and silencers are located upstream, downstream, or in the middle of the genes to control their expression (Lodish et al., 2007). The exact mechanisms of enhancement or repression by enhancers or silencers are not clear. However, it has been visually demonstrated by electronic microscopy that the bovine papillomavirus type 1 (BPV-1) enhancer protein E2 binding on an enhancer site directly interacts with the Sp1 protein binding on a Sp1 binding site (5’-GGGCCGG-3’) on an artificial DNA molecule (Li et al., 1991).

mRNA stability can also be regulated. For example, mRNAs can be stabilized by binding of protein factors or destroyed by binding of exactly matched micro RNAs (miRNAs) or small interfering RNAs (siRNAs) at their 3’ UTRs (Jacobson, 2004; Fred and Welsh, 2009).

Translation is conducted by the ribosome in the cytosol or attached to the ER membranes. Most mRNAs are translated via ribosome scanning mechanism following binding of eIF4F (eIF4F consists of eIF4E, eIF4G, and eIF4A) to the mRNA 5’-m7G cap structure (Komar and Hatzoglou, 2005). The 43S complex composed of the 40S subunit, the eIF2•GTP•Met-tRNA ternary complex, and eIF3 are recruited to the cap, which leads to the formation of the 48S complex by recruiting eIF1A. This is followed by scanning in
a 5' to 3' direction to search for the AUG codon and recruitment of the 60S ribosomal subunit (Komar and Hatzoglou, 2005). Translation initiation involves formation of the 80S initiation complex at the AUG translation start codon of specific mRNAs. The internal ribosome entry site (IRES)-mediated mechanism is initiated by recruitment of the 40S directly to the IRES. This is followed by scanning for the AUG codon by of 40S and the joining by 60S to form 80S (Komar and Hatzoglou, 2005). In some stress conditions, eIF2α is phosphorylated, which decreases the concentration of active ternary complexes and therefore decreases global rates of protein synthesis (Fernandez et al., 2002b). The IRES-mediated initiation mechanism may be used to initiate translation for some mRNAs under stressed conditions (Fernandez et al., 2002a; Yaman et al., 2003; Hatzoglou et al., 2004).

Translation can be regulated as well. For example, different translation initiation mechanisms can be used under different conditions (Hatzoglou et al., 2004). Translation can be repressed by binding of partial matched miRNA or siRNA. For a particular gene, transcription can be regulated at one or more levels. Gene expression can be measured by checking the corresponding RNA or protein levels.

**Transcriptional regulation of gene expression by amino acid starvation**

Amino acid starvation leads to eukaryotic initiation factor 2α (eIF2α) phosphorylation and subsequent inhibition of general protein synthesis by suppressing translation initiation (Hinnebusch, 1994; Fafournoux et al., 2000). Different stresses including amino acid starvation can induce eIF2α phosphorylation by activating different kinases (Wek et al., 2006). During amino acid starvation, the protein kinase, general control non-derepressible-2 (GCN2), is activated to phosphorylate eIF2α (Wek et al., 1995). GCN2 is
activated by direct interaction of its histidyl-tRNA synthetase-related sequences with the uncharged tRNA accumulated during amino acid starvation (Wek et al., 1995). GCN2 can also be activated by UV irradiation and proteasome inhibition (Wek et al., 2006). Besides GCN2, other kinases that phosphorylate eIF2α are PKR-like ER kinase (PERK)/pancreatic eukaryotic initiation factor 2α kinase (PEK), RNA-dependant protein kinase (PKR) and heme-regulated inhibitor (HRI). PERK is activated by endoplasmic reticulum (ER) stress, PKR is activated by virus infection, and HRI is activated by heme deprivation, oxidative stress, and heat shock (Wek et al., 2006). The central event caused by eIF2α phosphorylation is increase of the transcription factor ATF4, which is integral to the eIF2 kinase pathways (Wek et al., 2006). ATF4 induces transcription of many genes, such as asparagine synthetase (AS), C/EBPβ homologous protein gene (CHOP, or GADD153) and insulin-like growth factor binding protein-1 (IGFBP-1) (Siu et al., 2002; Ma et al., 2002; Averous et al., 2004; Fernandez et al., 2003; Marchand et al., 2006). (Regulation of cat-1 gene expression by amino acid starvation will be described in another section). It has been demonstrated that the transcription factors ATF4, ATF2, and C/EBPβ and the cis-acting elements amino acid starvation response element (AARE), nutrient sensing response element-1 (NSRE-1), and NSRE-2 in the promoter regions of the target genes are involved in transcriptional regulation of amino acid starvation induced genes (Fafournoux et al., 2000; Averous et al., 2004; Siu et al., 2002).

The CHOP gene encodes a basic leucine zipper transcription factor of the C/EBP family. CHOP gene expression is induced by both amino acid starvation and ER stress. The minimum core sequence of the CHOP AARE (5’-ATTGCATCA-3’) is similar to the ATF/cAMP response element and the binding site of C/EBP proteins. It has been
demonstrated that this element interacts with ATF4 and C/EBPβ to mediate CHOP transcriptional up-regulation by amino acid starvation (Averous et al., 2004). Another recent study indicates that ATF2 phosphorylation and increase of ATF4 are necessary for CHOP transcriptional up-regulation by amino acid starvation and they both interact with the AARE of the CHOP promoter in vitro (Averous et al., 2004).

Asparagine synthetase is responsible for the synthesis of asparagine from aspartate and glutamine. AS gene transcription is regulated by both amino acid starvation and ER stress (Barbosa-Tessmann et al., 1999). It was first found that the same region (-111 to -34) in the promoter of AS gene is responsible for both forms of regulation (Barbosa-Tessmann et al., 2000). Then it was found that, in the promoter region of the AS gene, two cis-acting elements NSRE-1 (5’-TGATGAAAC-3’) (nt -68 to -60) and NSRE-2 (5’-GTTACA-3’) (nt -48 to -43) are essential for transcriptional activation by both amino acid starvation and ER stress (Barbosa-Tessmann et al., 2000). Luciferase assay, electrophoretic Mobility Shift Assay (EMSA), and overexpression of dominant negative mutants indicated that activation of the AS gene by amino acid starvation and ER stress involves binding of ATF4 and C/EBPβ to the NSRE-1 site (Siu et al., 2001; Siu et al., 2002). The AS NSRE-1 element was defined as a AARE element by a earlier study and demonstrated that it mediates induction of AS gene transcription during amino acid starvation and ER stress (Guerrini et al., 1993). Transcriptional induction of mammalian System A amino acid transporter the sodium-coupled neutral amino acid transporter 2 (SNAT2) by amino acid starvation is mediated by ATF4 via interaction with the intronic cis-acting element AARE (5’-ATTGCATCAG-3’) (Palii et al., 2006).

**Regulation of cat-1 gene expression by amino acid starvation**
The cat-1 gene is located on chromosome 12 in the rat genome. The gene contains no TATA box in its promoter region. As an amino acid transporter, regulation of cat-1 gene expression by amino acid availability has been extensively studied during the last 15 years (Hatzoglou et al., 2004; Lopez et al., 2007). CAT-1 is the only member in the y+ System that is regulated by amino acid availability (White and Christensen, 1982). During amino acid starvation, induction of cat-1 gene expression is regulated at 3 different levels: A. Transcriptional enhancement by transcription factors ATF4 and C/EBPβ via interaction with the cat-1 AARE (5'-TGATGAAAC-3') in the first exon of the gene (Fernandez et al. 2003; Lopez et al., 2007); B. Increased cat-1 mRNA stability via the binding of the HuR protein to the 11 nucleotide AU rich element (ARE) in the 3’ UTR of the mRNA (Aulak et al., 1999; Yaman et al., 2002); C. Translational control of cat-1 mRNA via a cap-independent translation initiation mechanism via an IRES in the 5’ UTR of the mRNA (Komar and Hatzoglou, 2005; Fernandez et al., 2001; Fernandez et al., 2002a; Fernandez et al., 2002b; Yaman et al., 2003).

The dramatic increase of cat-1 mRNA during amino acid starvation is caused by both up-regulation of transcription and increased mRNA stability (Fernandez et al. 2003; Aulak et al., 1999; Yaman et al., 2002). (Transcriptional regulation of cat-1 gene expression by amino acid starvation is described in the next section.) Cat-1 gene transcribes a ~7.9 kb and a ~3.4 kb mRNA, the two mRNAs result from polyadenylation at two different sites within the 3’ untranslated region (UTR) (Aulak et al., 1999). Based on sequence homology, the two mRNAs encode for the same protein since they have the same open reading frame (ORF) encoding for a single CAT-1 protein. During amino acid starvation, the 7.9 kb mRNA stability is increased while the stability of the 3.4 kb mRNA
is not (Aulak et al., 1999). These data suggested that there is an RNA element that regulates cat-1 mRNA stability in the extra 4.5 kb fragment contained in the 3’ UTR of the 7.9 kb mRNA but is not in the 3.4 kb mRNA. By using 3’ UTR truncation and mutation analysis, it was found that a 11 nt AU-rich element is responsible for the stability regulation (Yaman et al., 2002). This AU-rich element interacts with the HuR protein during amino acid starvation (Yaman et al., 2002). HuR is a RNA binding protein that shuttles between the nucleus and the cytosol. Amino acid starvation induces transient accumulation of HuR in the cytosol (Yaman et al., 2002).

At the translational level, amino acid starvation causes increased cat-1 mRNA translation (Fernandez et al., 2001). Amino acid starvation induces eIF2α phosphorylation, which happens within an hour of the onset of amino acids starvation and suppresses cap dependent translation (Fernandez et al., 2001). Under eIF2α phosphorylation during ER stress, cat-1 mRNA translation is mediated by IRES-mediated translation initiation in a manner that requires both eIF2α phosphorylation and translation of the upstream open reading frame (uORF) (Fernandez et al., 2001; Fernandez et al., 2002a; Fernandez et al., 2002b; Fernandez et al., 2002c). The cat-1 uORF is located in the 5’ UTR of the cat-1 mRNA, which is translated into a predicted 48 amino acids peptide. The translation of the uORF opens an inhibitory structure in the 5’ UTR of the cat-1 mRNA, thus inducing a confirmational change that activates the IRES (Yaman et al., 2003). In addition, it has been demonstrated that the slower translation elongation rate of the uORF stimulates the translation of the downstream cat-1 ORF (Fernandez et al., 2005).

**Transcriptional regulation of cat-1 gene expression by amino acid starvation**
At the transcriptional level, cat-1 mRNA is induced to similar levels by depletion of all 20 amino acids or any single amino acid (Fernandez et al. 2003). Mutagenesis indicates that transcriptional induction of cat-1 gene expression by amino acid starvation in C6 cells is mediated by the AARE element located in the first exon of the gene (Fernandez et al. 2003). Transcription factors ATF4, C/EBPβ, and ATF3 are induced in the nucleus of C6 cells and MEFs by amino acid starvation (Fernandez et al. 2003; Lopez et al., 2007). In eIF2α(A/A) MEFs and ATF4 deficient MEFs, induction of cat-1 mRNA by amino acid starvation is diminished, which indicates that an increase of ATF4 and/or eIF2α phosphorylation is required for cat-1 gene transcriptional induction by amino acid starvation (Lopez et al., 2007). In ATF3 deficient MEFs, cat-1 mRNA is induced to a higher level in comparison to the wild type (WT) MEFs, which suggests that ATF3 inhibits cat-1 transcription during amino acid starvation (Lopez et al., 2007). These data have been confirmed using luciferase assays by contransfection of the cat-1 promoter luciferase reporter with individual or a combination of expression vectors. Individually transfected ATF4 or C/EBPβ activate cat-1 promoter activity, and the combination of ATF4 and C/EBPβ induces cat-1 promoter activity to an even higher level. ATF3 inhibits the cat-1 reporter activity induced by transfected ATF4, C/EBPβ, or the combination of ATF4 and C/EBPβ (Fernandez et al. 2003; Lopez et al., 2007). Eletrophoretic mobility shift assays (EMSA) demonstrated interaction between the AARE element with the transcription factors individually or in combinations of ATF4 and C/EBPβ or C/EBPβ and ATF3 (Lopez et al., 2007). The in vivo binding of the transcription factors with the cat-1 promoter in C6 cells was demonstrated by ChIP assays (Lopez et al., 2007). Taken together, these data indicate that ATF4 and C/EBPβ
bind to the AARE as an heterodimer to induce cat-l transcription, which followed by binding of ATF3 and C/EBPβ to the AARE to suppress cat-l transcription (Fernandez et al. 2003; Lopez et al., 2007). Since it has been demonstrated that induced ATF3 level by amino acid starvation or ER stress requires ATF4 and/or eIF2α phosphorylation (Jiang et al., 2003). This suggests that transient increase of cat-l transcription during amino acid starvation is via a feedback mechanism through induction of ATF3 by ATF4 (Lopez et al., 2007).

**Endoplasmic reticulum (ER) stress and unfolded protein response (UPR)**

In eukaryotic cells, endoplasmic reticulum (ER) is the organelle responsible for synthesis, modification, and delivery of secretory and membrane located proteins (Rutkowski and Kaufman, 2004). The newly synthesized and modified secretory proteins in the ER are sequestered into small vesicles (sacs) and transported to the Golgi complex for secretion, and the membrane proteins are directly inserted into the membrane. ER is also the organelle for the synthesis of sterols and lipids. Accumulation of unfolded or aggregated proteins in the ER lumen causes perturbations of normal ER function, this is termed ER stress. The coordinated signaling network activated by ER stress is called unfolded protein response (UPR) (Schroder and Kaufman, 2005a).

So far in mammalian cells, it has been revealed that there are 3 ER membrane associated proteins that sense luminal unfolded proteins in the ER and initiate the UPR signaling pathways, they are inositol requiring 1 (IRE1)/ or ER to nuleus signaling 1 (ERN1), PERK/PEK, and activating transcription factor 6 (ATF6) (Figure 1-1; Schroder and Kaufman, 2005a). IRE1 and PERK are type I transmembrane proteins, they both
Figure 1-1. Distinct components of the unfolded protein response (UPR) mediated by each of the sensors resident in the endoplasmic reticulum (ER). Alterations in the timing with which each pathway of the UPR can be activated, combined with positive and negative feedback loops, lead to distinct aspects of the response falling under the provenance of different sensors. The diagram shown conveys an approximate sense of timing, with events pictured farther from the ER sensing-molecules PERK, ATF6 and IRE1 taking place later than those pictured more proximal. For instance, the full activation of the IRE1 pathway requires the enhanced production of XBP1 mRNA by ATF6, resulting in an earlier induction of the transcription of genes downstream of ATF6 than those downstream of XBP1. The broken arrows represent feedback loops, both positive (green) and negative (red), known to act on the UPR. It should be emphasized that the functions of PERK, ATF6 and IRE1 are probably not as mutually exclusive as pictured here, and some genes require the action of more than one sensing pathway for their activation. (from Rutkowski and Kaufman, 2004; used with permission)
Figure 1-2. Principal architecture of the ER stress sensors IRE1, PERK, and ATF6. Orange bars represent regions sufficient for signal transduction or oligomerization. Blue bars represent regions binding to BiP. A black box represents the signal peptide and the hatched box depicts the region of limited homology between IRE1 and PERK. Abbreviations: bZIP – basic leucine zipper, GLS1 and GLS2 – Golgi localization sequences 1 and 2, TAD – transcriptional activation domain, and TM – transmembrane domain. Drawings are not to scale. (adapted from Schroder and Kaufman, 2005; used with permission)
FIGURE 1-2

ER luminal

IRE1

PERK

ATF6α

ATF6β

cytosolic- or nucleoplasmic

TM

kinase

RNase

TM

kinase

cytosolic- or nucleoplasmic

ER luminal

GLS1

GLS2

GLS2
contain luminal ER stress regulated oligomerization domains and cytosolic kinase domains, IRE1 also has a cytosolic RNase domain in addition (Figure 1-2). ATF6 is a type II transmembrane protein (Schroder and Kaufman, 2005a). It has two independent Golgi localization sequences (GLS) GLS1 and GLS2 (Shen et al., 2002). Immunoglobulin heavy chain-binding protein (BiP)/glucose regulated protein 78 (GRP78) is a chaperon protein located in the ER lumen. In unstressed condition BiP binds to the luminal domains of the sensor proteins (Berrtolotti et al., 2000; Shen et al., 2002). Upon onset of ER stress, unfolded proteins increase in the ER. BiP is totally saturated from the luminal domains of the ER stress sensor proteins by the binding of the huge excess of unfolded proteins, the ER sensor proteins are released from BiP binding. Both PERK and IRE1 are activated by autophosphorylation via oligomerization in a similar manner upon release of BiP (Shamu and Walter, 1996). PERK and IRE1 have a small homologous domain, which is conserved throughout all eukaryotes (Schroder and Kaufman, 2005a). The regions required for BiP binding, oligomerization, and signaling partially overlap each other in PERK but not in IRE1 (Figure 1-3; Schroder and Kaufman, 2005a). IRE1 has two isoforms IRE1α and IRE1β. IRE1α is ubiquitously expressed while IRE1β is only expressed in the gut (Tirasophon et al., 1998). ATF6 is activated by cleavage by site-1 protease (S1P) and Site-2 protease (S2P). After BiP is released from the luminal domain of ATF6, it translocates to the Golgi complex (Haze et al., 1999). S1P cleaves ATF6 in the luminal domain, then the N-terminal anchored on the membrane is cleaved off by S2P, the released N-terminal portion of ATF6 is a basic leucine zipper (bZIP) transcription factor (Haze et al., 1999; Chen et al., 2002). ATF6α and ATF6β/CREB-RP are two homologous proteins in mammalian cells (Haze et al.,
2001). It has been shown that ATF6β serves as a dominant negative transcriptional suppressor of ATF6α since it only has very weak transcriptional activity (Thuerauf et al., 2004). In general, three signaling pathways triggered by ER stress are PERK-eIF2α-ATF4, IRE1-XBP1s, and ATF6 pathways corresponding to the three ER stress sensors.

The significant effects of onset of UPR are attenuation of general protein synthesis, cell cycle arrest, and transcriptional induction.

1. Attenuation of general protein synthesis caused by inhibiting translation initiation via phosphorylation of the α unit of the eukaryotic translation initiation factor 2 (eIF2α). This is one of the earliest events during ER stress, the level of phosphorylated eIF2α reaches to its maximum level within 1 hour in both ER stress and amino acid starvation in most cell types (Fernandez et al., 2002; Schroder and Kaufman, 2005a). However, translation of certain specific mRNAs is induced, for example ATF4 mRNA translation is increased by a mechanism mediated by upstream ORFs and eIF2α phosphorylation (Vattem and Wek, 2004). These proteins are most involved in adaptation to ER stress. To recover from inhibition of general protein synthesis, phosphorylated eIF2α (eIF2α-P) needs to be dephosphorylated. eIF2α-P is dephosphorylated by the type 1 protein serine/threonine phosphatase (PP1) via two negative feedback loops during ER stress. PP1 is activated by its association with growth arrest and DNA damage inducible protein 34 (GADD34). Induction of GADD34 synthesis requires eIF2α-P at two steps, its transcriptional activation by ATF4 (Ma and Hendershot, 2003), and also a translational requirement of eIF2α-P (Lee et al., 2009). Inhibition of eIF2α-P dephosphorylation by Salubinal (Sal), a small molecule inhibitor of PP1-GADD34 phosphatase, protects PC12 cells from ER stress-induced apoptosis (Boyce et al., 2005).
2. Cell cycle arrest in G1 phase. Different studies have revealed that cell cycle arrest during ER stress is mediated by the expression levels or activity of cell cycle regulators, which include cyclin D1, cyclin-dependent kinase inhibitors p21 (Waf1/Cip1), the tumor suppressor p53. Cyclins are the regulatory subunits of cyclin-dependent kinases (CDKs). They form complexes with CDKs to regulate cell cycle progression from G1 phase to S phase and from G2 phase to M phase (Brewer and Diehl, 2000). Cyclin D1 is a critical modulator of G1/S transition via Rb phosphorylation and p21/p27 titration. It has been demonstrated that eIF2α phosphorylation by PERK during ER stress causes translational repression of cyclin D1, which subsequently causes cell cycle arrest in G1 phase (Brewer and Diehl, 2000). Excessive cyclin D1 expression is common in human cancers (Knudsen et al., 2006). P21 is a CDK inhibitor, it binds to cyclin E-Cdk2 complex inhibiting its kinase activity and blocking the transition from G1 to S phase (Caldon et al., 2006). Genes encoding transcription factors of the E2F family proteins are members of a family of genes controlled by complexes of CDKs. These proteins modulate cell cycle progression by regulating expression of genes needed for S-phase entry including cyclins (Dyson, 1998). In response to DNA damage, E2F1 coordinates cell cycle progression and apoptosis. It has been reported that deficiency of E2F1 in MEFs results in enhanced eIF2α phosphorylation and the cells are resistant to apoptosis triggered by the ER stress inducer thapsigargin (Tg) (Park et al., 2006). However, PERK−/− MEFs demonstrate an attenuated G1/S arrest that is not caused by cyclin D1 loss, which suggests that there might be cyclin D1-independent cell cycle arrest mechanisms in UPR. It has been shown that UPR triggered p53 accumulation and activation is involved in cell cycle inhibition (Zhang et al., 2006). UPR promotes enhanced interaction between the ribosomal proteins
(rpL5, rpL11 and rpL23) and Hdm2 in a PERK-dependent manner, and the association of ribosomal subunit with Hdm2 couples the unfolded protein response to p53-dependent cell cycle arrest (Zhang et al., 2006).

