Chapter 5
Gadd45 in the Liver: Signal Transduction and Transcriptional Mechanisms

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Abstract Injury and growth stimulation both remarkably increase the hepatic expression of Gadd45β. In liver cancer, promoter methylation frequently silences Gadd45β, demonstrating due to a suppressive function that is often proapoptotic. This contrasts with normal hepatocytes, where Gadd45β facilitates cell survival, growth, and proliferation. Gadd45β binds MKK7—downstream of TNFα and its receptors—to prevent this kinase from activating JNK2. Hence, the *Gadd45b−/−* genotype increases cell injury and decreases cell proliferation during liver regeneration (i.e., compensatory growth and proliferation). Liver hyperplasia (i.e., de novo growth and proliferation) is an alternate form of growth, caused by drugs that activate the nuclear receptor, CAR. As in regeneration, the *Gadd45b−/−* genotype considerably slows growth during hyperplasia. However, there is no injury and the slowing occurs because Gadd45β normally binds to CAR and activates its transcriptional stimulation. Thus, Gadd45β protects the liver through two entirely different processes: binding MKK7 to block damaging signal transduction or binding CAR to coactivate anabolic transcription.

Abbreviations

CAR	Constitutive androstane receptor
Gadd45	Growth arrest and DNA damage-inducible 45 proteins
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus

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IL6	Interleukin 6
JNK	c-Jun N-terminal kinase
MKK4/JNKK1	MAPK kinase 4/JNK kinase 1
MKK7/JNKK2	MAPK kinase 7/JNK kinase 2
NFκB	Nuclear factor kappa B
PH	Partial hepatectomy
TCPOBOP	1,4-bis[2-(3,5-dichloropyridyloxy)]benzene
TNFα	Tumor necrosis factor alpha
TNFR1, TNFR2	Tumor necrosis factor a receptor 1, 2

5.1 Introduction

The growth arrest and DNA damage 45 (Gadd45) family consists of three homologous acidic proteins (α, β, and γ). They are cellular responders to physiological and environmental stress (Liebermann and Hoffman 2008), and despite their small size (17–18 kDa), each protein has a surprising number of functions and binding partners. As part of its