3. Transcriptional induction of genes involved in adaptation of ER stress or apoptosis. All three signaling pathways triggered by ER stress are involved in up-regulation of transcription factors. Transcription factor ATF4 is induced by ER stress via increase of ATF4 mRNA translation by a reinitiation mechanism involving upstream ORFs and eIF2α phosphorylation (Vattem and Wek, 2004). ATF4 enhances two additional transcription factors CHOP and ATF3 (Averous et al., 2004; Lopez et al., 2007). Spliced XBP1 (XBP1s) is induced by unconventional splicing of XBP1 mRNA by ER stress activated IRE1. Cleaved ATF6α itself is an active transcription factor. These transcription factors are then involved in transcriptional up-regulation of genes for adaptation of ER stress or execution of apoptosis. ER chaperones for buffering unfolded proteins (such as BiP) and ER associated degradation (ERAD) pathways are up-regulated either through activation of transcription or by translational control (Rutkowski and Kaufman, 2004). This provides a quality control system associated with ER to ensure that only correctly folded proteins exit the ER and unfolded or misfolded proteins are retained in the ER and ultimately degraded. Cells with severe unfolded protein accumulation in the ER will eventually go through apoptosis (Schroder and Kaufman, 2005b).

ER stress occurs in disease conditions such as in pancreatic islets of patients with type II diabetes and mice with deficient ER membrane protein wfs (Shen et al., 2004; Yamada et al., 2006) (ER stress related diseases will be described in another section). ER stress can also naturally occur at normal unstressed conditions. For example, weak ER stress is
induced in pancreatic β cells when blood glucose level is low since ATP level is low and protein glycosylation is insufficient (Scheuner et al., 2001). This fluctuation of weak UPR contributes to glucose sensing.

ER stress can be induced in cell cultures by treatment of cells with chemicals or forced expression of mutant secretory proteins. Thapsigargin (Tg), tunicamycin (Tu), and dithiothreitol (DTT) are regularly used chemicals. Tg is an inhibitor of the ER Ca\textsuperscript{2+} ATPase pump. It induces ER stress by inhibiting Ca\textsuperscript{2+} uptake from the cytosol. A high Ca\textsuperscript{2+} concentration in the ER is required for it’s normal function. Tu is an inhibitor of protein N-glycosylation. Since most proteins synthesized in the ER are N-glycosylated, and N-glycosylation is important for protein folding. DTT is a reducing agent that disrupts disulfide bonds. Intramolecular or intermolecular disulfide bonds are formed for proper folding in the proteins synthesized in the ER. Injection of Tu can be used to induce ER stress in animal models (Yoshida, 2007).

**Transcriptional regulation of gene expression by ER stress**

It has been shown that the transcription factors ATF4, XBP1s, ATF6, CHOP, and ATF3, etc. are induced or activated by ER stress and are involved in transcriptional regulation during ER stress (Schroder and Kaufman, 2005a). ATF4 is induced by eIF2α phosphorylation. Both ATF3 and CHOP are downstream of ATF4. XBP1s is induced through increased XBP1 mRNA splicing by IRE1 and then transcriptional induction by ATF6 and XBP1s (Rutkowski and Kaufman, 2004). ATF6 is activated by proteolytic cleavage in Golgi complex as described in the last section. These factors mediate transcriptional up-regulation during ER stress by interacting with the cis-acting elements including AARE (consensus sequence: 5’-TGATGCAAT-3’), endoplasmic reticulum
response element (ERSE) (consensus sequence: 5’-CCAAT-N9-CCACG-3’), ERSE-II (consensus sequence: 5’-ATTGG-N-CCACG-3’), and UPRE (consensus: 5’-TGACGTGG/A-3’) in the promoters of the ER stress target genes (Li et al. 2000; Yamamoto et al., 2004).

ATF4 interacts with cis-acting element AARE to mediate transcriptional induction in response to either ER stress or amino acid starvation. CHOP gene transcription is induced by not only amino acid starvation but also ER stress. As stated earlier, during amino acid starvation, the minimum core sequence of the CHOP AARE (5’-ATTGCATCA-3’) interacts with ATF4 and C/EBPβ to mediate CHOP transcriptional up-regulation (Averous et al., 2004). Binding of ATF4 to the AARE also involved in induction of CHOP gene transcription during ER stress (Ma et al., 2002). In addition, other two pathways are also involved in induction of CHOP gene transcription (this will be described later) (Ma et al., 2002; Bruhat et al., 2002). IGFBP-1 is a member of the IGFBPs, which are a family of secreted proteins that bind to the insulin like growth factors (IGFs) with high affinity. Thus IGFBPs regulate availability of IGFs by regulating their transport and half-life. IGFBP-1 affects glucose homeostasis, its serum level decreases after food intake and increases between meals. It has been demonstrated that IGFBP-1 gene expression is regulated in response to amino acid starvation, ER stress, and hypoxia (Averous et al., 2005; Marchand et al., 2006). IGFBP-1 mRNA and protein levels are induced by various ER stress reagents in human liver-derived cells (Marchand et al., 2006). The enhancement of transcription by ER stress is mediated by a distal ATF4 binding element (5’-TTTACATCA-3’), which is located at about -6380 from the transcription start site (Marchand et al., 2006).
XBP1s mediates gene transcription via nuclear factor Y (NF-Y) dependent binding to ERSE or binding to UPRE or ERSE-II without requiring NF-Y (Yamamoto et al., 2004). In comparison to ATF6, XBP1s has stronger binding to ERSE-II and weaker binding to ERSE (Yamamoto et al., 2004). CCAAT/enhancer-binding protein beta (C/EBPβ) is a basic leucine zipper (bZIP) transcription factor. It is involved in regulation of gene transcription during amino acid starvation and ER stress (Siu et al., 2001; Lopez et al., 2007). Transcriptional induction of human C/EBPβ gene by ER stress is mediated via binding of XBP1s to a distal UPRE (Chen et al., 2004). Luciferase assays using reporter structures with deletions or mutations indicates that the transcriptional induction of human C/EBPβ gene by ER stress is mediated by a putative UPRE element (5’-TGACGCAA-3’) located downstream of the coding region (+1614 to +1621) (Chen et al., 2004). Electrophoretic mobility shift assay (EMSA) demonstrated that this element specifically interacts with ER stress induced proteins (Chen et al., 2004). Luciferase assays in XBP1 deficient MEFs and luciferase assays with reporter vectors cotransfected with XBP1s expression vectors indicate that the transcription factor XBP1s is responsible for transcriptional induction by ER stress (Chen et al., 2004). By using DNA array, it has been revealed that a subset of ER resident chaperone genes including BiP, EDEM, p58(IPK), and ERdj4 are regulated by XBP1 (Lee et al., 2003).

Active ATF6 (p50ATF6) mediates gene transcription via its NF-Y required binding to ERSE in the target genes (Li et al, 2000). Induction of CHOP gene transcription by ER stress also involves a ERSE (CCAAT-N9-CCACG) downstream of the AARE. During ER stress, binding of ATF4 to the AARE and the NF-Y required binding of ATF6 to the ERSE mediate transcriptional induction of CHOP gene expression (Ma et al., 2002;
Bruhat et al., 2002). The CCAAT sequence of the ERSE interacts nuclear factor Y (NF-Y), and the CCACG sequence of the ERSE interacts with AFT6 (Ma et al., 2002; Bruhat et al., 2002). And later, it was observed that XBP1 is required for full induction of CHOP transcription during ER stress (Donati et al., 2006). Probably, induction of CHOP transcription by XBP1 during ER stress is mediated by its binding to the CHOP ERSE. BiP, a chaperone protein located in the ER lumen, is involved in protein folding and buffering unfolded proteins. BiP gene transcription is regulated by ER stress and glucose depletion. During ER stress, TFII-I and a protein complex containing ATF6 and YY1 are recruited to the cis-acting element ERSE to induced BiP gene transcription (Hong et al., 2005; Baumeister et al., 2005). The protein complex is formed by interaction of the zinc finger domain of YY1 and the basic leucine zipper of the active ATF6 (Baumeister et al., 2005). YY1 is constitutively expressed, but it only binds to the promoter upon ER stress. ER stress induced TFII-I phosphorylation at Try^{248} correlates with its nuclear localization (Hong et al., 2005).

It has been shown that IRE1-XBP1 pathway is compensatory for the ATF6 pathway (Lee et al., 2003). This is probably because of the binding properties of the two transcription factors to the cis-acting elements and the existing crosstalk between the two pathways.

**ER stress and human diseases**

Most conformational diseases are typical ER stress related diseases (Schroder and Kaufman, 2005a). Conformational diseases are a collection of diseases caused by accumulation of misfolded cellular proteins, such as type 2 diabetes, Alzheimer’s disease, Parkinson’s disease, and atherosclerosis (Yoshida, 2007). Since misfolded proteins in the
ER triggers ER stress, it has been speculated that ER stress is involved in most of conformational diseases. Under normal conditions, protein folding capacity is well balanced with the protein synthesis process in the ER. Under some circumstances, the amount of protein synthesized exceeds the folding ability of the organelle, which causes onset of ER stress. Diabetes mellitus is characterized by hyperglycemia caused by impaired secretion or action of insulin (Hansen et al., 2007). It has been shown in mouse models that ER stress is the link between obesity insulin resistance and type 2 diabetes (Ozcan et al., 2004). Obesity causes ER stress that in turn activates c-Jun N-terminal kinase (JNK), and subsequent phosphorylation of insulin receptor substrate-1 (IRS-1) by JNK causes insulin resistance (Ozcan et al., 2004). Chemical chaperone 4-phenyl butyric acid (PBA) is a low molecular weight compound. It improves ER folding capacity, stabilizes protein conformation, and facilitates the trafficking of misfolding proteins. Pretreatment of hepatoma cells with PBA suppressed Tu induced PERK and eIF2α phosphorylation and JNK activation (Ozcan et al., 2006). Oral administration of PBA to leptin deficient ob/ob mice reduced peripheral blood glucose to normal levels, restored systemic insulin sensitivity (Ozcan et al., 2006). Evidence has suggested that ER stress is involved in pancreatic β cells death during the development of type 2 diabetes. It has been demonstrated that pancreatic islets of an ER membrane protein Wolfram syndrome 1 (wfs1) deficient mice exhibit an enhanced ER stress response, which is associated with increased expression of the cyclin-dependent kinase inhibitors p21 in wfs1-deficient islets. Treatment of mouse pancreatic islets with the ER stress inducer Tg causes induction of p21. Forced overexpression of p21 in MIN6 cells (β cell line) results in reduced cell numbers, which suggests that increased p21 expression induced by ER stress
is involved in β cell loss in the mutant islets (Yamada et al., 2006). Many reports have shown that ER stress is involved in neurodegenerative diseases Alzheimer’s and Parkinson’s (Lindholm et al., 2006).

Human diseases can also be caused by defects or inhibition of UPR signaling (Schroder and Kaufman, 2005a). Wolcott-Rallison syndrome is a rare autosomal recessive disease associated with permanent neonatal insulin-dependent diabetes, it is caused by mutations in the EIF2AK3 (PERK) gene (Durocher et al., 2006).

The revelation of further aspects of UPR signaling pathways and mechanisms of gene regulation by ER stress will lead to the diagnosis and treatment of other ER stress related human diseases.

**Rationale of the thesis research**

The objective of the thesis is to study the mechanisms of transcriptional regulation of cat-1 gene during ER stress. It is determined here that the cat-1 mRNA level increases to about 5 fold after 9 hours of treatment with the ER stress inducer Tg and then decreases to about 2 fold after 24 hours of treatment. To study the mechanism of the transcriptional regulation, the following hypotheses were tested: 1). There are ER stress induced or activated transcription factors involved in cat-1 transcriptional up-regulation at the early stage of ER stress, and there are cis-acting elements in the cat-1 promoter region interacting with the transcription factors; 2). There are suppressors involved in down-regulation of cat-1 gene transcription during late ER stress; 3). Inducible transcription factors are regulated by ER stress. If a novel transcription factor is involved, the regulation of the transcription factor will be studied; 4). If a transcription factor is involved in transcriptional regulation of cat-1 gene expression during ER stress, it may be
involved in transcriptional regulation of other ER stress target genes.

It is shown here that transcription factor ATF4 interacts with the cis-acting element AARE and XBP1s interacts with a putative cis-acting element ERSE-II to induce cat-1 gene transcription at the early stage of ER stress. At the late stage of ER stress, C/EBPβ LIP isoform down-regulates cat-1 gene transcription by specifically suppressing the up-regulation mediated by AARE via binding of ATF4. ATF4-mediated transcriptional induction of SNAT2 and AS is also suppressed by C/EBPβ LIP during ER stress. The down-regulation of C/EBPβ LIP level at the early stage of ER stress is mediated by proteasome-mediated degradation and eIF2α phosphorylation. The dramatic increase of C/EBPβ LIP at the late staged of ER stress is caused by both increased mRNA translation and stability of the protein.
CHAPTER 2

EIF2α PHOSPHORYLATION IS REQUIRED FOR INDUCTION OF CAT-1 GENE TRANSCRIPTION MEDIATED BY ATF4 AND XBP1S DURING ER STRESS

INTRODUCTION

As described in chapter 1, in mammalian cells, the 3 UPR signaling pathways are involved in induction or activation of transcription factors mediating transcriptional induction of ER stress target genes. These transcription factors interact with AARE, ERSE, ERSE-II, or UPRE to induce gene transcription (Li et al., 2000; Yamamoto et al., 2004). The only crosstalk revealed among these 3 signaling pathways is the enhancement of XBP1 transcription by ATF6, which is between the IRE1-XBP1 pathway and the ATF6 pathway (Schroder and Kaufman, 2005a).

ATF4 interacts with cis-acting element AARE to mediate transcriptional induction in response to either ER stress or amino acid starvation (Guerrini et al., 1993; Barbosa-Tessmann et al., 2000; Siu et al., 2001; Siu et al., 2002). XBP1s mediates gene transcription via nuclear factor Y (NF-Y) dependent binding to ERSE or binding to UPRE or ERSE-II without requiring NF-Y (Yamamoto et al., 2004). In comparison to ATF6, XBP1s has stronger binding to ERSE-II and weaker binding to ERSE (Yamamoto et al., 2004). CCAAT/enhancer-binding protein beta (C/EBPβ) derived factors belong to the bZIP transcription factor family. It has been observed that they are involved in regulation of AS gene transcription during amino acid starvation and ER stress (Siu et al., 2001).
Cat-1 gene expresses ubiquitously and its expression level varies in different tissues and cells (Wu et al., 1994; Aulak et al., 1996). Expression of cat-1 gene can be modulated by stimuli including ER stress, availability of nutrients, cell proliferation, growth factors, and hormones (Aulak et al., 1996; Fernandez et al., 2001). The transcriptional up-regulation of the cat-1 gene by amino acid starvation is mediated by the transcription factor ATF4 via the cis-acting AARE in the first exon of the cat-1 gene (Fernandez et al., 2003; Lopez et al., 2007). It has been demonstrated that cat-1 mRNA levels increase during ER stress (Fernandez et al., 2003), but the mechanism of cat-1 transcriptional induction by ER stress has not been addressed. We shown here that the cat-1 mRNA level increases to about 5 fold after 9 hours of treatment with ER stress inducer thapsigargin (Tg) and then decreases to about 2 fold after 24 hours of treatment. During ER stress, ATF4 binds to the cis-acting element AARE and XBP1s binds to the putative cis-acting element ERSE-II in the cat-1 promoter to mediate cat-1 transcriptional up-regulation. The finding that a target gene of ER stress is coordinately regulated by both ATF4 and XBP1s is novel. Induction of cat-1 gene transcription by ER stress requires eIF2α phosphorylation due to induction of ATF4 requires and induction of XBP1s largely depends on eIF2α phosphorylation. To our knowledge, the crosstalk between the PERK-eIF2α-ATF4 and the IRE1-XBP1 pathways of the 3 UPR signaling pathways is being demonstrated here for the first time. This is another example of the same cis-acting element AARE mediating both ER stress and amino acid starvation induced transcription.
MATERIALS AND METHODS

Plasmid constructs

PA1.4N luciferase reporter vector was constructed previously in the lab (Fernandez et al., 2003). muAARE was also called PA1.4N(mut2), which is the PA1.4N with the AARE (5’-TGATGAAAC-3’) (+45 to +53) and the upstream cytosine (C) (+44) mutated to 5’-ATGT'TAAAC-3’ as described earlier (Figure 6A; Fernandez et al., 2003). muERSE is the PA1.4N with the mutated putative ERSE-II element (5’-ATTGGTGCTGG-3’) (-25 to -11). muUPRE is the PA1.4N with the putative UPRE element (5’-TGACCCA-3’) (-145 to -138) mutated to 5’-CCTATTCG-3’, and the muAAER is the PA1.4N with mutated both the AARE and the putative ERSE-II element.

Cell culture and DNA transfections

C6 rat glioma cells were grown in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and 5% calf serum (CS). Mouse embryonic fibroblasts (MEFs) were grown in 10% FBS supplemented high glucose DMEM. To induce ER stress, cells were incubated with medium containing 400 nM thapsigargin (Tg) for the indicated times. Amino acid starvation was induced by incubation of cells in Met- and Cys-free high glucose DMEM supplemented with 10% dialyzed FBS as described previously (Lopez et al., 2007). To inhibit protein synthesis or proteasome mediated degradation, cells were incubated with medium containing 10 µg/ml cycloheximide (CHX) or 10 µM MG132 for the indicated times, respectively. Cells were transfected with plasmid DNA using FuGENE 6 (Roche Applied Science). The medium containing transfection reagent was changed to regular medium about 24 hours after transfection. For reporter assays, cells were harvested about 48 hours after
transfection. Transfection efficiency in transient transfection experiments was controlled by cotransfection of plasmids expressing either β-galactosidase or Renilla luciferase. Luciferase and β-galactosidase activities were measured as described previously (Fernandez et al., 2001). Stably transfected mass cultures were generated by selection of transfectants in medium containing 0.1% G418. Stable transfected cells were grown in medium containing 0.04% G418 and shifted to drug-free medium 1 day before experiments.

**Chromatin immunoprecipitation (ChIP) assay**

ChIP assay was performed as described previously (Lopez et al., 2007). Briefly, C6 glioma cells in 150-mm dishes were grown up to a density of 2.5 x 10^7 cells, then treated with ER stress inducer Tg and subsequently cross-linked with 1% formaldehyde added in medium. Nuclei were isolated as described earlier in western blot analysis and sonicated to 100-500 bp fragments in nuclear lysis buffer (50 mM Tris-Cl pH 8.0, 10 mM EDTA, 1 % SDS). Each immunoprecipitation reaction contains precleared 100 µl of sonicated chromatin and 2 µl of antibodies in 1 x immunoprecipitation buffer (40 mM Tris-Cl pH 8, 300 mM NaCl, 0.02% SDS, 2% Triton X-100, 4 mM EDTA). After the reactions were incubated at 4 °C for 2 hours, 30 µl mass volume of Protein G Dynabeads (Dynal Biotech) was added in each reaction and continue to incubate for 1 more hour. After washes, the beads were collected with a magnet stand and washed. DNA fragments pulled down were eluted and purified using QIAquick gel extraction kit (Qiagen) and used as templates for PCR analysis. The PCR products were run on a 2.5% agarose gel and visualized with ethidium bromide (Eth-Br) staining. The Eth-Br stained bands were scanned and quantified by a computer program (ImageJ). Normal rabbit IgG (sc-2027),
anti-ATF4 antibodies (C-20, sc-200), anti-XBP1 antibodies (M-186, sc-7160), and anti-Pol II antibodies (N-20, sc-899) were purchased from Santa Cruz Biotechnology. Primers used for PCR are listed in Table 2-1).

**Northern blot analysis**

Total RNA samples were separated by 1% denaturing agarose gel containing 5% formaldehyde, and then transferred to nitrocellulose membranes by capillary blotting. Specific mRNAs were detected by hybridizing the membranes with $\gamma^{32}$P-labeled DNA probes. A 7 kb fragment of the cat-1 cDNA was used to detect cat-1 mRNA (Fernandez et al., 2003). A 900 bp fragment of the AS cDNA was used to detect AS mRNA (Hutson and Kilberg, 1994). A 5.8 kb DNA fragment containing the 18S mouse ribosomal RNA gene was used to detect 18S ribosomal RNA (Katz et al., 1983).

**Western blot analysis**

Western blots were done as described previously (Li et al., 2007). Briefly, treated cells were washed twice with phosphate-buffered saline (PBS) and lysed in lysis buffer containing 20 mM HEPES pH 8.0, 380 mM NaCl, 6 mM MgCl$_2$, 1% deoxycholic acid. The supernatants was collected as total cell lysates after centrifugation at 17,000 x g for 10 min at 4 °C. To prepare nuclear extracts, cells were resuspended in cytosolic extraction buffer (CEB) (10 mM HEPES pH 8.0, 3 mM MgCl$_2$, 20 mM KCl, 0.5 mM DTT, 5% glycerol, 0.5% NP-40) and lysed by passing through a 23G needle 5-10 times. The supernatants were collected as cytosolic extracts after centrifugation at 4,000 x g for 10 min at 4 °C. The pellets were resuspended in nuclear extraction buffer (NEB) (20 mM HEPES pH 8.0, 225 mM NaCl, 1 mM EDTA, 3 mM MgCl$_2$, 0.5 mM DTT, 10% glycerol, 0.5% NP-40) and lysed by passing through a 26G needle 5-10 times. The supernatants
were collected as nuclear extracts after centrifugation at 17,000 x g for 10 min at 4 °C. Proteins were transferred to polyvinylidene difluoride membranes (PVDF) membranes after total cell lysates (20 µg protein) or nuclear extracts (10 µg protein) were separated by SDS-PAGE, the membranes were then probed with antibodies and exposed to films. Antibodies against the following proteins were purchased from Santa Cruz Biotechnology: ATF4 (C-20, sc-200), BiP (H-129, sc-13968), C/EBPβ C terminus (C-19, sc-150), CHOP (B-3, sc-7351), HDAC1 (H-11, sc-8410), Pol II (N-20, sc-899), and XBP1 (M-186, sc-7160). Antibodies against FLAG (PRB-132P) were from Covance. Antibodies against eIF2α-P (44–728G) were from BIOSOURCE. Monoclonal antibody against tubulin (T9026) was from Sigma. Anti-eIF2α antibodies were custom-made by Quality Controlled Biochemicals. Antibodies against PERK were a gift from Dr. Antonis E. Koromilas, Department of Oncology, McGill University, Canada.

**Reverse transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR)**

Total RNA was isolated using RNeasy Mini Kit (Qiagen). SuperScript III (Invitrogen) was used to synthesize the first-strand cDNA from the total RNA samples. Poly(T) primers were used to synthesize a mixture of total cDNAs. In qRT-PCR, 18S cDNA was amplified to use as an internal control. The PCR primers used for RT-PCR and qRT-PCR are listed in Table 2-1 (XBP1 and EDEM) or Table 4-1 (cat-1, CHOP, BiP, and 18S).
Table 2-1. DNA primers used in polymerase chain reaction (PCR), reverse transcription polymerase chain reaction (RT-PCR), and quantitative RT-PCR (qRT-PCR).
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer name</th>
<th>DNA sequence of the primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat-1</td>
<td>F-AARE</td>
<td>5’-ATTGGTGCCTGGGAAGCTGA-3’</td>
</tr>
<tr>
<td></td>
<td>R-AARE</td>
<td>5’-GGGGAGGGAGGCGTCAGGATGC-3’</td>
</tr>
<tr>
<td>Cat-1</td>
<td>F-ERSE</td>
<td>5’-TCGGTTGGGCTGCTGAGGACCAA-3’</td>
</tr>
<tr>
<td></td>
<td>R-ERSE</td>
<td>5’-TTTCATCAGCGCGCGCGCCT-3’</td>
</tr>
<tr>
<td>XBP1</td>
<td>Forward</td>
<td>5’-ACACGCTTTGGGAATGGACAC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-CATGGGAAGATGTCTGGG-3’</td>
</tr>
<tr>
<td>EDEM</td>
<td>Forward</td>
<td>5’-AGTCAAATGTGGATATGCTACGC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-ACAGATATGATATGGCCCTCAGT-3’</td>
</tr>
</tbody>
</table>
RESULTS

Cat-1 mRNA levels are induced by ER stress

It has been demonstrated previously that endogenous cat-1 mRNA is induced in C6 cells by ER stress (Fernandez et al, 2003). To quantitatively determine the kinetics of the cat-1 mRNA levels in C6 cells during this stress, qRT-PCR was used to evaluate cat-1 mRNA levels using total cDNA from C6 cells treated with thapsigargin (Tg) (Figure 2-1A). As expected, cat-1 mRNA is increased to about 5 fold after 9 hours of treatment and then declined to about 2 fold after 24 hours (Figure 2-1A, left panel). As a control for cell treatment and qRT-PCR, it is seen that CHOP mRNA level was dramatically induced to about 25 fold in the same samples (Figure 2-1A, right panel).

At the early stage of ER stress, eIF2α is phosphorylated to attenuate general protein synthesis by inhibiting cap-dependent translation initiation (Rutkowski and Kaufman, 2004). Western blot analysis was used to check eIF2α-P levels in C6 cells treated with Tg. Phosphorylated eIF2α increased after 1 hour of treatment and started declining at 6 hours (Figure 2-1B). The pattern of eIF2α phosphorylation is consistent with previous findings (Fernandez et al., 2002b).

We wanted to determine the transcription factors involved in cat-1 transcriptional induction by ER stress. It is known that the transcription factors ATF4, XBP1s, ATF6, and C/EBPβ are involved in transcriptional regulation of ER stress target genes, and some of these, such as ATF4 can be induced by eIF2α phosphorylation (Schroder and Kaufman, 2005a). Western blot analysis was used to determine the levels of putative transcription factors in the nucleus of ER stress-treated C6 cells. The data demonstrate that protein levels of the active transcription factors ATF4 and XBP1s are transiently
**Figure 2-1. Cat-1 mRNA levels are induced by ER stress.** A, Cat-1 (left panel) and CHOP (right panel) mRNA levels were monitored by qRT-PCR using same group of total RNA samples from C6 cells treated with Tg for ER stress for the indicated times. Data were normalized to the 18 S ribosomal RNA levels. B and C, Western blot analysis of total cell lysates (B) or nuclear extracts (C) from C6 cells treated with Tg for the indicated times. Blots were probed with antibodies for eIF2α-P, ATF4, XBP1s, or the indicated proteins. Tubulin was used as a loading marker for total cell lysates. HDAC1 was used as a loading marker for nuclear extracts. D, XBP1 mRNA splicing was monitored by RT-PCR using primers flanking the splicing region and total RNA samples from C6 cells treated with Tg for the indicated times. The PCR products were separated on 2% agarose gel and visualized with ethidium bromide (Eth-Br) staining.
FIGURE 2-1

A

B

C

D

RT-PCR using C6 cells

M

CON

0.5 1 2 4 6 9 (h)

C6 cell lysates

eIF2α-P

eIF2α

tubulin

0 1 3 6 (h)

C6 nuclear extracts

ATF4

XBP1s

CHOP

HDAC1

0 3 6 9 12 18 24 (h)
induced in the nucleus of C6 cells by ER stress, while dramatically up-regulated CHOP indicates the proper ER stress treatment of the cells (Figure 2-1C). It would be expected that the kinetics of eIF2α phosphorylation correlate with ATF4 accumulation since the ATF4 mRNA is only well translated at the time that eIF2α is phosphorylated. However, ATF4 accumulated to a high level after 3 hours treatment and peaked again at 12 hours treatment, suggesting additional mechanisms regulating expression of the ATF4 gene. It is shown that XBP1s level increased in the nucleus of C6 cells treated for ER stress (Figure 2-1C). To confirm that ER stress is the cause of the induction of XBP1s, RT-PCR was used to check if ER stress induces splicing of the XBP1 mRNA. The data demonstrate that, during ER stress, a small fragment (~26 nt) is spliced out from the XBP1 mRNA in C6 cells (Figure 2-1D). The kinetics of accumulation of spliced XBP1 mRNA correlates with the increase of XBP1s in the nucleus of C6 cells (Figure 2-1, C and D).

**Induction of cat-1 gene transcription during ER stress is mediated by ATF4 and XBP1s**

Our previous studies indicate that the *cis*-acting elements mediating induction of cat-1 gene transcription by ER stress are within -1.4 kb to +270 bp of the transcription start site of the gene (Fernandez et al., 2003). The cat-1 promoter luciferase reporter construct PA1.4N contains this region and the downstream luciferase gene is controlled by the promoter of the cat-1 gene, so it was used to determine the putative transcription factors that induce cat-1 gene transcription during ER stress. Luciferase reporter assays were used to determine whether expression of ATF4 or XBP1s in untreated cells can induce cat-1 promoter activity. C6 cells were cotransfected with PA1.4N reporter and
expression vectors for ATF4, or XBP1s, and analyzed for luciferase activity. It is shown that ATF4 or XBP1s induced luciferase activity by 7 and 5-fold over the control, respectively (Figure 2-2A). These data were further supported by a 3-fold induction by transfection of IRE1α (splicing enzyme for the XBP1 mRNA) and only 1.5-fold induction by transfection of the unspliced XBP1 (XBP1u) (Figure 2-2A). The limiting factor or XBP1 mRNA splicing in unstressed C6 cells is the amount of IRE1α, since there is already unspliced XBP1 mRNA in untreated C6 cells (Figure 2-1D). This result suggests that ATF4 and XBP1s are the putative transcriptional activators involved in mediating cat-1 transcriptional induction by ER stress in C6 cells.

It is known that ATF4 mediates cat-1 gene transcriptional induction by amino acid starvation in C6 cells (Fernandez et al, 2003). If it is also involved in mediating cat-1 transcriptional induction by ER stress, a dominant negative form of ATF4 would suppress cat-1 promoter activation by ER stress. When PA1.4N was cotransfected with expression vector of a ATF4 dominant negative form into C6 cells and subsequently treated with Tg for ER stress, ER stress induced luciferase activity is suppressed (Figure 2-2B). The luciferase activity goes lower when ATF4 dominant form is increased (data not shown).

The effect of enforced expression of a transcription factor on the cat-1 gene promoter activity in unstressed cells might be very different than the ER stress treatment, because ER stress induces or inhibits expression levels of many transcription factor genes at the same time. To further determine whether XBP1s and ATF4 are responsible for cat-1 transcriptional induction by ER stress, we used luciferase reporter assays with XBP1- and ATF4-deficient MEFs transfected with PA1.4N reporter vector treated for ER stress. Our
Figure 2-2. Induction of cat-1 gene transcription during ER stress is mediated by ATF4 and XBP1s. A–D, C6 cells (A and B), ATF4 deficient MEFs ATF4−/− and the control MEFs ATF4+/+ (C), or XBP1 deficient MEFs XBP1−/− and the control MEFs XBP1+/+ (D) were transfected with the cat-1 promoter luciferase reporter vector PA1.4N and treated with Tg for ER stress (B and C), treated for ER stress or amino acid starvation (D), or transfected along with expression vectors for the indicated transcription factors (A and B). “ATF4(DN)” stands for dominant negative form of ATF4. Luciferase activity of cell extracts was measured after 48 hours of transfection. Protein concentration was measured to normalize for transfection efficiency. E, Cat-1 mRNA levels were monitored by qRT-PCR using total RNA samples from ATF4−/−, XBP1−/−, and wild type control MEFs treated with Tg for the indicated times. Data were normalized to the 18 S ribosomal RNA levels. The cell samples for qRT-PCRs (E) were treated by Yi Li, the qRT-PCRs were performed by Elena Bevilacqua.
data show that both XBP1s and ATF4 are involved in cat-1 transcription up-regulation by ER stress (Figure 2-2, C and D). ER stress-induced PA1.4N luciferase activity is almost totally abolished in both ATF4−/− and XBP1−/− MEFs (Figure 2-2, C and D). It is also indicates that induction of cat-1 promoter activity by amino acid starvation is independent of XBP1 (Figure 2-2D), and in agreement with our previous findings that ATF4 is the transcription factor that induces cat-1 transcription during amino acid starvation (data not shown). These results suggest that both XBP1s and ATF4 are involved in induction of cat-1 transcription by ER stress. Because depletion of either ATF4 or XBP1 abolished induction of cat-1 promoter activity by ER stress, these data also suggest that ER stress may modulate the levels/activity of these transcription factors via a common signaling pathway.

To further confirm that ATF4 and XBP1s mediate induction of cat-1 gene transcription during ER stress, the endogenous cat-1 mRNA levels were determined by qPCR in ATF4 or XBP1 deficient MEFs treated for ER stress. The data demonstrate that induction of cat-1 mRNA levels by ER stress were attenuated in both ATF4 and XBP1 deficient MEFs (Figure 2-2E), which indicates that both ATF4 and XBP1 are required for induction of cat-1 gene transcription by ER stress. These data well correlate with the results obtained by luciferase assay in the ATF4- and XBP1-deficient MEFs (Figure 2-2, C and D).

**ATF4 and XBP1s act via independent cis-acting DNA elements within the cat-1 gene promoter/regulatory region to induce cat-1 gene transcription during ER stress**

Our data suggest that ATF4 and XBP1s are the putative transcription factors mediating induction of cat-1 transcription in C6 cells during ER stress. Then luciferase reporter
assay was used to determine the interaction sites of ATF4 and XBP1s in the cat-1 gene promoter. We knew that ATF4 mediates cat-1 transcriptional up-regulation through the AARE element in the cat-1 promoter in C6 cells by amino acid starvation (Fernandez et al., 2003) and we therefore expected to find that ATF4 also binds to the same element during ER stress. By searching the DNA sequence of the cat-1 promoter region in PA1.4N for putative XBP1s binding sites, we found two putative sites. We noticed including a ERSE-II-like element (5’-ATTGGTGCCCTGG-3’) (-25 to -11) and a putative UPRE (5’-TGACCCA-3’) (-145 to -138) (Figure 2-3A). This ERSE-II-like element is similar to the ERSE-II consensus sequence 5’-ATTGG-N-CCACG-3’ (Yamamoto et al., 2004). To analyze whether the putative ERSE-II is involved in mediating induction of cat-1 gene transcription during ER stress, this site in PA1.4N was mutated by PCR-based mutagenesis. When the mutated PA1.4N luciferase vector construct (muERSE) was transfected into C6 cells and the cells subsequently subjected to ER stress, mutation of the ERSE-II-like element significantly reduced luciferase activity induced by ER stress (Figure 2-3B). This result indicates that the ERSE-like element is partially required for induction of cat-1 gene transcription by ER stress.

To analyze whether XBP1s is acting via the putative ERSE-II or the UPRE element, the putative ERSE-II-mutated or the UPRE-mutated luciferase reporter construct was cotransfected with an XBP1s expression vector into C6 cells and we conducted luciferase assays. The results demonstrate that XBP1s might act via the ERSE-II-like site since mutation of the ERSE-II-like element significantly reduced the induction of the luciferase activity by XBP1s (Figure 2-3C). As expected, ATF4 cotransfection with the ERSE mutant reporter caused an increase in luciferase activity, although it’s lower than the WT
**Figure 2-3.** ATF4 and XBP1s act via independent *cis*-acting DNA elements within the cat-I gene promoter region to induce cat-1 gene transcription during ER stress.

*A*, Schematic of the cat-1 promoter region showing the relative locations of the *cis*-acting AARE, the putative ERSE-II and UPRE, and the primer locations used for chromatin immunoprecipitation (ChIP) assay. *B*, C6 cells were transfected with the cat-1 promoter luciferase reporter vector PA1.4N or the mutated vector containing mutated putative ERSE-II (muERSE) and then treated with Tg for ER stress for 16 hours. *C*, C6 cells were transfected with PA1.4N, muERSE, or the mutated vector containing mutated putative UPRE (muUPRE) along with expression vectors for ATF4 or XBP1s as indicated. Luciferase activity of cell extracts was measured 48 hours after transfection. Protein concentration was measured to normalize for transfection efficiency. *D*, ChIP assay for analyzing binding of ATF4 and XBP1s to cat-1 promoter region using C6 cell treated with Tg for indicated times. AARE primers as indicated in *A* were used to amplify fragments pulled down by anti-ATF4 or Pol II antibodies, ERSE primers as indicated in *A* were used to amplify fragments pulled down by antibodies against XBP1 proteins. PCR products were separated on 2.5% agarose gel and visualized with Eth-Br staining.
FIGURE 2-3

A

F-ERSE
-162 -141
F-AARE
-37 -18

UPRE
-145 -138
ERSE
-25 -11

+45 +53
AARE
+45 +66
R-ERSE
R-AARE
+136 +154

B

C

D

PCR products of ChiP
Input
IgG
ATF4
XBP1
Pol II

0 1 3 6 9 12 (h)
reporter (Figure 2-3C).

To demonstrate the binding of ATF4 and XBP1s to the cat-1 gene promoter in vivo, chromatin immunoprecipitation (ChIP) assay was conducted using C6 cells treated for ER stress (Figure 2-3D). Chromatin fragments pulled down by antibodies against ATF4, XBP1s, or Pol II were amplified by PCR using primers flanking the AARE (for anti-ATF4 and Pol II pulled down fragments) or primers flanking the ERSE-II-like element (for anti-XBP1 pulled down fragments). The primer locations are indicated in Figure 2-3A. The ChIP assay results indicate that ATF4 binding to the cat-1 promoter peaks at 3 hour during ER stress, and this is correlated with the binding of Pol II. Binding of XBP1s to the promoter peaks at 6 hours during ER stress (Figure 2-3D). These data are in agreement with the hypothesis that the sequential binding of ATF4 and XBP1 to the cat-1 gene promoter is required for sustained transcription of the cat-1 gene during ER stress.

**Phosphorylation of the translation initiation factor eIF2α is required for induction of cat-1 gene transcription by ER stress**

Because eIF2α is transiently phosphorylated by ER stress (Figure 2-1B), eIF2α(A/A) MEFs were used to check whether eIF2α phosphorylation affects cat-1 gene expression. The amino acid residue of eIF2α that is phosphorylated by ER stress and amino acid starvation is serine 51. Serine 51 is mutated to alanine in eIF2α(A/A) cells, so that eIF2α can not be phosphorylated in these cells. As expected, eIF2α-P and ATF4 are not up-regulated in eIF2α(A/A) cells by ER stress (data not shown). Luciferase assay was used to check whether PA1.4N could be activated in eIF2α(A/A) MEFs by ER stress. Induction of PA1.4N in eIF2α(S/S) MEFs by ER stress and amino acid starvation are about 5 fold, which is consistent with endogenous mRNA induction in C6 cells (Figure 2-
4A, Figure 2-1A), confirming that the *cis*-acting elements mediating cat-1 transcription are located in the region between -1.4kb and +270 bp. Activation of PA1.4N in eIF2α(A/A) MEFs by ER stress and AA starvation is totally abolished (Figure 2-4A). The loss of induction in eIF2α(A/A) MEFs can be due to either loss of transcriptional or translational control. We therefore determined cat-1 mRNA levels in eIF2α(A/A) cells treated with ER stress by using Northern blot analysis. Cat-1 mRNA could not be detected in both ER stress and AA starvation treated eIF2α (A/A) MEFs (Figure 2-4B). These data suggest that eIF2α phosphorylation is required for induction of the cat-1 gene transcription by ER stress.

**Induction of XBP1s is largely dependent on eIF2α-P but not ATF4 during ER stress**

To investigate whether eIF2α phosphorylation is related to XBP1s induction, Western blot analysis was used to check XBP1s levels in eIF2α (A/A) MEFs treated for ER stress. As expected, eIF2α-P and ATF4 are not detectable in eIF2α (A/A) MEFs (Figure 2-5A). To our surprise, XBP1s levels were significantly lower in eIF2α (A/A) MEFs (Figure 2-5A). These data support our hypothesis that induction of ATF4 and XBP1s require a common signaling pathway. We show here that this pathway involves eIF2α phosphorylation. To further confirm this hypothesis, we tested the levels of XBP1s in PERK-deficient MEFs treated with ER stress (Figure 2-5B). PERK phosphorylates eIF2α during ER stress. Similar to the eIF2α (A/A) MEFs, PERK-deficient MEFs did not show an accumulation of the XBP1s protein (Figure 2-5B). BiP induction was similar in both wild type and the PERK deficient MEFs, and CHOP induction was lower in PERK deficient MEFs, in agreement with the loss of ATF4 induction (Figure 2-5B). These data suggest that eIF2α phosphorylation is required for accumulation of the XBP1s protein.
Figure 2-4. Phosphorylation of the translation initiation factor eIF2α is required for induction of cat-1 gene transcription by ER stress. A, eIF2α mutant MEFs eIF2α(A/A) and the wild type control MEFs eIF2α(S/S) were transfected with the cat-1 promoter luciferase reporter vector PA1.4N and then treated for ER stress or amino acid starvation. Protein concentration was measured to normalize for transfection efficiency. Luciferase activity of cell extracts was measured after 48 hours of transfection. B, Cat-1, SNAT2, and AS mRNA, and ribosomal RNA levels in eIF2α(A/A) and eIF2α(S/S) MEFs treated for ER stress or amino acid starvation were monitored by Northern blot analysis. Cells were treated for indicated times. Gene-specific probes were used to detect the RNAs. “star” stands for amino acid starvation; “ribo” stands for 18S ribosomal RNA. The DNA probes for Northern blot analysis were prepared by Yi Li the Northern blot analysis (B) was performed by Chuanping Wang.
FIGURE 2-4

A

![Graph showing luciferase activity compared to protein concentration for PA1.4N with different conditions: CON for AA starvation, AA starvation, -Tg, and +Tg. The x-axis represents S/S and A/A conditions.]

B

Northern blot

<table>
<thead>
<tr>
<th></th>
<th>CON 3</th>
<th>6</th>
<th>3</th>
<th>6</th>
<th>CON 3</th>
<th>6</th>
<th>3</th>
<th>6</th>
<th>(h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNAT2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ribo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A/A S/S
Figure 2-5. Induction of XBP1s is largely dependent on eIF2α-P but not ATF4 during ER stress. A–C, Western blot analysis of total cell lysates from eIF2α mutant MEFs eIF2α(A/A) and the wild type control MEFs eIF2α(S/S) (A), PERK-deficient MEFs and the wild type control MEFs (B), or the ATF4-deficient MEFs and the wild type control MEFs (C) treated with Tg for the indicated times. Blots were probed with antibodies for the indicated proteins. Tubulin was used as a loading marker for total cell lysates.
FIGURE 2-5

A

total cell lysates

<table>
<thead>
<tr>
<th></th>
<th>XBP1s</th>
<th>ATF4</th>
<th>eIF2α-P</th>
<th>eIF2α</th>
<th>tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S/S</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

total cell lysates

<table>
<thead>
<tr>
<th></th>
<th>PERK</th>
<th>XBP1s</th>
<th>ATF4</th>
<th>BiP</th>
<th>CHOP</th>
<th>tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PERK&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PERK&lt;sup&gt;−&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

total cell lysates

<table>
<thead>
<tr>
<th></th>
<th>XBP1s</th>
<th>ATF4</th>
<th>tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATF4&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATF4&lt;sup&gt;−&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
during ER stress. Since ATF4 is induced by eIF2α-P during ER stress, one possible involvement of eIF2α-P to XBP1s induction is that transcription of the XBP1 gene may be induced by ATF4. However, XBP1s levels were similar in ATF4 deficient and wild type MEFs (Figure 2-5C), which indicates that ATF4 is not involved in XBP1s induction and it is not the reason causing different levels of XBP1s in eIF2α (A/A) and eIF2α (S/S) MEFs during ER stress.

**Proteasome-mediated degradation is involved in XBP1s turnover during ER stress**

The different levels of XBP1s in eIF2α(S/S) and eIF2α(A/A) MEFs treated for ER stress can be due to differences in the degradation rate of the protein. It is known that proteasome-mediated ER stress associated protein degradation pathway (ERAD) is up-regulated by ER stress. When the proteasome pathway was inhibited by MG132 in C6 cells during ER stress, XBP1s levels were increased (Figure 2-6A), which indicates that XBP1s turn over is mediated by proteasome pathway during ER stress. Furthermore, it is shown that XBP1s has a relative short half-life of about 30 minutes (Figure 2-6A).

Next, we tested whether the rate of proteasome-mediated degradation of XBP1s is different in eIF2α(S/S) and eIF2α(A/A) MEFs, which produces different levels of XBP1s. When MG132 was applied to inhibit proteasome-mediated XBP1s degradation after it was induced to a high level after 8 hours ER tress treatment, XBP1s levels are still different in eIF2α(S/S) and eIF2α(A/A) MEFs (Figure 2-6B). This indicates that proteasome mediated degradation is not the cause of different levels of XBP1s in eIF2α(S/S) and eIF2α(A/A) MEFs, XBP1s levels would be similar in the two cell lines after blockage of proteasome pathway if proteasome mediated protein degradation causes different levels of XBP1s in the two cell lines.
**Figure 2-6.** Proteasome-mediated degradation is involved in XBP1s turn over during ER stress. *A* and *B*, Western blot analysis of total cell lysates from C6 cells (*A*) or eIF2α mutant MEFs eIF2α(A/A) and the wild type control MEFs eIF2α(S/S) (*B*). Cells were treated with Tg for 8 hours (T8) before addition of MG132 or CHX for indicated times. Blots were probed with antibodies for XBP1s and tubulin.
**eIF2α phosphorylation is partially required for XBP1 mRNA splicing during ER stress**

To further investigate the mechanism that causes different levels of XBP1s in eIF2α(S/S) and eIF2α(A/A) MEFs, we looked into the splicing event of XBP1 mRNA in the two cell lines by using RT-PCR. eIF2α(S/S) and eIF2α(A/A) MEFs were treated as indicated hours (Figure 2-7A), and total RNA samples were isolated from the untreated control and treated cells. A mixture of total cDNAs were synthesized by reverse transcription using poly(T) primers. To monitor the splicing of XBP1 mRNA, the splicing regions of XBP1 cDNAs were amplified by RT-PCR using the XBP1 specific primers flanking the splicing region. Levels of two other mRNAs were monitored by RT-PCR using gene-specific primers. As expected, cat-1 mRNA levels were lower in eIF2α(A/A) MEFs while BiP mRNA levels were similar in the two cell lines (Figure 2-7A). Induction of BiP gene transcription by ER stress is mainly mediated by ATF6 pathway (Hong et al., 2005; Baumeister et al., 2005). XBP1 mRNA was spliced in eIF2α(A/A) MEFs, although with somewhat lower efficiency in comparison with eIF2α(S/S) MEFs. As indicated in the chart, the percentage of unspliced XBP1 mRNA in eIF2α(A/A) MEFs is about 3 times of the percentage in eIF2α(S/S) MEFs (Figure 2-7A). These data suggest that eIF2α-P is partially required for splicing of the XBP1 mRNA. To further confirm the partial dependence of XBP1 mRNA splicing on eIF2α phosphorylation, we tested XBP1 mRNA splicing in PERK-deficient MEFs. In agreement with the eIF2α(A/A) MEFs, XBP1 mRNA splicing was partially inhibited (Figure 2-7B). These data suggest that eIF2α-P may be required for sufficient XBP1 mRNA splicing and full induction of XBP1 levels during ER stress.
Figure 2-7. eIF2α phosphorylation is partially required for XBP1 mRNA splicing during ER stress. A and B, XBP1 mRNA splicing was monitored by RT-PCR using primers flanking the splicing region and total RNA samples from eIF2α mutant MEFs eIF2α(A/A) and the wild type control MEFs eIF2α(S/S) (A) or PERK deficient MEFs PERK<sup>-/-</sup> and the wild type control MEFs PERK<sup>+/+</sup> (B) treated with Tg for the indicated times. And mRNA levels of indicated genes were also monitored by RT-PCR using gene specific primers and the same group of total RNA samples from eIF2α(A/A) and eIF2α(S/S) MEFs (A) or PERK<sup>-/-</sup> and PERK<sup>+/+</sup> MEFs (B). The PCR products were separated on 2% agarose gel and visualized with Eth-Br staining. The Eth-Br stained bands of unspliced XBP1 mRNA or both the unspliced and spliced were quantified. The percentage of unspliced mRNA in total XBP1 mRNA of each time point was calculated by using intensity of the bands.
FIGURE 2-7

A

![Western blot images of XBP1, cat1, BiP in S/S and A/A cell lines](image)

<table>
<thead>
<tr>
<th>Cell</th>
<th>S/S</th>
<th>A/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>h</td>
<td>CON</td>
<td>1</td>
</tr>
<tr>
<td>U/T%</td>
<td>93.1</td>
<td>18.1</td>
</tr>
</tbody>
</table>

B

![Western blot images of XBP1, cat1, CHOP, EDEM, BiP in PERK+/+ and PERK−/− cell lines](image)

<table>
<thead>
<tr>
<th>Cell</th>
<th>PERK+/+</th>
<th>PERK−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>h</td>
<td>CON</td>
<td>1</td>
</tr>
<tr>
<td>U/T%</td>
<td>86.7</td>
<td>16.5</td>
</tr>
</tbody>
</table>
**XBPI gene expression during ER stress may help cells to survive during ER stress**

Specific transcription factors have been found function in specific physiological processes. For example, it has been found that CHOP functions in ER stress induced apoptosis process (Zinszner et al., 1998). Cells will eventually go through apoptosis if they can’t survive ER stress. In order to find whether XBPI gene functions to help cells to survive or assists cells to go to apoptosis during ER stress, XBPI-deficient and the wild type control MEFs were treated with 400 nM Tg for 24 hours and checked for viable cells by microscopy. Both type of cells were observed to go to apoptosis during ER stress. However, after 24 hours treatment, only a few of XBPI-deficient MEFs are alive while a significant number of wild type control MEFs survived (Figure 2-8, lower panels). This suggests that induction of XBPI gene expression helps cells to survive in ER stress. The survived cells are much larger than the untreated same type of cells (Figure 2-8, left panels). The enlargement of the cells is probably resulted from adaptation to the stress.
Figure 2-8. XBP1 gene expression during ER stress may help cells to survive during ER stress. XBP1 deficient MEFs (XBP1−/−) or the wild type control MEFs (XBP1+/+) were incubated with medium containing 400 nM Tg (Tg) or only medium (CON) for 24 hours. Then phase-contrast photos were taken by using a camera connected to a microscope.
FIGURE 2-8

CON

Tg

treated for 24 hours
DISCUSSION

We have demonstrated that induction of cat-1 gene transcription by ER stress is mediated by ATF4 via its binding to the cis-acting element AARE and XBP1s via its binding to the putative cis-acting ERSE-II element in the cat-1 gene promoter. eIF2α phosphorylation is required to induce cat-1 gene transcription by ER stress due to induction of both ATF4 and XBP1s. Our data suggest that there is a crosstalk between the PERK-eIF2α-ATF4 pathway and the IRE1-XBP1 pathway of the 3 UPR signaling pathways. We have shown here that XBP1 mRNA splicing and accumulation of XBP1 mRNA levels require a signaling pathway mediated by eIF2α-P.

Cat-1 gene putative ERSE-II element

We have shown that both the cis-acting AARE and the putative ERSE-II element in the cat-1 gene promoter are involved in induction of cat-1 gene transcription by ER stress. To regulate transcription of ER stress target genes, ERSE elements interact with XBP1s or ATF6 with or without the transcription factor NF-Y. The core sequence of the cat-1 ERSE-II-like element is 5’-ATTGGTGCCCTGG-3’ (-25 to -11), which contains a NF-Y binding consensus 5’-ATTGG-3’ and a putative XBP1s or ATF6 binding site 5’-CCTGG-3’. We have shown here that XBP1s but not ATF6 induce cat-1 gene transcription. Therefore the CCTGG at the 3’-end of the putative ERSE-II element is likely to bind XBP1s. The NF-Y consensus sequence 5’-ATTGG-3’ is consistent with the NF-Y consensus sequence contained in the cis-acting ERSE-II element 5’-ATTGG-NCACG-3’. The putative XBP1s binding site 5’-CCTGG-3’ is similar with the antisense strand (5’-CGTGG-3’) of the XBP1s or ATF6 binding site consensus sequence (5’-CCACG-3’) in the consensus ERSE-II (Yamamoto et al., 2004). The ERSE consensus
(5’-CCAAT-N9-CCACG-3’) contains 9 base pairs between the NY-F and the ATF6 or XBP1s binding site consensus sequences. The cat-1 putative ERSE-II element can be described as 5’-ATTGG-N-CCTGG-3’. We have shown that the cat-1 ERSE-II-like element is partially responsible for activation of the cat-1 promoter by expression of XBP1s or Tg induced ER stress (Figure 2-3B and C). This is consistent with previously demonstrated phenomenon that XBP1s has stronger binding to ERSE-II and weaker binding to ERSE in comparison to ATF6 (Yamamoto et al., 2004). ERSE-II was first identified in the promoter region of Herp gene, which encodes for a ER membrane protein (Kokame et al., 2001). The Herp gene promoter contains the Herp ERSE-II (5’-ATTGG-N-CCACG-3’) and a ERSE, the both elements are bound by ATF6 to induce Herp gene transcription by ER stress.

**The crosstalk between the eIF2α-ATF4 and the IRE1-XBP1 pathways**

The only demonstrated crosstalk between the three UPR pathways is the connection between the IRE1-XBP1 and the ATF6 pathways via activation of XBP1 gene transcription by p50ATF6. Therefore, activation of the ATF6 pathway may subsequently induce or enhance activation of the XBP1 pathway. The evidence in the literature suggest that additional crosstalk may exist between these two pathways. It has been shown that enforced expression of ATF6α induces a number of ER stress target genes. However those UPR target genes can be induced to the similar levels in ATF6-deficient cells. These data suggest that compensatory mechanisms for ATF6 are present in the UPR signaling pathways (Lee et al., 2003). Since cells deficient in both XBP1 and ATF6α have almost totally impaired induction of the ER stress target genes, it suggests that XBP1 can functionally compensate for ATF6 (Lee et al., 2003).
We have shown here that cat-1 transcriptional induction by ER stress requires eIF2α phosphorylation due to induction of ATF4 and induction of XBP1s in a manner dependent on eIF2α phosphorylation. This indicates there is a crosstalk between the eIF2α-ATF4 and the IRE1-XBP1 pathways. The mechanism of ATF4 induction by eIF2α phosphorylation has been revealed (Vattem and Wek, 2004). We investigated the mechanism of XBP1s induction on eIF2α-P. We found that both spliced XBP1 mRNA and total XBP1 mRNA (confirmed by qPCR data not shown, qPCR by Mithu Majumder using cDNA samples and primers from Yi Li) is less in ER stress treated eIF2α (A/A) MEFs in comparison to treated eIF2α (S/S), which may indicate that XBP1 gene transcription and the XBP1 mRNA splicing partially requires eIF2α-P. One possible explanation is that the unspliced XBP1 mRNA is unstable in Tg-stressed cells before engaged in splicing. A reasonable hypothesis is that eIF2α phosphorylation enhances splicing of the XBP1 mRNA and in its absence the unspliced mRNA is being degraded. This hypothesis is supported by our findings of inefficient splicing of the XBP1 mRNA in A/A cells. Our working hypothesis is that inefficient splicing may be the cause of less total XBP1 mRNA in eIF2α (A/A) MEFs. An alternative explanation for the decreased total XBP1 mRNA levels in A/A cells is reduced transcription of the XBP1 gene. Transcriptional control of XBP1 during ER stress is via the transcription factor ATF6. However, as a ATF6 dependent gene during ER stress, BiP mRNA levels are similar in eIF2α (S/S) and eIF2α (A/A) MEFs (Figure 2-7A), which indicates that dependence of XBP1s induction on eIF2α-P is unlikely related with transcriptional regulation. This is further supported by BiP levels in PERK deficient and the wild type control MEFs (Figure 2-7A).
Regulation of cat-1 mRNA levels during ER stress

It has been determined here that the cat-1 mRNA level increases to 5-fold after 9 hours of treatment by ER stress and declines thereafter. Similar regulation was observed previously for the accumulation of cat-1 mRNA levels during amino acid starvation (Fernandez et al., 2003). Induction of cat-1 gene transcription during amino acid starvation is mediated by ATF4, and attenuation of transcriptional activation is mediated via the transcriptional repressor ATF3 (Lopez et al., 2007). During ER stress, induction of cat-1 gene transcription involves ATF4 and XBP1S. Because binding of ATF4 to the cat-1 gene promoter proceeds binding of XBP1s, we conclude that XBP1s mediates sustained transcriptional activation of the cat-1 gene during ER stress.

In the three UPR signaling pathways, the ATF6 pathway targets rapid responses while the IRE1-XBP1 pathway is responsible for a sustained response (Yamamoto et al., 2004). The eIF2α-ATF4 pathway covers the early response and possibly the late response to ER stress. Inhibition of general protein synthesis and cell cycle arrest are critical early events happening immediately after the onset of ER stress, which is mediated by phosphorylation of eIF2α by PERK. Apoptosis is the last event if the intensity of stress exceeds the adaptive response of the cells. Our data indicate that the transcription factors ATF4 and XBP1s are involved in transcriptional induction of the cat-1 gene during ER stress. Theses two transcription factors bind to the cat-1 promoter region sequentially to induce cat-1 gene transcription.

Besides inducing CHOP transcription, ATF4 also targets genes involved in the adaptive response to stress. AS gene is a direct target of ATF4 during ER stress (Siu et al., 2001; Siu et al., 2002). By supplying amino acids, asparagine synthase helps cells to
adapt to ER stress. Transcription of eIF4E binding protein 1 (4E-BP1) gene is induced by ATF4 during ER stress (Yamaguchi et al., 2008). 4E-BP1 binds to eukaryotic initiation factor 4E (eIF4E) to suppress cap-dependent translation. 4E-BP1 has been shown to contribute to the maintenance of pancreatic beta cell homeostasis during ER stress (Yamaguchi et al., 2008). It has been demonstrated that XBP1s mainly regulates transcription of ER resident chaperon genes such as BiP, p58(IPK), ERdj4, as well as EDEM (Lee at al., 2003). In agreement with the recent finding by Peter Walter’s group that sustained activation of IRE1 promotes cell survival (Lin et al., 2007), we have observed that XBP1s function to help cells to survive ER stress. After 24 hours of Tg treatment, there were more surviving wild type MEFs as compared to XBP1 deficient MEFs (Figure 2-8).

**Physiological functions of CAT-1 during ER stress**

CAT-1 protein is induced during ER stress. As a response of adaptation of ER stress, induced CAT-1 may supply cationic amino acids for the needs of synthesis of necessary proteins. However, the absolute importance of CAT-1 induction during ER stress remains unclear. Because cat-1 gene up-regulation is maintained at late ER stress, it’s likely that CAT-1 protein is not related with availability of amino acids in the cells, but has additional functions. Since eIF2α phosphorylation results in translational repression of cyclin D1 and subsequently cell cycle arrest in the G1 phase during ER stress, the CAT-1 protein might be involved in cell cycle reentry if the cells can survive ER stress, or alternatively cells may go into apoptosis if they can’t survive ER stress in a NO-dependent pathway. It is known that the level of CAT-1 protein increases in proliferating cells (Yoshimoto et al., 1992). However, because CAT-1 is a transporter for arginine
which is the substrate for NO-synthesis by the NO synthetases (NOS), it’s possible that increased CAT-1 protein levels during ER stress cause increased NO levels, which may lead cells to apoptosis. To further investigate the physiological function of CAT-1, fluorescence activated cell sorting (FACS) can be used to analyze cell cycle progression of MEFs deficient for cat-1 during ER stress, and NOS activities and CAT-1 level can be analyzed to check if there is a correlation among CAT-1 level, intracellular NO amount, and cell cycle progression or apoptosis.

**Future studies**

Based on what we have shown in this chapter, we will investigate the following questions in next chapter: 1) the transcription factor involved in down-regulation of cat-1 gene transcription; 2) the mechanism of down-regulation of cat-1 gene transcription at late stage of ER stress.
CHAPTER 3

C/EBPβ LAP ISOFORM ACTIVATES AND LIP ISOFORM INHIBITS CAT-1 GENE TRANSCRIPTION VIA THE AARE DURING ER STRESS

INTRODUCTION

Induction of cat-1 gene transcription by amino acid starvation is mediated by ATF4 via its binding to the cis-acting element AARE in the proximal region of the cat-1 gene promoter (Fernandez et al., 2003; Lopez et al., 2007). As it demonstrated in chapter 2, induction of cat-1 gene transcription by ER stress is mediated by the transcription factors ATF4 and XBP1s, ATF4 binds to the AARE and XBP1s binds to the putative ERSE-II.

According to our hypothesis, positively acting transcription factors bind to the cat-1 promoter region to up-regulate transcription during the early stage of ER stress which yields high cat-1 mRNA levels. Negatively acting transcription factors bind later in the stress response, in consistent with lower cat-1 mRNA levels. This mechanism of transcriptional induction followed by transcriptional attenuation during ER stress is a common theme of regulating the levels of important stress-response proteins. We have previously shown that ATF3 is the transcription factor to down-regulate cat-1 gene transcription during amino acid starvation (Lopez et al., 2007). The mechanism of down-regulation of cat-1 gene transcription during ER stress is yet to be determined.

Transcription factors can be regulated by stress at the levels of transcription, translation, nuclear localization, post-translational modification, and stability (Schroder and Kaufman, 2005a). The transcription factor ATF2 is activated by post-translational modification during amino acid starvation (Averous et al., 2004); ATF4 is induced by
translational up-regulation during both ER stress and amino acid starvation (Vattem and Wek, 2004); XBP1s is induced by up-regulated splicing of its mRNA during ER stress (Rutkowski and Kaufman, 2004). CHOP is involved in transcriptional regulation of genes involved in the events during late stage of ER stress, its transcription is activated by ATF4 (Schroder and Kaufman, 2005).

C/EBPβ has been shown to be involved in transcriptional regulation of ER stress target genes in combination with the major activators. Transcription of the mammalian C/EBPβ gene generates a single transcript that can encode several N-terminal truncated C/EBPβ isoforms (Calkhoven et al., 2000; Xiong et al., 2001). It has been revealed that all the isoforms can be generated by translation initiated from different AUG start codons (Figure 3-1; Calkhoven et al., 2000). It has also been suggested that the small isoforms can be generated by proteolytic cleavage of the large isoforms (Baer and Johnson, 2004). C/EBPβ proteins belong to the leucine zipper transcription factor family. The C-terminus of every C/EBPβ isoform contains a leucine zipper domain for dimerization with another leucine zipper protein and a basic region for DNA binding (Baer and Johnson, 2004). Each of the LAP isoforms contains an activation domain at their N-terminus while the LIP isoform does not since LIP is N-terminal truncated. It has therefore suggested that LAP activates gene transcription and LIP inhibits gene transcription. We have previously shown that C/EBPβ, in combination with ATF4, is involved in induction of cat-1 gene transcription during amino acid starvation (Lopez et al., 2007). It has also been shown that C/EBPβ is involved in mediating activation of AS gene transcription by both amino acid starvation and glucose deprivation (Siu et al., 2001). Furthermore, it has been
Figure 3-1. Amino acid sequence of the full length C/EBPβ and the predicted translation start sites. Every red “M” stands for the start amino acid for a predicted C/EBPβ isoform generated by translation initiated from a corresponding “AUG” codon in the mRNA sequence. The estimated molecular weight by SDS-PAGE for each of the predicted isoforms is labeled.
FIGURE 3-1

The Isoforms of C/EBPβ

MHRLLAWDAACLPPPAAFRPMEVANFYYPEDCRAYGAKAARAAPRAA 1st (38 kDa) 2nd (34 kDa)
EPAIGEHERAIDFSPYLEPLAPADAAPAPAHHDFLSDLFADDYGAKPSKK

PADDYGYVSLGRAGAKAAPPACPFPNPAAALKAEPGFEPADCKRADDAPAM 3rd (20 kDa)
AAGFPFALRAYLGYQATPSGSGSLSTSSSSPPGTPSPADAKAAPAACF
AGPAPPAKAKKTVDLSEYKMRRERNIAVRKSRDKAKMNRLTQH 4th (14 kDa)
KVLELTAEQLQKVEQLSRELSTLRNFLKQKLPELLASAGHC*
demonstrated that forced expression of LAP increases AS gene promoter activity, while forced expression of LIP inhibits amino acid and glucose deprivation-induced AS promoter activation (Siu et al., 2001). Recently, LAP was shown to also inhibit AS gene promoter activity (Thiaville et al., 2008). We tested the hypothesis that LIP may be involved in attenuation of induction of cat-1 gene transcription by ER stress.

Our studies show the following: (i) The Levels of C/EBPβ LIP isoform is dramatically up-regulated during late stage of both ER stress and amino acid starvation while the level of LAP isoform is increased slightly; (ii) The LAP isoform activates and the LIP isoform inhibits cat-1 promoter activity via the AARE element; (iii) Electrophoretic mobility shift assay indicates that C/EBPβ isoforms interact with the cat-1 AARE in vitro; (iv) LIP expression during late ER stress promotes cell survival.
MATERIALS AND METHODS

Plasmid constructs

The PA1.4N luciferase reporter vector was described in chapter 2. Cat-1 AARE luciferase reporter (also called pGL3-3xAARE) and cat-1 AARE(mut2) luciferase reporter (also called pGL3MUT2) vectors were described previously (Lopez et al., 2007). The LIP expression vector was constructed by ligating a PCR-amplified DNA fragment containing the LIP ORF into pcDNA3.1(−). The LAP expression vector expresses C/EBPβ LAP2 isoform, it was a gift from Dr. Richard Hanson (Department of Biochemistry, Case Western Reserve University).

Cell culture and transfection

C6 rat glioma cells and mouse embryonic fibroblast (MEF) cells were cultured and transfected as described in chapter 2.

Western blot analysis

Western blot analysis was done as described in chapter 2. Monoclonal antibody against the N-terminus of C/EBPβ LAP was from Biolegend (Cat. No., 626002). Other antibodies are as described in chapter 2.

Cell viability assay

Cells were grown up to about 90% confluence in 6-well plates, and then ER stress was induced by incubating cells in fresh medium containing 1 µg/ml ER stress inducer tunicamycin (Tu) for indicated times. Triplicate wells for each time point were set up. Wells without cells were set up as negative controls to determine the background fluorescence. Wells with untreated-cells were used as vehicle controls. Resazurin was used to measure cell viability. Briefly, after taking off the medium containing ER stress...
inducer, cells were washed twice with warm PBS and incubated in cell culture medium containing no serum but 0.011 mg/ml resazurin for 2 hours at 37 °C. Immediately after incubation, 100 µl of the incubated medium was transferred from each well of the 6-well culture plates to a well of a 96-well plate containing 50 µl 3% SDS for stabilizing generated fluorescence. For recording data, fluorescence intensity was read in a plate reader with fluorescence filter setting as 535 nm filter for excitation and 590 nm filter for emission. During the assay process, the plates were always kept in dark to avoid prolonged exposure to light for reducing background fluorescence since resazurin is light-sensitive.

**Electrophoretic mobility shift assay (EMSA)**

EMSA assay was performed as described previously (Lopez et al., 2007). Briefly, a double-stranded DNA oligonucleotide containing the wild type rat cat-1 AARE (sense strand sequence: 5'-GCGCGGCTGATGAAACCGGCCTCGGAT-3') or mutated rat cat-1 AARE (sense strand sequence: 5'-GCGCGGCTGAATAGCCGCTCGGAT-3') was labeled with [γ-32P] and used as the probe. Nuclear extracts were isolated as described in Western blot analysis in chapter 2 from C6 cells treated with 400 nM ER stress inducer Tg. The labeled oligonucleotide (1.5 pmol) was incubated with 5 µg of nuclear extracts in 10 µl of 1 x binding buffer containing 20 mM Hepes-KOH (pH 7.4), 100 mM NaCl, 1 mM EDTA, 2.5% glycerol and 0.01 mg/ml poly(dI-dC) for 30 minutes at 4 °C. After the binding reaction, the reaction mixtures were resolved on 4% non-denaturing polyacrylamide gels containing 0.5 x TBE containing 5 mM Tris-HCl (pH8.0), 44.5 mM boric acid and 1 mM disodium EDTA. Gels were dried and exposed to films. In the case of antibodies are needed, 2 µg of C/EBPβ polyclonal antibodies (C-19, sc-150; Santa
Cruz Biotechnology) were added to binding reaction mixtures and incubated for 30 minutes at 4 °C prior to addition of the labeled oligonucleotide.
RESULTS

C/EBPβ LIP is up-regulated at late stage of ER stress and amino acid starvation

The protein levels of the C/EBPβ isoforms in C6 cells treated for ER stress were monitored by Western blot analysis. We used antibodies against a common peptide fragment of the C-terminus of the proteins to detect all the isoforms, and antibodies against a peptide fragment in the N-terminus of the larger isoforms to detect the LAP isoforms. Two major and one minor protein bands were detected by the anti-C-terminus antibodies from both total lysates and the nuclear extracts from C6 cells treated with Tg (400 nM) for ER stress or amino acid starvation. The estimated molecular weight of the proteins corresponding to the major bands are 34 kDa and 16 kDa in comparison to the protein markers (Figure 3-2A and B). There was only one major protein band detected by the anti-N-terminus antibodies, at the same size of 34 kDa that was detected by the anti-C-terminus antibodies (Figure 3-2, A and B). This indicates that the 16 kDa protein is a N-terminal truncated isoform in comparison to the 34 kDa protein. The minor band about 38 kDa was seen at longer exposures of the films (data not shown). The 38 kDa and the 34 kDa proteins are well matched with the LAP isoforms according to the literature. The 16 kDa protein has been confirmed corresponding to the LIP isoform that is generated by translation initiated from the third AUG start codon (explained in detail in chapter 4). The LIP isoform is dramatically increased in the nucleus at 18 and 24 hour ER stressed or amino acid starved cells while the levels of the LAP isoform are increased slightly in comparison with the controls (Figure 3-2B). This suggests that the LIP isoform may function at the late stage of ER stress. Its higher levels correlate with down-regulation of expression of ER stress-induced genes. The regulation of the CHOP protein was shown
Figure 3-2. C/EBPβ LIP is up-regulated at late stage of ER stress and amino acid starvation. A and B, Western blot analysis of total cell lysates (A) or nuclear extracts (B) from C6 cells treated for ER stress or amino acid starvation for the indicated times. Blots were probed with antibodies against C-terminus of C/EBPβ isoforms for detecting all the C/EBPβ isoforms, antibodies against N-terminus of LAP for detecting the long isoforms, or antibodies for CHOP or the indicated proteins. Tubulin was used as a loading marker for total cell lysates, HDAC1 was used as a loading marker for nuclear extracts. “AA starvation” stands for amino acid starvation.
FIGURE 3-2

A
C6 cell total lysates

anti-C-terminus
C/EBPβ
anti-N-terminus
CHOP
Tubulin

0 6 18 24 0 6 18 24 (h)
Tg AA starvation

B
C6 nuclear extracts

anti-C-terminus
C/EBPβ
anti-N-terminus
CHOP
HDAC1

0 6 18 24 0 6 18 24 (h)
Tg AA starvation
as a control for treatment of the cells.

**C/EBPβ LAP activates cat-1 promoter activity and LIP inhibits ER stress induced cat-1 promoter activity**

The LAP isoforms contain activation domains at their N-terminus while the LIP isoform does not since LIP is N-terminal truncated. So it has been suggested that the LAP activates gene transcription and the LIP inhibits gene transcription. However, it has been shown that both the LAP and the LIP inhibit AS gene promoter activity (Thiaville et al., 2008). To investigate the possible functions of the isoforms in cat-1 gene transcription, the cat-1 luciferase reporter PA1.4N was cotransfected with either individual expression vector expressing ATF4, LAP, LIP, both C/EBPβ isoforms or each of the expression vectors in combination with ATF4 expression vector as indicated (Figure 3-3A). Our data indicate that the LAP isoform induces luciferase activity driving by cat-1 promoter to the similar fold as induced by ATF4, but cotransfection of LAP with ATF4 does not have synergistic effect to cat-1 promoter activity, and the LIP isoform inhibits ATF4 induced cat-1 promoter activity (Figure 3-3A). Interestingly, when PA1.4N is cotransfected with C/EBPβ vector expressing both LAP and LIP isoforms, the luciferase activity is between the only LAP and only LIP expression vector cotransfection. This indicates that these two isoforms have opposite effects on cat-1 promoter activity. To further investigate the effect of the LIP isoform to cat-1 promoter activity during ER stress, PA1.4N was transfected into C/EBPβ-deficient (C/EBPβ−/−) and the wild type control MEFs (C/EBPβ+/+)/, then treated for indicated times and assayed for luciferase activities (Figure 3-3B). The result shows that the induction of the luciferase activity by ER stress is suppressed in the wild type MEFs after 24 hours ER stress and LIP is elevated at this
Figure 3-3. C/EBPβ LAP activates cat-1 promoter activity and LIP inhibits ER stress induced cat-1 promoter activity. A and B, C6 cells were transfected with PA1.4N along with expression vectors for indicated proteins (A), or either C/EBPβ deficient MEFs C/EBPβ−/− (B, right panel) or the wild type control MEFs C/EBPβ+/+ (B, left panel) was transfected with PA1.4N and treated for ER stress for indicated times. Expression plasmid for β-galactosidase (β-gal) was included or protein concentration was measured to normalize for transfection efficiency as indicated. Enzymatic activities of cell extracts were measured after 48 hours of transfection.
FIGURE 3-3

A

![Graph A showing relative luciferase activity for different conditions](image)

B

![Graph B showing relative luciferase activity for C/EBPbeta+/- and C/EBPbeta-/-](image)
time (Figure 3-3B, left panel), the suppression is reversed in the C/EBPβ deficient MEFs that do not express LIP at all (Figure 3-3B, right panel). The expression pattern of the C/EBPβ isoforms in MEFs is similar to C6 cells (see Chapter 4). LIP isoform is dramatically increased at the late stage of ER stress while LAP level is increased slightly. This indicates the LIP isoform functions to inhibit cat-1 gene transcription during ER stress.

**C/EBPβ LIP inhibits ATF4-induced and the LAP activates expression of a reporter containing the cat-1 AARE in the enhancer site**

We next investigated whether LAP and LIP function via the cat-1 AARE or a different element in cat-1 promoter region. pGL3-promoter luciferase vector contains a SV40 promoter and the vector is commonly used to test whether a putative enhancer or silencer is functional. If LAP or LIP regulates the cat-1 promoter via an element other than the cat-1 AARE, then LAP or LIP would not regulate SV40 promoter activity when the AARE is placed at the enhancer position of the pGL3-promoter vector. When cat-1 AARE reporter was cotransfected with both ATF4 and LIP expression vectors, LIP suppressed ATF4-induced luciferase activity (Figure 3-4A). The luciferase activity decreases along with increase in the amount of LIP, indicating that ATF4 induced promoter activity is specifically suppressed by the LIP isoform. When the cat-1 AARE reporter was cotransfected with LAP, the luciferase activity increased while LAP does not affect the empty pGL3-promoter vector activity. These data indicate that LAP induces SV40 promoter activity via the cat-1 AARE (Figure 3-4B). When the AARE element is mutated in the cat-1 AARE reporter, there is no suppression when there is no ATF4 mediated induction (Figure 3-4C). This further confirmed that LIP negatively
Figure 3-4. Both C/EBPβ LAP activates the heterogeneous SV40 promoter and LIP inhibits ATF4 induced SV40 promoter via the cat-1 AARE.

A-C, C6 cells were transfected with cat-1 AARE luciferase reporter (A-C) or cat-1 AARE(mut2) luciferase reporter (C) along with expression vectors for C/EBPβ isoforms or in combination with ATF4 expression vector as indicated. Expression plasmid for β-galactosidase (β-gal) was included or protein concentration was measured to normalize for transfection efficiency as indicated. Enzymatic activities of cell extracts were measured after 48 hours of transfection.
FIGURE 3-4

A

relative luciferase activity (Fluc/Gal)

B

cat-1 AARE reporter

C

cat-1 AARE reporter

relative luciferase activity (Fluc/protein)

C

cat-1 AARE reporter

relative luciferase activity (Fluc/protein concentration)

89
regulates SV40 promoter activity induced by ATF4 via the cat-1 AARE.

**C/EBPβ isoforms bind to the cat-1 AARE element in vitro**

Since C/EBPβ LAP and LIP can affect SV40 promoter activity via the cat-1 AARE element, it’s likely that LAP and LIP may interact with this element. EMSA was used to investigate if C/EBPβ isoforms directly interact with the cat-1 AARE element. The double stranded-DNA probe either containing a wild type AARE or a mutated AARE was used to check whether C/EBPβ isoforms in nuclear extracts from C6 cells treated for ER stress can specifically form complexes with the AARE. The results demonstrate that the cat-1 AARE element specifically forms two different size complexes with nuclear protein factors in C6 cells subjected to ER stress, a larger heavy complex and smaller lighter complex as indicated by arrowhead (Figure 3-5). The larger complex likely contains more factors. When the antibodies against the C-terminus of the C/EBPβ isoforms were added in the reaction mixtures, both complexes formed without antibodies decrease and two new complexes were formed as indicated by empty arrowheads (Figure 3-5). This means binding of the antibodies to the C/EBPβ proteins prevents forming of the complexes formed without antibodies, the two larger complexes formed contain antibodies (Figure 3-5). This indicates that C/EBPβ isoforms are contained in the both complexes formed without adding antibodies. Forming of two different complexes without antibodies may reflect that different C/EBPβ isoforms are involved in forming different complexes. According to the pattern of cat-1 mRNA levels and the patterns of shifted complexes by the antibodies, the larger complex may contain the LIP isoform and the smaller complex may contain the LAP isoform. To absolutely confirm which isoform is contained in which complex, specific antibodies against the N-terminus of the LAP
Figure 3-5. C/EBPβ isoforms bind to the cat-1 AARE element in vitro. Radioactive labeled wild type or mutated cat-1 AARE and nuclear extracts from C6 cells treated for ER stress for indicated times were used for electrophoretic mobility shift analysis (EMSA). Antibodies against C-terminus of C/EBPβ isoforms were added as indicated to check for their involvement.
FIGURE 3-5

AARE probe: + - + + + + + + + +
Mutant probe: - + - - - - - - -
C/EBPβ antibody: - - - - - + + + +
C6 nuclear extract: - ⬤ 3 9 18 24 ⬤ 3 9 18 24 (h)

lighter exposure
isoform can be used for EMSA.

**C/EBPβ LIP mediates better survival of cells during ER stress**

To determine the physiological function of LIP induction during ER stress, C/EBPβ deficient MEFs (C/EBPβ−/−), stable LIP expressing C/EBPβ−/− [C/EBPβ−/−(LIP)], and the wild type control MEFs (C/EBPβ+/+) were treated with 1 μg/ml Tunicamycin (Tu) for 36 hours and viability of each type of cells was monitored by microscopy and the reazurin assay method. The expression patterns of the C/EBPβ isoforms in C/EBPβ+/+, C/EBPβ−/−, and C/EBPβ−/−(LIP) MEFs are demonstrated in Chapter 4. Since LIP expression pattern in C/EBPβ−/−(LIP) MEFs is well correlated with its expression pattern in C/EBPβ+/+ during ER stress. Viability of C/EBPβ−/−(LIP) MEFs can reflect the LIP function during cell survival. It is clearly observed by microscope that there are more surviving C/EBPβ−/−(LIP) MEFs than C/EBPβ−/− MEFs. Furthermore, C/EBPβ−/− MEFs survived better than control C/EBPβ+/+ after 36 hours of ER stress induced by 1 μg/ml Tu (Figure 3-6A). To quantitate the viability of each type of cells, the reazurin viability assay method was used. When cell culture medium containing reazurin is incubated with cells, viable cells can reduce reazurin to resorufin. Resorufin has different light absorbance and fluorescence properties than reazurin. Thus either absorbance or fluorescence measurements can be used to monitor the amount of resorufin. Viable cells are expected to have higher fluorescent activity and absorbance. The viability assay indicated that about 20% more C/EBPβ−/−(LIP) MEFs survived in comparison with the wild type control MEFs, which is consistent with microscopy results (Figure 3-6B). All together, these data indicate that LIP may help cells to survive during ER stress.
Figure 3-6. C/EBPβ LIP functions to help cells to survive during ER stress. A, C/EBPβ deficient MEFs C/EBPβ−/−, stable LIP expressing C/EBPβ−/− C/EBPβ−/− (LIP), or the wild type control MEFs C/EBPβ+/+ were incubated with medium containing 1 µg/ml tunicamycin (Tu) or empty vehicle (CON) for 36 hours. Then phase-contrast photos were taken by using a camera connected to a microscope. B, After treated with 1 µg/ml Tu for 36 hours, viability of the cells were measured by resazurin method as described in the “materials and methods”. The viable value of the control cells of each type cells were set up as 100%, and the percentage of viable value of treated each type of cells to the control cells were plotted.
FIGURE 3-6

A

CON

Tu (1µg/ml)

C/EBPβ<sup>+/+</sup> C/EBPβ<sup>-/-</sup> C/EBPβ<sup>-/-</sup>(LIP)

B

relative viability (% of the control)

C/EBPβ<sup>+/+</sup> C/EBPβ<sup>-/-</sup> C/EBPβ<sup>-/-</sup>(LIP)

CON

1 µg/ml Tu
DISCUSSION

We have found that (i) C/EBPβ LIP levels increase during late ER stress; (ii) The LAP isoform activates and the LIP isoform inhibits cat-1 promoter activity via the AARE element; (iii) It may means that the LIP isoform inhibits cat-1 promoter activity by competing with ATF4 to bind to the AARE; and (iv) physiologically, the LIP isoform helps cell survival during ER stress.

Generation of C/EBPβ LIP

The LIP isoform we have seen here is likely generated by translation initiated from a AUG codon. If this is true, the translation would be initiated from the third or fourth AUG codon since the LIP isoform is estimated about 16 kDa on our SDS-PAGE. Protein translated from the third mouse C/EBPβ AUG start codon was estimated as 20 kDa and from the forth AUG codon was estimated as 14 kDa. In chapter 4, we will determine whether the 16 kDa C/EBPβ isoform is generated by translation initiation from the third or fourth AUG codon. And the translation mechanism can be further studies to investigate how LIP is generated. It has been shown that generation of the truncated isoforms (21 and 14 kDa) is C/EBPα dependent in liver tissue in vivo by using C/EBPα knockout mice, whereas they are present in the liver of the wild-type animals (Burgess-beusse et al., 1999). More interestingly, it has been shown that increase of C/EBPα expression leads to an increase of the 21 kDa truncated C/EBPβ isoform production in cultured cells (Burgess-beusse et al., 1999). It also has been revealed that the expression of truncated C/EBP isoforms requires the small ORF between the first and second AUG start codon and enhanced eIF activity as well (Calkhoven et al., 2000). A mRNA binding protein, CUG-repeat RNA-binding protein 1 (CUGBP1), is required for IFNβ mediated
induction of the C/EBPβ LIP (Dudaronek et al., 2007). CUGBP1 has been shown to form complex with eIF2 in liver tissue (Wang et al., 2008). It would be very interesting to find out the mechanism of LIP generation during ER stress besides determining whether it is generated by translation or not.

**Inhibition of cat-1 gene transcriptional induction**

We have demonstrated that C/EBPβ LAP activates SV40 promoter activity via the cat-1 AARE element and the LIP isoform inhibits ATF4-induced SV40 promoter activity. It has also been demonstrated that both the LAP and the LIP isoforms bind to the cat-1 AARE element. It has been questioned that the transcriptional inhibition by LIP isoform is via its interaction with the AARE or ATF4. The direct interaction in the C/EBP family has been demonstrated by immunoprecipitation (Nerlov, 2007). It’s possible that C/EBPβ LIP forms heterodimers with another leucine zipper transcription factor ATF4, XBP1s, or ATF6 (Figure 3-8). By prediction, ATF4 has the strongest affinity with the C/EBPβ isoforms (Figure 3-8). However, theoretically, homodimers formed by leucine zipper domains are more stable than heterodimers formed by leucine zipper domains. It would be minor population if heterodimers of ATF4 and C/EBPβ LIP do exist. On one single AARE molecule, it’s likely that C/EBPβ LIP homodimers compete with homodimers of ATF4 or other positive regulators to inhibit transcriptional induction. However, as a key issue of transcriptional regulation by transcription factors, it needs to be further confirmed exactly that what isoforms of C/EBPβ interact with the cat-1 AARE. By EMSA assay, we have seen that two different size of C/EBPβ isoforms bind to the cat-1 AARE. This will be further investigated in chapter 4.

It has been previously demonstrated that C/EBPβ is involved in mediating up-
Figure 3-7. Predicted interaction between the leucine zipper domain of C/EBPβ isoforms and the leucine zipper domain of another ER stress involved bZIP transcription factor ATF4, XBP1s, or ATF6. The number after each transcription factors indicates the position of the leucine zipper domain in the full amino acid sequence of the protein. The stars indicate identical amino acid residues in the two sequences comparing. The rectangles in red line indicate possible interaction and the red rectangle in red dashed line indicates possible weaker interaction between the two sequences.
FIGURE 3-7

with ATF4

C/EBPβ (257-285): LTAENERLQKVEQLSRELSTLRLNFLKQQL
ATF4 (304-332): LTGECKELEKNEALKEKADSLAKEIQYL

C/EBPβ (257-275): LTAENERLQKVEQLSRELSTL

with XBP1s

C/EBPβ (257-275): LTAENERLQKVEQLSRELSTL
XBP1s (112-133): LLEQVVDLEENQKLLEQQL

with ATF6

C/EBPβ (257-275): LTAENERLQKVEQLSRELSTL
ATF6 (343-364): LEARLKAALSEQKKEKENGSL

C/EBPβ (257-275): LTAENERLQKVEQLSRELSTL
ATF6 (295-316): LSVTKPVIQSATRSMGDIAVL
regulation of cat-1 gene transcription during amino acid starvation (Lopez et al., 2007). By functional assay and confirming interaction of the C/EBPβ isoforms with the cat-1 AARE, we have confirmed that the involvement of C/EBPβ isoforms in cat-1 gene regulation during ER stress. It has been known that the transcription coactivator p300 plays a transactivation role mediated by C/EBPβ. P300 and its close-related homologue protein CREB-Binding Protein (CBP) function to link individual transactivators via protein-protein interactions to the basal transcription machinery (Mink et al., 1997). It would be interesting to investigate whether p300/CBP is involved in cat-1 gene transcriptional regulation by ER stress.

Physiological function of LIP during ER stress

Our data indicates that the LIP isoform help cells to survive during ER stress. This is consistent with other previous findings. It has been shown that C/EBPα and C/EBPβ isoforms control differentiation and proliferation in a number of cell types (Calkhoven et al., 2000). Several studies revealed that C/EBPα has a dominant anti-proliferation function (Diehl et al., 1996; Wang et al., 2008). The C/EBPβ LIP appears to be associated with increased tissue growth and cell proliferation, that elevated levels of LIP in mouse mammary tumors and human breast cancer have been seen (Zahnow et al., 1997). Forced constitutive expression of truncated C/EBPβ or C/EBPα in 3T3-L1 cells indicates that up-regulation of truncated C/EBP isoforms prevents proliferation arrest and contact inhibition, and furthermore interferes with terminal adipogenic differentiation (Calkhoven et al., 2000). It’s possible that up-regulated LIP isoform during stress conditions may function to increase cell proliferation property and thus help cells to survive. It seems to be controversial that both C/EBPβ−/− MEFs and the C/EBPβ−/−(LIP)
MEFs survive better than the wild-type control C/EBPβ+/+ MEFs after 36 hours ER stress treatment. It would be expected that wild type C6 cells survive better than C6 cells with C/EBPβ gene expression knockdown since LAP expression is only increased slightly in C6 cells. What we have observed based on MEFs is related with LAP expression in the cell type. To clearly address this question, only LAP expressing MEFs [C/EBPβ−/−(LAP)] have to be established. Attempts were made to establish stable C/EBPβ−/−(LAP) MEFs with constant LAP expression, but the cells were not viable. This is probably related to the function of the LAP isoform. A inducible expression vector can be tried to establish cell lines for further investigation.

Future studies

Based on what we have revealed in this chapter, the following questions will be studied in next chapter: 1) the regulation of C/EBPβ isoforms in animal models, to confirm that the regulation of the C/EBPβ isoforms actually happens in vivo; 2) the mechanism of generation of the LIP isoform; 3) the mechanisms of regulation of protein levels C/EBPβ isoforms; 4) whether induction of other genes mediated by AARE by ER stress are also inhibited by LIP.
CHAPTER 4
DIFFERENTIAL CONTROL OF THE C/EBPβ PRODUCTS LAP AND LIP
AND THE REGULATION OF GENE EXPRESSION DURING
THE RESPONSE OF ER STRESS

INTRODUCTION

The accumulation of unfolded proteins in the endoplasmic reticulum triggers a complex regulatory program that involves regulation of both transcription and translation (Schroder and Kaufman, 2005a). In the early part of the unfolded protein response (UPR), there is a decrease in protein synthesis and increased expression of proteins that protect cells from stress (Schroder and Kaufman, 2005a). In contrast, during prolonged stress, the UPR can change to a proapoptotic program, leading to the expression of proteins that promote cell death (Eizirik et al., 2008; Yoshida, 2007; Zhang and Kaufman, 2006).

The regulatory events in the UPR are linked to a key sensor within the ER lumen, the chaperone GRP78/BiP (Bertolotti at al., 2000). In stressed cells, the binding of BiP to unfolded proteins lowers the level of free BiP, promoting the activation of three signaling pathways. (i) PERK protein kinase phosphorylates the α subunit of the translation initiation factor, eIF2 (eIF2α), to attenuate global translation initiation. Interestingly, the translation of some mRNAs increases (Harding et al., 2000; Vattem and Wek, 2004); increased translation of the bZIP transcription factor ATF4 mRNA leads to transcriptional activation of important stress-response genes (Siu et al., 2002; Ohoka et al., 2005). (ii) The transmembrane protein ATF6, which is normally retained in the ER, transits to the Golgi, where it is cleaved to produce an active transcription factor. Its target genes
include ER chaperones that assist with protein folding (BiP, GRP94, and calreticulin) (Yamamoto et al., 2007). (iii) The endonuclease IRE1 is activated, initiating the cytoplasmic splicing of XBP1 mRNA to a form that encodes a potent transcription factor (Calfon et al., 2002). XBP1 forms heterodimers with ATF6 or directly induces gene transcription (Yamamoto et al., 2007). The level of downstream gene expression in these signaling pathways determines cell fate during the ER stress response (Marciniak et al., 2004), as recently shown for IRE1 signaling (Lin et al., 2007).

Expression of the bZIP transcription factor C/EBPβ is induced by stress conditions, such as ER stress and amino acid starvation (Chen et al., 2005; Chen et al., 2004). C/EBPβ heterodimerizes with other bZIP factors, including ATF4 and CHOP, which are also induced during the UPR (Sok et al., 1999). CHOP is a C/EBP family member that has been associated with induction of apoptosis, as well as many diseases (Zhang and Kaufman, 2006). During translation of the C/EBPβ mRNA, initiation at alternate in-frame AUG codons gives rise to at least three isoforms, LAP-1, LAP-2, and LIP (see Figure 4-1C) (Calkhoven et al., 2000). LAP-1 and -2 contain an N-terminal transcription transactivation domain, a central DNA binding domain, and a C-terminal leucine zipper domain that homo- or heterodimerizes with other bZIP proteins (Nerlov, 2007). LIP is missing the transactivation domain but can dimerize and bind DNA. As a consequence, LAP-1 and -2 are mostly transcriptional activators, whereas LIP acts as a repressor (Zahnow et al., 1997). C/EBPβ is expressed in many tissues (Nerlov, 2007), and high LIP levels are associated with aggressive tumors, suggesting that LIP is oncogenic (Zahnow et al., 1997). In addition, characterization of C/EBPβ−/− mice provides clues to its functions. These mice have two phenotypes, A and B (Croniger et al., 2001). Mice with the B
phenotype die in the perinatal period because of hypoglycemia and inability to mobilize glycogen. Mice with the A phenotype survive to adulthood but have defective glucose and lipid homeostasis. They have fasting hypoglycemia, lower hepatic glucose production, as well as reduced plasma insulin and free fatty acids. The importance of the individual C/EBPβ isoforms in vivo has not been demonstrated. However, in a recent report, mice containing a knock-in mutation of the LAP-2 translation initiation codon did not have the metabolic defects of the C/EBPβ–/– mice, suggesting important roles for LAP-1 and LIP in the cellular metabolism (Uematsu et al., 2007).

The importance of C/EBPβ in the ER stress response has been shown by the reduced apoptosis of C/EBPβ+/– MEFs (Zinzsner et al., 1998), which suggested that this protein might play a role in the regulation of gene expression during the response. In fact, C/EBPβ has been associated with regulation of the asparagine synthase (AS) gene (Siu et al., 2001) and genes downstream of CHOP (DOCs) during ER stress (Ohoka et al., 2005; Wang et al., 1998). Because there are no clear consensus DNA-binding sites for the bZIP heterodimers induced during ER stress, regulation of individual genes needs to be determined experimentally. The opposing roles of LIP and LAP in the regulation of gene expression (Siu et al., 2001), the induction of C/EBPβ expression during ER stress (Chen et al., 2004), and the potential of LAP and LIP to form heterodimers with other bZIP transcription factors led us to undertake the studies in this report.

We explored the regulation of LIP and LAP levels during ER stress and the physiological significance of C/EBPβ in the stress-response program. We found that LIP levels decrease during the first few hours of ER stress, followed by a large increase, whereas LAP levels showed a steady increase. The result was a decline and then a large
increase in the LIP/LAP ratio. Both LIP and LAP were found to be short lived proteins that are degraded by the proteasome. Our results suggest that changes in the rates of synthesis and degradation are important for regulating LIP and LAP levels. We also show that LIP can inhibit the transcription of stress-response genes that are activated by the binding of ATF4 to an amino acid-response element (AARE). In support of this idea, the expression of these genes was altered in C/EBPβ−/− MEFs. In wild-type cells and in C/EBPβ−/− MEFs expressing LIP (but not LAP), expression increased early in the UPR and then declined, whereas the expression remained elevated in C/EBPβ−/− MEFs. In contrast, a subset of genes that also require AAREs for induction of transcription during ER stress were attenuated in the absence of C/EBPβ. The transcription factor CHOP has been implicated in the regulation of these genes (Ohoka et al., 2005; Marciniak et al., 2004). Our data suggest differential regulation of AARE-mediated transcription during ER stress by C/EBPβ. The findings in this study are consistent with the notion that the LIP/LAP ratio is an important regulator of the stress response, and that the high levels of LIP during the late response correlate with attenuated expression of ATF4-regulated genes.
MATERIALS AND METHODS

Plasmid constructs

pGL3-3xUPRE and pGL3-2xERSE luciferase reporter vectors were gifts from Dr. R. Kaufman. PA1.4N and pGL3-3xAARE luciferase reporter vectors were described previously (Fernandez et al., 2003; Lopez et al., 2007). FLAG-tagged mouse C/EBPβ expression vectors carrying mutations in AUG codons were generated from the mouse C/EBPβ expression vector pcDNA3.1(–)mC/EBPβ-5'-UTR (Baer and Johnson, 2000) by inserting a DNA fragment encoding a FLAG tag (5’-GAC TAC AAA GAC GAT GAC GAC AAG-3’) into the mouse C/EBPβ open reading frame (ORF) just before the stop codon and mutating the third or fourth ATG codons to ATC using PCR methods. To construct the LIP expression vector, a fragment containing the LIP ORF was amplified by PCR and inserted into pcDNA3.1(–).

Cell culture and DNA transfections

C6 rat glioma cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and 5% calf serum. WT and C/EBPβ−/− mouse embryonic fibroblasts (MEFs) (Sebastian et al., 2005) were maintained in high glucose DMEM supplemented with 10% FBS. Plasmid DNAs were transfected into C6 cells using FuGENE 6 (Roche Applied Science). Stably transfected mass cultures were generated by selection in 0.1% G418. Transfected cells were grown in medium containing 0.04% G418 and shifted to drug-free medium 1 day before experiments. To control for transfection efficiency in transient transfection experiments, cells were cotransfected with plasmids expressing either β-galactosidase or Renilla luciferase (Fernandez et al., 2001). To induce ER stress, cells were incubated with
medium containing 400 nM thapsigargin (Tg) or 2 µg/ml tunicamycin (Tu) for the indicated times. Amino acid starvation was induced by incubation of cells in Met-, Cys-free high glucose DMEM supplemented with 10% dialyzed FBS as described previously (Lopez et al., 2007). To inhibit protein synthesis or the proteasome, cells were incubated with medium containing 10 µg/ml cycloheximide (CHX), 1 µM hippuristanol (Hipp), 10 µM MG132, or 10 µM epoxomycin, for the indicated times. Luciferase and β-galactosidase activities were measured as described previously (Fernandez et al., 2001).

Animal studies

Breeding of C/EBPβ−/− and wild-type mice was described previously (Millward et al., 2007). To induce ER stress, 8–10-week-old mice were injected intraperitoneally with Tu (1 µg/g body weight). After 24 h, livers, kidneys, and pancreata were harvested and homogenized in lysis buffer (20 mM Tris, pH 7.6, 0.1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 250 mM sucrose) as described previously (Yaman et al., 2002). The homogenates were centrifuged at 17,000 x g for 30 min at 4 °C, and the supernatants were discarded. The pellets were sonicated in 0.4 ml of lysis buffer/g of tissue and centrifuged at 17,000 x g for 10 min at 4 °C, and the supernatants were collected as nuclear extracts.

Western blot analysis

Cells were scraped in phosphate-buffered saline, collected by centrifugation, and suspended in 20 mM HEPES, pH 8.0, 380 mM NaCl, 6 mM MgCl2, 1% deoxycholic acid, incubated on ice for 15 min with occasional mixing, and centrifuged at 17,000 x g for 10 min at 4 °C. The supernatants were collected as total cell lysates. Nuclear extracts were isolated as described previously (Yaman et al., 2002). Total cell lysates (20 µg of protein) or nuclear extracts (10 µg of protein) were separated by SDS-PAGE, transferred
to polyvinylidene difluoride membranes, and probed with antibodies using standard procedures. Antibodies for the following proteins were purchased from Santa Cruz Biotechnology: ATF4 (C-20, sc-200), BiP (H-129, sc-13968), C/EBPβ C terminus (C-19, sc-150), CHOP (B-3, sc-7351), HDAC1 (H-11, sc-8410), and XBP1 (M-186, sc-7160). Anti-FLAG antibodies (PRB-132P) were from Covance. Antihistone H3 antibodies (sc-9715) were from Cell Signaling. Anti-eIF2α-P antibodies (44–728G) were from BIOSOURSE. Monoclonal anti--tubulin antibodies (T9026) were from Sigma. Anti-eIF2α antibodies were prepared by Quality Controlled Biochemicals.

**Polysome profiles and RNA isolation**

Control or Tg-treated C6 cells were incubated for the last 5 min of treatment with 100 µg/ml CHX. Cells were then washed twice with phosphate-buffered saline, scraped in phosphate-buffered saline, and centrifuged at 1,200 x g for 5 min. The cell pellets were suspended and homogenized by 15 passages through a 25-gauge needle in 500 µl of 10 mM HEPES-KOH, pH 7.4, 2.5 mM MgCl₂, 100 mM KCl, 1 mM dithiothreitol, 0.25% Nonidet P-40, 200 units/ml RNase inhibitor, 100 µg/ml CHX, and 1 tablet of EDTA-free protease inhibitor mixture/10 ml (Roche Applied Science). Lysates were centrifuged at 17,000 x g for 15 min; the supernatants were collected, and absorbances were measured. About 7 A₂₆₀ units of the cytosolic extracts were layered over 10–50% sucrose gradients and centrifuged at 16,000 rpm in a Beckman SW28 rotor for 19 h at 4 °C. After centrifugation, fractions (1.2 ml each) were collected. RNA was extracted with TRIzol reagent (Invitrogen) and used to determine the distribution of ribosomal RNAs (by agarose gel electrophoresis) and mRNAs (by qRT-PCR).

**Metabolic labeling and immunoprecipitation**
C6 cells were incubated for 1 h in L-Met-, L-Cys-deficient DMEM (Invitrogen catalog number 21013) containing 10% dialyzed FBS with or without 400 nM Tg. Cells were then incubated in Met-, Cys-deficient DMEM with $^{35}$S-Met and -Cys (100 μCi/ml Expre$^{35}$S$^{35}$S Protein labeling mix, PerkinElmer Life Sciences) with or without Tg for an additional hour. Cells were scraped in cold phosphate-buffered saline and lysed in RIPA buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1 mM NaF, 1 mM Na$_3$VO$_4$, 2 mM imidazole) supplemented with protease inhibitor mixture (Roche Applied Science). Lysates (500 μg of protein) were incubated overnight with anti-C/EBPβ (2 μg, Santa Cruz Biotechnology, sc-150). Samples were separated on 12% SDS-polyacrylamide gels, dried, and analyzed by autoradiography.

**DNA affinity pulldown**

A biotinylated double-stranded DNA oligonucleotide containing two copies of the cat-1 AARE

1

was used to isolate proteins from C6 cell nuclear extracts as described previously (Chodosh, 2001) with minor modifications. Extracts (500 μg of protein) were incubated streptavidin magnetic particles (Roche Applied Science) for 30 min at 4 °C; the beads were removed, and 100 μg/ml poly(dI-dC) was added to the supernatants for 30 min, followed by 100 pmol of the biotinylated oligonucleotide in binding buffer (12 mM HEPES-NaOH, pH 7.9, 4 mM Tris-HCl, pH 7.9, 12% glycerol, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol) for 1 h at 4 °C. Streptavidin magnetic particles were added, incubated for 30 min at 4 °C, and the particles isolated. The particles were washed twice in binding buffer and once with binding buffer supplemented with 40 mM KCl at room
temperature. The isolated proteins were separated on 12% SDS gels and analyzed by Western blotting.

**Quantitative real time RT-PCR (qRT-PCR)**

First-strand cDNA samples were synthesized from RNA samples using SuperScript III (Invitrogen). The PCR primers used are listed in Table 4-1.
Table 4-1. Primers used for qRT-PCR.
# TABLE 4-1

Primers used for qRT-PCR

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Sequence:</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-tubulin</td>
<td>Forward 5'-CAACAAACTGGGCTAAGGGAATGCTACTAC-3'</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>Reverse 5'-CACCACATCCAAGCAGAGTCAACC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Forward 5'-TTGACGGAAGGGCACCACAG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GCACCACCACCCACGGAATCG-3'</td>
</tr>
<tr>
<td>AS</td>
<td>Forward 5'-TTGACCGCGCTGTTGGAATG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CGGCTTGGTGTAAGATTCCCAC-3'</td>
</tr>
<tr>
<td>ATF4</td>
<td>Forward 5'-GTG TGA CTT CGA TGC TCT GTT TC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GGG CTC CTT ATT AGT CTC TCG G-3'</td>
</tr>
<tr>
<td>C/EBPβ</td>
<td>Forward 5'-CGGGTTCGGGACTTGAT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GCCCGGGCTGACAGTTACAC-3'</td>
</tr>
<tr>
<td>cat-1</td>
<td>Forward 5'-CTTGGATTCCTCTCTGGGTTCGTC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GTTCTTGACTTCTCCCTCTGGG-3'</td>
</tr>
<tr>
<td>CHOP</td>
<td>Forward 5'-CTGGAAGCCCTGGATGAGAT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CAGGGTCAAGAGATGGGAGCT-3'</td>
</tr>
<tr>
<td>GADD34</td>
<td>Forward 5'-GACACAGAAGAGAGAGGACAG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CAGCAGACAGAAAGGAAATGGAC-3'</td>
</tr>
<tr>
<td>GRP78 (BiP)</td>
<td>Forward 5'-ACTTGGGACCACCTTCTTCT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-ATCGCCAATCGACGGCTCC-3'</td>
</tr>
<tr>
<td>SNAT2</td>
<td>Forward 5'-TAAATGACGATCGAGAGTTG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-AGATGGACGAGTATAGCGAAA-3'</td>
</tr>
<tr>
<td>TRB3</td>
<td>Forward 5'-GCAAACCGCGCTGATGCTTG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-AGAGTCGCGTAAGGTTATCTG-3'</td>
</tr>
</tbody>
</table>
RESULTS

Differential regulation of the transcriptional activator LAP and transcriptional repressor LIP during ER stress

Although C/EBPβ has been implicated in the transcriptional control of stress-response genes (Chen et al., 2005; Chen et al., 2004), its role in the adaptive response of cells to ER stress is not clear. Because translation of the C/EBPβ mRNA produces two transcriptional activators, LAP1 and -2, and a transcriptional repressor, LIP (Figure 4-1E), we first determined the levels of LAP and LIP in C6 cells treated with Tg for 0–24 h, which acts by depleting ER Ca²⁺ stores (Harding et al., 2000). As expected, Tg caused ER stress, as shown by the induction of key modulators of the stress response (Schroder and Kaufman, 2005a). These include the transcription factors ATF4, CHOP, and the spliced form of XBP1 in nuclear fractions (Figure 4-1A), as well as the phosphorylated form of the translation initiation factor eIF2α in whole cell lysates (Figure 4-1B). In contrast, the levels of histone deacetylase 1 and tubulin, which were used as loading controls for nuclear and whole cell fractions, respectively, were constant.

Tg caused a small increase in nuclear LAP2 levels (Figure 4-1A). In contrast, LIP levels decreased in nuclear extracts from 0 to 3 h, followed by an increase from 9 to 24 h, with the final level greatly exceeding that in untreated cells. The levels of LIP and LAP were also examined in whole cell lysates (Figure 4-1B). Similar to nuclear fractions, LIP declined and then showed a large increase; in contrast, LAP levels increased continuously throughout the experiment.

The LIP/LAP ratio has been proposed to be a critical modulator in the expression of C/EBPβ-regulated genes (Zahnow, 2002; Descombes and Schibler, 1991; Raught et al.,
Figure 4-1. Differential regulation of LIP and LAP levels during ER stress. A, Western blot analysis of nuclear extracts (A) or whole cell extracts (B) from C6 cells treated with Tg for the indicated times. Blots were probed with antibodies for C/EBPβ or the indicated proteins. C, Western blots in three independent experiments were quantified and used to calculate the LIP/LAP ratio. Means ± S.E. are shown. D, C/EBPβ mRNA levels were monitored by qRT-PCR using total RNA samples from C6 cells treated with Tg for the indicated times. Data were normalized to the 18 S ribosomal RNA signal. E, schematic of the translation products of C/EBPβ. The arrows show the mutations of the AUG codons in the AUG3mut and AUG4mut constructs. F, Western blot analysis of cell extracts from Tg-treated C6 cells stably expressing mutant FLAG-tagged CEBPβ proteins. Blots were probed with antibodies for C/EBPβ and the FLAG epitope. G, Western blot analysis of nuclear extracts from the indicated tissues of WT and C/EBPβ−/− mice following 24 h treatment with Tu. Blots were probed for antibodies for C/EBPβ and the indicated proteins. For the Western blot analysis of animal tissues in panel G, animals were treated and nuclear extracts were isolated from tissues by Yi Li, Western blot analysis was performed by technical assistance of Chuanping Wang,
FIGURE 4-1

A. C8 nuclear extracts
   LAP
   LIP
   ATF4
   XBP1s
   CHOP
   HDAC1
   (Tg) 0 3 6 12 18 24 (h)

B. C8 cell lysates
   LAP
   LIP
   eIF2α-P
   eIF2α
   CHOP
   Tubulin
   (Tg) 0 3 6 12 18 24 (h)

C. LIP/LAP
   (fold change)
   Tg Treatment (h)

D. C/EBPβ mRNA
   (fold change)
   Tg Treatment (h)

E. mouse C/EBPβ mRNA
   AUG1  AUG2  AUG3  AUG4  AUG5  STOP
   5' SOFR
   LAP1  LAP2  LIP
   mut  mut

F. anti-C/EBPβ
   LAP  LIP  tubulin
   untransfected  AUGmut  AUGmut
   mutated  AUGmut
   flag-LAP  flag-LIP
   Tg (24 h)

G. nuclear extracts
   LAP  LIP  ATF4  CHOP  BiP  Histone H3
   liver  kidney  pancreas
   Tu (24h) - + - + - + - + - + - + - +
   C/EBPβ +/- +/- +/- +/- +/- +/-
During ER stress, the LIP/LAP ratio in total cell lysates decreased by 90% in the early response (3–6 h) and then increased by 30-fold from the minimum (Figure 4-1C). This finding suggests that modulation of LAP and LIP levels may be required for the adaptive response to ER stress. Similar results were obtained in cells treated with Tu, which causes ER stress by blocking N-glycosylation (not shown). We also examined the levels of C/EBPβ mRNA during Tg treatment by qRT-PCR (Figure 4-1D). The levels rose by 4-fold after 3 h of treatment and remained elevated. This finding can explain the increased levels of both LIP and LAP in whole cell extracts but not the differential accumulation of these proteins during late ER stress (Figure 4-1B). Because LIP and LAP are synthesized from the same mRNA, the changes in the relative levels of these proteins must be due to independent regulation of their synthesis and degradation.

LIP is expressed by translation initiation at an in-frame AUG codon (AUG3) in the C/EBPβ ORF (Figure 4-1E). To confirm that the LIP protein expressed during ER stress is the authentic p20 C/EBPβ isoform, we mutagenized AUG codons in an expression vector that encodes mouse C/EBPβ with a C-terminal FLAG epitope. These mutations preserve the C/EBPβ ORF. LAP, but not LIP, was expressed from the AUG3mut construct as seen in blots probed with anti-C/EBPβ and anti-FLAG antibodies (Figure 4-1F), whereas both forms were expressed from the AUG4mut construct, demonstrating that C6 cells express authentic LIP that is translated from AUG3. These data exclude the possibility that the LIP isoform is a product of proteolysis in our cells, as suggested previously (Baer and Johnson, 2000).

We next determined if nuclear C/EBPβ levels are regulated in mice treated with Tu, which is a rapid inducer of ER stress in vivo. Tu has been shown to cause an acute ER
stress response in the kidney (Zinszner et al., 1998). Among the other tissues, pancreas and to a lesser extent liver are most susceptible to ER stress (Scheuner et al., 2005). To evaluate the role of C/EBPβ in the ER stress response, we compared the induction of stress-response genes in wild-type (WT) and C/EBPβ−/− mice at 24 h of treatment. This time of treatment has been associated with kidney damage (Zinszner et al., 1998). In WT mice, Tu caused an induction of LAP and LIP levels in liver, kidney, and pancreas (Figure 4-1G). The LIP/LAP ratio increased by 2.5-fold in the liver and decreased 3- and 7-fold in kidney and pancreas, suggesting differential responses of the three tissues to ER stress. As expected, LAP and LIP were not expressed in C/EBPβ−/− mice.

To correlate the LIP/LAP ratio with the stress response, we examined induction of three stress-response proteins, ATF4, CHOP, and BiP, in nuclear extracts. ATF4 is a transcription factor that has been implicated in supporting either survival or death of stressed cells (Ohoka et al., 2005; Fels and Koumenis 2006). CHOP is a transcription factor that promotes cell death (Marciniak et al., 2004), whereas BiP is a chaperone within the ER and nuclear envelope that has pro-survival functions (Li and Lee, 2006). Liver showed the highest induction of BiP in WT and mutant mice (Figure 4-1E), in agreement with previous reports that BiP induction depends on the ATF6 signaling pathway (Li et al., 2000). In contrast, ATF4 and CHOP were expressed in the liver of wild-type but not C/EBPβ−/− mice, although these proteins were induced in kidney and pancreas of mutant animals. These data suggest that the ER stress gene expression program varies in different tissues. The absence of ATF4 and CHOP and the presence of BiP in C/EBPβ−/− livers may suggest that the liver uses the XBP1/ATF6 rather than the PERK/eIF2α pathway for response to late ER stress, as shown for pancreatic beta cells.
that lack PERK signaling (Oyadomari et al., 2002). Interestingly, the decreased LIP/LAP ratios in pancreas and kidney are correlated with higher tissue injury. Given the importance of ATF4, CHOP, and C/EBPβ in gene regulation by forming heterodimers among them (Ohoka et al., 2005; Sok et al., 1999; Wang et al., 1998), our data also suggest that LIP/LAP ratios may play a role in modulating the ER stress-response program.

**LAP and LIP are degraded by the proteasome during ER stress**

We have shown that LIP levels increased by 30-fold between 3 and 18 h of Tg treatment (Figure 4-1C). In contrast, LAP levels showed only a 1.5-fold increase. These differences may be due to differences in the rates of synthesis or degradation. We therefore determined the half-lives (t) of LAP and LIP by measuring protein levels in the presence of the protein synthesis inhibitor, CHX, in cells after either 2 or 18 h of Tg treatment (Figure 4-2). CHX caused decreases in these proteins in both nuclear extracts (Figure 4-2, A and B) and whole cell extracts (Figure 4-2, C and D). LAP in nuclear extracts decreased with t of 6 and 3 h at 2 and 18 h, respectively, whereas the t for LIP was 2 h at both times. Similar results were obtained from the analysis of total cell extracts. The levels of the long lived proteins proliferating cell nuclear antigen and HDAC1 did not show large changes during CHX treatment (Figure 4-2, A and B). These data suggest that the increases in LIP levels between 2 and 18 h of Tg treatment are because of increased synthesis, rather than a decrease in degradation. In contrast, the decline in the t of LAP during late ER stress may contribute to the fact that LAP shows smaller increases than LIP during prolonged stress.

We next determined if the proteasome is involved in LAP and LIP degradation during
Figure 4-2. Differential regulation of LIP and LAP levels during ER stress by inhibitors of the proteasome and protein synthesis. Western blot analysis of nuclear extracts (A and B) and total extracts (C and D) from C6 cells. Cells were treated with Tg for 2 h (A and C) or 18 h (B and D) before addition of CHX or MG132 as indicated. Blots were probed with antibodies for C/EBPβ, HDAC1, proliferating cell nuclear antigen (PCNA), and tubulin. E, cells treated with Tg for 1 h or control (CON) cells were labeled with $^{35}$S-Met/Cys for 1 h and immunoprecipitated (IP) with anti-C/EBPβ antibody. Samples of cell lysates and the immunoprecipitates were analyzed on SDS gels, and radioactivity was detected by autoradiography. Metabolic labeling in panel E was conducted by Calin-Bogdan Chiribau.
ER stress by treating cells with Tg and the proteasome inhibitor MG132. Inhibition of the proteasome resulted in increased levels of LAP in early and late stress in both whole cell and nuclear extracts (Figure 4-2, A to B), indicating that proteasomal degradation of this protein is an important route of turnover. MG132 caused increased LIP accumulation during the early phase (Figure 4-2, A and C) but not in the late phase (Figure 4-2B and D). These data suggest that the rate of LIP synthesis exceeds its degradation in late ER stress, consistent with the large accumulation of LIP between 18 and 24 h (Figure 4-1A). Interestingly, MG132 resulted in a dramatic increase of total LIP levels but a smaller increase of nuclear levels (compare Figure 4-2, A and C), consistent with cytoplasmic degradation of the protein. The mechanism of degradation of LIP is under investigation in our laboratory. We also used MG132 to show that the proteasome degrades LAP and LIP in unstressed cells (Figure 4-3A). There is a striking accumulation of LIP and LAP in cells treated with both Tg and MG132 for 2 h (Figure 4-3A). It is worth noting that MG132 abolished accumulation of phospho-eIF2α during ER stress (Figure 4-3A), in agreement with some reports (Wojcik et al., 2006) but in disagreement with others (Yerlikaya et al., 2008; Jiang and Wek, 2005; Mazroui et al., 2007), possibly because of the short treatment and low MG132 concentration. Longer times and higher concentrations increased eIF2α phosphorylation (data not shown). We therefore conclude that LAP and LIP are degraded by the proteasome in untreated and ER-stressed cells and that the proteasome is involved in the decline in LIP during the first 2 h of stress (Figure 4-3A). Similar results were obtained with epoxomycin, another proteasome inhibitor (not shown).

An early event in the cellular response to ER stress is a decrease in global protein
Figure 4-3. Decreased LIP levels during early ER stress require eIF2α phosphorylation and an active proteasome. Western blot analysis of cell extracts from C6 cells (A) or S/S and A/A MEFs (B) treated with Tg and MG132 for the indicated times. Blots were probed with antibodies for C/EBPβ, eIF2α-P (Ser-51), and tubulin. C and D, C6 cells were treated with MG132 and/or the translation inhibitor hippuristanol (Hipp) for the indicated times. Proteins in whole cell lysates were analyzed on Western blots. CON, control. Western blot analysis in panel D was conducted by Calin-Bogdan Chiribau.
synthesis, which is caused by the phosphorylation of eIF2α (Schroder and Kaufman, 2005a). Consequently, we assessed the contribution of this change to the decreased LIP levels in early stress. Cells were treated with Tg for 1 h and then labeled with \(^{35}\text{S}\)-Met and -Cys for an additional hour. SDS gel analysis of whole cell lysates revealed the expected 50% decrease in protein synthesis (Figure 4-2E). Immunoprecipitation of LIP and LAP revealed that similar amounts of LAP1 and -2 were synthesized in Tg-treated and control cells, whereas the amount of LIP declined by 50%. Therefore, decreased global protein synthesis may contribute to the lower LIP levels during early stress. However, the constant levels of LAP suggest that synthesis of these proteins may involve translation regulation.

We next tested directly the role of eIF2α phosphorylation in the regulation of LIP levels early in the stress response. This was accomplished by comparing WT MEFs (S/S) and MEFs with a S51A mutation in eIF2α (A/A) that cannot be phosphorylated. LIP levels decreased in WT MEFs treated with Tg for 2 h (Figure 4-3B, S/S). In contrast, the decrease was not observed in mutant MEFs (Figure 4-3B, A/A). These data demonstrate that eIF2α phosphorylation is required for the decreased LIP levels during early ER stress.

As an additional test of the relative half-lives of LIP and LAP, we tested the effects of Hipp, which blocks initiation by inhibiting eIF4A (Lindqvist et al., 2008), in contrast to CHX, which inhibits elongation. Hipp treatment of C6 cells caused a large decrease in LIP levels, with a much smaller change in LAP (Figure 4-3C). This decrease was prevented by MG132 (Figure 4-3D), further supporting the idea that LIP is a short lived protein that is degraded by the proteasome in both stressed and unstressed cells.
The results in Figures 4-2 and 4-3 suggest that changes in synthesis rates contribute to the changes in LIP and LAP levels during ER stress. To evaluate this hypothesis, we examined the association of C/EBPβ mRNA with heavy polysomes as a measurement of translational efficiency. Samples were analyzed by gradient centrifugation, and mRNA levels were measured by quantitative RT-PCR. Control mRNAs included ATF4, because ER stress is known to increase the translation efficiency of this mRNA (Vattem and Wek, 2004; Lu et al., 2004), as well as tubulin mRNA.

The effect of ER stress on translation is indicated by the distribution of rRNAs (Figure 4-4). In control cells, most of the rRNAs are in heavy polysomes (fractions 10–12); ER stress causes a large decrease in the heavy polyosome fraction and a corresponding increase in lighter polysomes and free subunits. In unstressed C6 cells, 65% of the C/EBPβ mRNA was in the fractions (Yamamoto et al., 2007; Calfon et al., 2002; Marciniak et al., 2004) containing heavy polysomes (Figure 4-4A), and the distribution was similar to that of tubulin mRNA. Tg treatment for 1 h caused a shift of the C/EBPβ mRNA to lighter fractions (Figure 4-4B), consistent with less efficient translation. Starting at 3 h, the C/EBPβ mRNA returned to heavier polysomes, and by 18 h, 50% of the mRNA was in these fractions (Figure 4-4D). In contrast, tubulin mRNA was translated efficiently in untreated cells and less efficiently in stressed cells. The distribution of ATF4 mRNA also showed characteristic changes. In unstressed cells, only 20% of the ATF4 mRNA was in heavy polysomes (Figure 4-4A). In contrast, at 1 h of stress, 60% of the ATF4 mRNA was associated with heavy polysomes (Figure 4-4B) and then declined at later times, consistent with its efficient translation in early stress (Harding et al., 2000).
Figure 4-4. The C/EBPβ mRNA associates with heavy polysomes during prolonged ER stress. C6 cells were treated with Tg for the indicated times, and cytoplasmic extracts were analyzed by sucrose gradient centrifugation as described under "Experimental Procedures." Total RNA samples were run on agarose gels to analyze ribosomal RNAs (upper panels). qRT-PCR was used to monitor the distribution of C/EBPβ, tubulin, and ATF mRNAs (lower panels). CON, control. The cells were treated by Yi Li, the analysis of polysome profile was done by Mithu Majumder.
FIGURE 4-4

A

B

C

D

127
These results suggest that changes in translation efficiency of C/EBPβ during ER stress play a role in regulating the levels of LIP and LAP. However, because both are translated from the same mRNA, this experiment does not reveal which protein is synthesized from the polysome-associated mRNA. Nevertheless, the large sustained increase in LIP levels during late ER stress is consistent with a selective increase in LIP synthesis at this time.

**LIP levels increase during amino acid starvation but the LIP/LAP ratio shows a small change**

C/EBPβ has been implicated in the integrated stress response of cells to both ER stress (Chen et al., 2004) and nutrient limitation (amino acids or glucose, see Chen et al., 2005). We therefore tested the levels of LIP and LAP in C6 cells during amino acid starvation (Figure 4-5A). First, we showed that the response in C6 cells followed the previously reported gene expression pattern (Chen et al., 2004). This response involves phosphorylation of eIF2α by GCN2 kinase, which leads to the induction of the stress-response mediator ATF4. Figure 4-5 shows that eIF2α phosphorylation increases after 1 h and then declines, followed by a small increase during prolonged starvation, as reported previously (Fernandez et al., 2002). In addition, CHOP expression, which is regulated by ATF4 (Averous et al., 2004), is induced during prolonged starvation. Similar to ER stress, LIP and LAP levels decreased during early starvation and increased during prolonged starvation (Figure 4-5A). However, in contrast to ER stress, the changes of the LIP/LAP ratio during amino acid starvation were less than 2-fold (Figure 4-5B) (Oyadomari and Mori, 2004). Similar changes of LIP and LAP levels in nuclear extracts were also observed (not shown). These data suggest that involvement of LIP and LAP in stress response programs may vary with the type of stress (Chen et al., 2005; Chen et al., 2004).
Figure 4-5. Amino acid starvation induces LIP and LAP levels but has a small effect on the LIP/LAP ratio. A, Western blot analysis of extracts from C6 cells incubated in amino acid-deficient medium for the indicated times. Blots were probed with antibodies for the indicated proteins. B, quantification of the LIP/LAP ratio from three independent Western blot analysis shown in A. The cells were treated by Yi Li, Western blot analysis was performed by technical assistance of Chuanping, Wang.
LIP attenuates transcription mediated by ATF4 but not XBP1 or ATF6

The induced transcription of stress-response genes during ER stress involves either ATF4 or XBP1 and ATF6 (Siu et al., 2002; Oyadomari and Mori, 2004; Donati et al., 2006; Baumeister et al., 2005). Consensus DNA sequences specific for ATF4 (AAREs), ATF6 (ERSEs), and XBP1 (UPREs) have been described (Schroder and Kaufman, 2005a). Because LIP associates with CHOP (Zinszner et al., 1998) and because CHOP expression is stimulated by ATF4 (Averous et al., 2004), the increased LIP levels during ER stress might attenuate transcription mediated by these pathways. There are no studies demonstrating heterodimers of LIP with ATF4, ATF6, or XBP1 in vivo. We therefore compared the effect of LIP on ATF4- and XBP1/ATF6-mediated transcription. We used luciferase expression vectors with the SV40 early promoter and 2–3 copies of the stress-response elements downstream of the luciferase ORF (Figure 4-6A). The effect of cotransfecting LIP with ATF4, ATF6, or XBP1 was examined. LIP cotransfection with ATF4 inhibited luciferase expression from the construct containing the cat-1 AARE (Figure 4-6B). In contrast, neither ATF6- nor XBP1-mediated luciferase expression from the constructs containing ERSEs or UPREs was affected by coexpression of LIP (Figure 4-6C). As an additional test of the role of LIP in attenuating ATF-stimulated transcription, we examined its effect on the promoter of the gene encoding cat-1, which contains the AARE in the first exon (Fernandez et al., 2003). We used a construct with 1.4 kb of the promoter region and the 5'-UTR of the cat-1 mRNA linked to a luciferase reporter (Figure 4-6A). Luciferase expression from this construct was not induced by LIP, but ATF4-stimulated expression was inhibited by the coexpression of LIP (Figure 4-6D). These results demonstrate that LIP attenuates ATF4-mediated gene transcription and
Figure 4-6. LIP attenuates induction of transcription by ATF4 via the cat-1 AARE enhancer element. A, schematic of luciferase (LUC) expression vectors containing AAREs, ERSEs, or UPREs (top) and the cat-1 promoter-containing expression vector (bottom). B–D, C6 cells were transfected with the expression vectors in A along with expression vectors for the indicated transcription factors. Expression plasmids for β-galactosidase (β-gal) or Renilla luciferase were included to normalize for transfection efficiency. Enzymatic activities were measured in cell extracts 48 h after transfection and normalized as indicated. E, DNA affinity pulldown using nuclear extracts from control and Tg-treated C6 cells was conducted as described under "Experimental Procedures." The biotin-labeled DNA probe contained two copies of the cat-1 AARE. Samples were analyzed for C/EBPβ and ATF4 on Western blots. CON, control.
suggests that C/EBPβ regulates expression of genes that are directly or indirectly induced by the ATF4-mediated stress response program.

To test the DNA binding ability of active transcription factors in stressed cells, DNA pulldown experiments were performed. A biotinylated double-stranded oligonucleotide containing two copies of the cat-1 AARE was incubated with nuclear extracts from control and stressed cells. The oligonucleotide-protein complexes were isolated, and LIP, LAP, and ATF4 were analyzed by Western blotting (Figure 4-6E). LAP binding was high in all the extracts, consistent with the constant levels of this protein in nuclear extracts (Figure 4-1A). LIP showed strong binding in controls, in contrast to the low levels of this protein in nuclear extracts. This suggests that LIP can bind preferentially under these conditions, consistent with its proposed role as a transcriptional repressor of the gene encoding cat-1. The level of binding then declined and increased, mirroring the changes in LIP protein levels in nuclear extracts. ATF4 binding was low in controls, consistent with the low level of this protein in the extracts. The level increased strongly during stress, but then declined at 18 h, even though levels in the extracts remained high. This is probably because of competition with LIP, although the involvement of another protein cannot be excluded.

**C/EBPβ attenuates the induction of gene expression mediated by the PERK-ATF4 pathway but not the XBP1/ATF6 pathway during ER stress**

To test the hypothesis that C/EBPβ regulates expression of genes that are induced by the ATF4 (Ohoka et al., 2005; Marciniak et al., 2004; Chen et al., 2004; Palii et al., 2004; Ma and Hendershot, 2003) and not the ATF6/XBP1 (Baumeister et al., 2005; Shang and Lehrman, 2004; Lee at al., 2003) pathways, we compared the induction of stress-response
genes in wild-type and C/EBPβ−/− MEF cells during ER stress. In addition, to examine the role of LIP expression, we analyzed C/EBPβ−/− (LIP) cells that constitutively expressed LIP from a transfected plasmid. This plasmid contained the LIP ORF without any 5′- or 3′-flanking sequences.

First, we measured the induction of LIP and LAP by ER stress. The results in WT MEFs (Figure 4-7, A and C) were similar to those observed in C6 cells, with large increases in LIP and LAP levels following prolonged stress, and with the LIP/LAP ratio showing a transient decrease followed by a large increase (Figure 4-7C). The changes in the LIP/LAP ratio were similar in nuclear and total extracts of Tg-treated cells. C/EBPβ mRNA levels showed a smaller increase (2–3-fold) than protein levels during ER stress (Figure 4-7F), as reported previously (Chen et al., 2004). The C/EBPβ−/− (LIP) cells expressed LIP at all times, although there was a decrease in early stress followed by a large transient increase in late stress (Figure 4-7D). Because the LIP expression construct only contains the LIP ORF (Figure 4-1E), these changes are probably not because of translational control; changes in LIP stability are a more likely explanation. Furthermore, the sustained LIP levels in WT MEFs in late ER stress suggest efficient translation of the LIP ORF in the endogenous C/EBPβ mRNA but not in the transfected LIP expression plasmid.

Next, mRNA levels of stress-response genes were analyzed by quantitative RT-PCR analysis on cells stressed with Tg (Figure 4-8). Three patterns of expression were observed. 1) Some genes (AS and the amino acid transporters cat-1 and SNAT2) showed higher expression in C/EBPβ−/− cells than in wild-type cells, particularly after prolonged stress. These genes are known to be induced by ATF4 (Chen et al., 2004; Lopez et al.,
Figure 4-7. Induction of stress-response proteins in C/EBPβ+/+, C/EBPβ−/−, and C/EBPβ−/− (LIP) MEFs. WT, mutant, and C/EBPβ−/− cells stably expressing LIP (C/EBPβ−/− (LIP)) were treated with Tg for the indicated times. A and C–E, Western blot analysis of nuclear (A) or total (C–E) cell extracts probed with antibodies for the indicated proteins. B, quantification of the LIP/LAP ratio from A. F, quantification of C/EBPβ mRNA levels in MEFs treated with Tg for the indicated times. Data from qRT/PCR analysis of total RNA using C/EBPβ specific primers were normalized to the signal of 18 S ribosomal RNA. CON, control. Western blot analysis was performed by technical assistance of Chuanping Wang.
The attenuation of transcription was also seen in C/EBPβ−/− (LIP) cells (Figure 4-9A). Similar regulation was seen for the AS gene (not shown). These observations are consistent with the inhibition of ATF4-induced transcription by elevated LIP during prolonged stress. 2) Some genes had lower expression levels in C/EBPβ−/− cells than in WT cells, suggesting that C/EBPβ is required for the induction of transcription. These genes encode CHOP (Bruhat et al., 2002), GADD34 (Marciniak et al., 2004), and TRB3 (Ohoka et al., 2005), which are either downstream of ATF2/ATF4 (CHOP) or CHOP (GADD34 and TRB3). This is probably an effect of LAP, because C/EBPβ−/− and C/EBPβ−/− (LIP) cells were similar (data not shown). 3) BiP and ER degradation enhancing -mannosidase-like protein (EDEM) were expressed at similar levels in mutant and WT cells. The lack of induction is consistent with the regulation of these genes by ATF6 and XBP1, rather than ATF4. These data support our hypothesis that the expression of genes downstream of ATF4 is modulated by C/EBPβ. 4) The accumulation of ATF4 mRNA was increased more in C/EBPβ−/− (LIP) cells than in WT and C/EBPβ−/− cells (Figure 4-9B), suggesting that LIP has stimulatory effects on Atf4 gene expression in the absence of LAP. The mechanism of this regulation needs further investigation.

**C/EBPβ−/− MEF cells have lower levels of GADD34 and CHOP proteins and reduced eIF2α dephosphorylation**

We have shown that the induction of CHOP and GADD34 mRNAs during ER stress is lower in C/EBPβ−/− than in WT cells (Figures 4-8 and 4-9). To demonstrate that the changes in mRNA levels cause changes in protein levels, we measured these proteins by Western blotting during ER stress in WT and mutant cells. As expected, CHOP was
Figure 4-8. C/EBPβ differentially regulates expression of genes in the PERK/ATF4 pathway during ER stress. Quantification of mRNA levels for the indicated genes in C/EBPβ<sup>−/−</sup> and C/EBPβ<sup>+/+</sup> MEFs treated with Tg for the indicated times. Data from qRT/PCR analysis of total RNA using gene-specific primers were normalized to the 18 S ribosomal RNA signal. The cells used for qRT-PCR were treated by Yi Li, the qRT-PCR was performed by the help of Elena Bevilacqua.
FIGURE 4-8

A. cat-1 mRNA

B. SNAT2 mRNA

C. AS mRNA

D. EDEM mRNA

E. BIP mRNA

F. CHOP mRNA

G. TRB3 mRNA

H. GAD6534 mRNA

Time (h)
induced more slowly in mutant cells and accumulated to lower levels (Figure 4-7A). In contrast, BiP levels were induced to a similar extent in both cells, consistent with the similar induction in mRNA levels (Figure 4-8). GADD34 levels were also 2-fold lower in mutant cells (Figure 4-7E). In agreement with the decreased levels of GADD34, eIF2α phosphorylation was sustained in C/EBPβ−/− and C/EBPβ−/− (LIP) cells. In contrast, WT MEFs showed a transient decrease in eIF2α phosphorylation (Figure 4-7C), which is a hallmark of the stress response in many cell types, including C6 cells (Fernandez et al., 2002). ATF4 levels were higher in the mutant and C/EBPβ−/− (LIP) cells, consistent with the sustained eIF2α phosphorylation. Furthermore, the levels of the transcriptional repressor ATF3, which is downstream of ATF4, were also higher in the mutant cells. These data are consistent with the hypothesis that LAP modulates the ER stress response by enhancing expression of genes encoding proteins that promote cell death (CHOP, GADD34, and TRB3) and attenuating expression of genes encoding proteins involved in amino acid synthesis and transport (cat-1, AS, and SNAT2).
Figure 4-9. Attenuation of cat-1 gene transcriptional activation in C/EBPβ−/− (LIP) cells. MEF cells (C/EBPβ+/+, C/EBPβ−/−, and C/EBPβ−/− (LIP)) were treated with Tg for the indicated times, and cat-1, ATF4, and GADD34 mRNA levels were analyzed by qRT-PCR as in Figure 4-8. B shows results from all three cell lines, whereas A and C show only the C/EBPβ+/+ and C/EBPβ−/− (LIP) cells. The cells used for qRT-PCR were treated by Yi Li, the qRT-PCR was performed by the help of Elena Bevilacqua.
FIGURE 4-9

A. cat-1 mRNA

B. ATF4 mRNA

C. GADD34 mRNA
DISCUSSION

C/EBP transcription factors are involved in a variety of physiological processes, such as metabolic regulation, cellular differentiation, and stress responses (Chen et al., 2004; Chen et al., 2005; Bezy et al., 2007; Seagroves et al., 1998). In the case of C/EBPβ, translational control leads to synthesis of the transcriptional activators LAP-1 and LAP-2 and the transcriptional repressor LIP from a single mRNA (Descombes and Schibler, 1991; Ossipow et al., 1993). The cellular LIP/LAP ratio has been suggested to be an important determinant of regulation of gene expression (Raught et al., 1995; Gingra et al., 1999; Hu et al., 2004). It is shown in this study that LAP and LIP levels are regulated during ER stress. The findings can be summarized as follows. (i) Total LIP levels decrease during the early response (0–3 h), resulting in a 10-fold decrease in the LIP/LAP ratio. This decrease requires proteasome activity and the phosphorylation of eIF2α; it is paralleled by a decrease in C/EBPβ mRNA translation. (ii) Total LIP levels increase during the late ER stress response (>9 h), leading to a 30-fold increase of the nuclear LIP/LAP ratio because of a more efficient translation of the C/EBPβ mRNA and increased LIP protein. (iii) Expression of C/EBPβ proteins during ER stress has two opposite roles; it attenuates expression of some genes involved in amino acid metabolism (via LIP) and it enhances expression of proapoptotic genes, such as CHOP and its downstream targets (via LAP).

Differential Regulation of LAP and LIP Levels during ER Stress

A novel finding of this study is the complex changes in LIP and LAP levels during ER stress. This observation raises questions about the mechanisms that change the synthesis and degradation rates of these proteins and their physiological significance. C/EBPβ
mRNA translation decreases during early stress and recovers during late stress. This is explained at least in part by the effects of eIF2α phosphorylation, which causes a decline of global protein synthesis in early stress (Schroder and Kaufman, 2005a). Moreover, because LIP has a shorter half-life than LAP (2 h for LIP and 6 h for LAP), decreased translation of C/EBPβ mRNA can account for the more pronounced reduction in LIP during the early period. During late stress, the efficient translation of C/EBPβ mRNA contributes to the increases in both LIP and LAP. However the increase in the LIP/LAP ratio suggests that there is preferential accumulation of LIP. Because these proteins are synthesized from a single mRNA, the stress response may regulate translation. This novel mode of regulation is an avenue that we will pursue in the future. A previous report suggested that CUGBP1 preferentially increases LIP translation (Dudaronek et al., 2007). However, we found that depletion of CUGBP1 did not affect the accumulation of LIP during late ER stress (not shown).

Degradation also plays a role in the regulation of both LIP and LAP during ER stress. Our experiments with MG132 suggest that these proteins are degraded by the proteasome. The shorter half-life of LIPs could be due to differences in the N termini of the proteins or to differences in their molecular interactions, as has been shown for the homologue proteins of C/EBPβ and TRB3 in Drosophila (Ohoka et al., 2005). Cyclin D1 is also degraded by proteasomes during ER stress (Raven et al., 2008). Although eIF2α phosphorylation is required for the decreased levels of both proteins, the decline is accompanied by decreased mRNA translation of LIP but sustained translation of cyclin D1.

Our data on LIP levels during ER stress present a paradox. Measurements of LIP
stability with CHX showed that half-lives were similar in early and late stress. In contrast, MG132 increased LIP levels during early but not late stress, suggesting that LIP is more stable in late stress. Because the LIP level increases in late stress, our data could be explained by the fact that the rate of synthesis exceeds the rate of degradation, making the effects of MG132 difficult to detect. It is also possible that the drugs have indirect effects and alter LIP stability by their actions on other proteins. This could be the case in early stress if CHX indirectly inhibits LIP degradation. Although metabolic labeling to examine the LIP half-life would address this question, the labeling conditions will alter amino acid pools and induce stress by amino acid starvation. A striking finding was the accumulation of LIP protein during stress in C/EBPβ−/− (LIP) cells. The changes in LIP levels during ER stress in these cells paralleled those seen for protein expressed from the C/EBPβ mRNA in wild-type cells with one difference; accumulation of LIP in late ER stress was transient in C/EBPβ−/− (LIP) and continuous in C/EBPβ+/+ cells. These data suggest that LIP accumulation during ER stress is controlled in part by degradation of the protein, with rapid degradation during early stress and slower degradation in late stress. This conclusion appears to contradict the half-lives measured using inhibitors of protein synthesis, which gave similar values in early and late stress. The most likely explanation for this discrepancy is that a short-lived protein is involved in the control of LIP degradation during ER stress and that protein synthesis inhibitors interfere with normal regulation. We are currently investigating this hypothesis.

The proteasome plays a key role in the ER stress response. It has a pro-survival role in the early phase by degrading accumulated unfolded proteins and a pro-apoptotic role in the late phase by degrading pro-survival proteins such as Bcl-2 (Kondratyev et al., 2007;
Basu and Haldar, 2002). It is therefore possible that changes in LIP levels involve proteasome-mediated degradation of LIP in the pro-survival phase and protection of degradation by the proteasome in the proapoptotic phase of stress.

**LIP attenuates transcriptional induction of the PERK/eIF2α/ATF4 arm of the cellular response to ER stress**

What is the physiological significance of the regulation of LIP levels during ER stress? We show that amino acid starvation, another stress that involves eIF2α phosphorylation, only caused small changes in the LIP/LAP ratio during prolonged stress (Figure 4-5), suggesting that stress-specific mechanisms are involved in the regulation of C/EBPβ gene expression. Because LIP levels decrease during the pro-survival phase of stress and increase during the pro-apoptotic phase, we can speculate on the significance of its function as a mediator of pro-survival or pro-apoptotic processes. In at least one report, it was shown that LIP can bind DNA with higher affinity than LAP (Descombes and Schibler, 1991) and may form heterodimers with other members from the bZIP transcription factor family (Vinson et al., 2002). Heterodimers in cell extracts have been suggested with LAP or CHOP (Wang et al., 1998). Because LIP is missing the transactivation domain, it is believed to act as a transcriptional repressor by heterodimerizing with transcriptional activators and either sequestering them away from DNA target sites or binding the DNA targets and conferring reduced transcriptional activation. Because of these properties, the ratio of LIP to potential partner activators can be critical for gene regulation (Zahnow et al., 1997; Descombes and Schibler, 1991; Raught et al., 1995). This is clearly demonstrated by the decreased ability of ATF4 in cell extracts with increased LIP to bind the AARE (Figure 4-6E).
The early response of cells to ER stress involves the activation of the PERK/eIF2α signaling, which attenuates global protein synthesis and increases translation of specific mRNAs, among them the mRNA for the transcription factor ATF4 (Vattem and Wek, 2004). The PERK/eIF2α/ATF4 pathway has been implicated in the induction of genes involved in amino acid transport (cat-1 and SNAT2) and biosynthesis (asparagine synthase), glutathione biosynthesis, and protection against oxidative stress (Schroder and Kaufman, 2005a). We found that LIP attenuated ATF4-mediated transcription from the gene encoding cat-1, in agreement with its function as a transcriptional repressor. It is therefore likely that reduced LIP levels early in the ER stress response enable ATF4-mediated transcriptional induction of target genes.

In an attempt to determine the role of C/EBPβ in the ER stress response, we compared mRNA and protein levels of genes that are targets of the PERK/eIF2α/ATF4 pathway in wild-type and C/EBPβ−/− MEFs. This signaling pathway targets pro-survival genes early in ER stress (Yoshida, 2007) as mentioned above. However, it also induces pro-apoptotic genes during prolonged stress (Puthalakath et al., 2007). Among the pro-apoptotic genes are the ones shown to require CHOP for their induction during stress, such as those encoding TRB3 and GADD34 (Ohoka et al., 2005; Marciniak et al., 2004). We observed delayed and reduced accumulation of CHOP mRNA and protein during ER stress (Figures, 4-7 and 4-8). It has been documented that PERK/eIF2α signaling is an absolute requirement for induction of CHOP gene transcription during ER stress and that ATF4 may activate CHOP transcription during the early stress response by binding to an AARE (Harding et al., 2000; Averous et al., 2004). Our data show that induction of CHOP expression did not occur in the early stress response in C/EBPβ−/− cells despite high levels
of ATF4 and induction of other ATF4 target genes (Figures, 4-7 and 4-8). We can also speculate that eIF2α phosphorylation early in ER stress induces CHOP expression in two ways as follows: (i) phosphorylation increases ATF4 expression; (ii) phosphorylation decreases LIP levels, allowing ATF4 to form heterodimers with the activator LAP. In agreement with increased transcriptional activity of LAP during the early stress response is its increased phosphorylation at Thr-188 (rat sequence; data not shown) as shown previously in other systems (Kim et al., 2007). Regulation of CHOP gene expression via ATF4-C/EBPβ heterodimers binding to the CHOP AARE has been suggested (Chen et al., 2004). The CHOP gene promoter is also a target for the IRE-1 and ATF6 pathway, which has been implicated in induction of CHOP transcription in late ER stress (Yoshida et al., 2000). Our data are consistent with C/EBPβ inducing transcription of the CHOP gene early but not late stress response and are supported by a recent report that C/EBPβ binds to the CHOP gene promoter in vivo earlier than XBP-1 during ER stress (Donati et al., 2006).

The disruption of the C/EBPβ gene had opposite effects on CHOP, GADD34, and TRB3 expression as compared with AS, cat-1, and SNAT2 during ER stress. The induction of the latter mRNAs was significantly increased in C/EBPβ−/− cells during the late response, suggesting a loss of a transcriptional repressor during this phase of ER stress. We have shown in this study that LIP attenuates ATF4-mediated cat-1 transcriptional activation via the AARE. ATF3 is also a transcriptional repressor of these genes, and it inhibits ATF4-mediated transcription via their AAREs (Hai et al., 1999; Wolfgang et al., 1997). However, in C/EBPβ−/− cells, in contrast to the higher levels of these mRNAs, ATF3 levels were higher than the WT cells (Figure 4-7). This can be
explained by the overexpression of ATF4 and the fact that ATF3 is downstream of ATF4 (Jiang and Wek, 2004). Therefore, we can speculate that LIP levels, which increase dramatically during late ER stress, attenuate transcription of ATF4-mediated transcription of genes via AAREs. C/EBPβ has been suggested to be a negative regulator of expression of the AS gene, at a time when AS mRNA levels were declining during ER stress (Siu et al., 2001). Our findings can explain this correlation by LIP being the attenuator of positive regulation of AARE/ATF4-mediated gene transcription during late ER stress. A model summarizing the key finding of this work is shown in Figure 4-10. It was recently shown that LAP may also inhibit induction of AS gene expression during amino acid starvation (Thiaville et al., 2007). Our studies showed that the expression of LIP only in MEFs resulted in attenuation of ATF4-mediated cat-1 transcription. The regulation of AARE-containing genes by LAP and LIP during stress needs further study to clarify these issues.

**C/EBPβ is required for expression of genes downstream of CHOP**

Induction of CHOP has been associated with pro-apoptotic functions (Zinszner et al., 1998). These functions include down-regulation of Bcl-2 and up-regulation of genes that either compromise cell survival or promote apoptosis (Puthalakath et al., 2007; McCullough et al., 2001). CHOP can form heterodimers with either ATF4 or C/EBPβ proteins and induce gene expression during ER stress (Ohoka et al., 2005; Sok et al., 1999). Two of its target genes encode the activator of protein phosphatase 1, GADD34, which is induced by CHOP as a heterodimer with either ATF4 or C/EBPβ, and TRB3, which is induced by CHOP/ATF4 heterodimers (Ohoka et al., 2005; Sok et al., 1999). We show here that GADD34 and TRB3 expression was significantly reduced in C/EBPβ−
Figure 4-10. LIP modulates the PERK/ATF4-mediated gene expression program during ER stress. Diagrammatic representation of the regulation and functional significance of LIP during ER stress.
cells. This is in agreement with a recent report that C/EBP\(\beta\) induces TRB3 expression in mitogen-activated lymphocytes (Selim et al., 2007). The reduction of GADD34 is consistent with the sustained eIF2\(\alpha\) phosphorylation in C/EBP\(\beta^{-/-}\) cells during ER stress (Figure 4-7). This extended phosphorylation can explain the observation that ATF4 levels are higher in C/EBP\(\beta^{-/-}\) MEFs than in wild-type cells. However, the increased ATF4 levels may also be due to decreased levels of TRB3, which has been shown to attenuate ATF4-mediated transcription (Jousse et al., 2007).

Our data support the idea that C/EBP\(\beta\) is required for maximum induction of CHOP and its downstream targets. However, these genes were induced in C/EBP\(\beta^{-/-}\) cells, although at lower levels and with delayed kinetics. This is in contrast to a previous report on a different group of downstream genes called DOCs (Wang et al., 1998); induction of DOCs during ER stress was abolished in C/EBP\(\beta^{-/-}\) cells. It is therefore likely that GADD34 and TRB3 transcription is activated by homo- or heterodimers of ATF4 and CHOP in C/EBP\(\beta^{-/-}\) cells. However, because of the higher expression of these proteins in wild-type cells, it is likely that heterodimers of C/EBP\(\beta\) with other transcription factors are the most efficient activators of these genes. We prepared MEFs that express LIP in the absence of LAP, and we showed that LIP attenuates expression of genes involved in amino acid metabolism. We also plan to develop LAP-expressing cells in the absence of LIP. The successful development of these cell types will be a valuable tool to dissect the importance of LAP and LIP on pro-apoptotic gene expression during ER stress. It is therefore left in future studies to determine whether LIP has pro-survival functions during late ER stress by regulating the concentrations of transcription factors that compete for crucial DNA-binding sites.
Physiological significance of LAP/LIP ratios during ER stress and future directions

Our studies have demonstrated a physiological role of LIP in attenuation of ATF4-mediated transcription, in agreement with the role of ATF4 in the induction of genes required for amino acid metabolism and transport. Our studies will also shed light on diseases of bone formation, where high protein synthesis rates supported by high ATF4 contribute to skeletal abnormalities (Elefteriou et al., 2006).

Finally, we have shown that Tu induced ER stress in mice and caused accumulation of LAP and LIP after 24 h of treatment in liver, kidney, and pancreas. Although the significance of the different LIP/LAP ratios in the three ER-stressed organs is not known, it is clear that ER stress regulates C/EBPβ levels in animals and the stress-response gene expression program is tissue-specific. The most striking observation is that induction of CHOP is abolished in the liver but maintained in the kidneys of C/EBPβ−/− mice. In view of the recent reports on the importance of ER stress response pathways in cell fate (Lin et al., 2007), future studies in mice can address the differential response of tissues to ER stress and the importance of the C/EBPβ transcription factor.

Previous studies in mice have shown that Tu treatments, similar to the one used here, caused impairment in renal function and death of kidney cells (Zinszner et al., 1998). In agreement with the implication of CHOP and GADD34 in apoptosis during the ER stress response, mice lacking these proteins showed protection from kidney damage following treatment with Tu (Marciniak et al., 2004; Zinszner et al., 1998). Because C/EBPβ is the major heterodimerization partner of CHOP (Sok et al., 1999; Ron and Habener, 2006), its expression has also been implicated in promoting cell death (Kapadia et al., 2006). In fact, ER stress-induced apoptosis was lower in C/EBPβ−/− MEFs than WT MEFs.
(Zinszner et al., 1998). Based on these findings, we conclude that changes in LAP and LIP levels during ER stress may be important modulators of the integrated response to stress and may participate in decisions of cell fate during severe stress conditions.
APPENDIX

Contents in Table 1-1, Figure 1-1, and Figure 1-2 from indicated references were used with permissions.

Table 1-1
Physiol. Rev. 88: 249-286, 2008; doi:10.1152/physrev.00018.2006
APPROVED by pripta at 8:44 am, Mar 25, 2009

Figure 1-1
Licensee: Yi Li
License Date: Mar 24, 2009
License Number: 2155490327390
Publication: Trends in Cell Biology
Title: A trip to the ER: coping with stress
Type Of Use: Thesis / Dissertation
Total: 0.00 USD

Figure 1-2
Licensee: Yi Li
License Date: Mar 24, 2009
License Number: 2155460658035
Publication: Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis
Title: ER stress and the unfolded protein response
Type Of Use: Thesis / Dissertation
Total: 0.00 USD
BIBLIOGRAPHY


Fred R, and Welsh N. (2009). The importance of RNA binding proteins in preproinsulin


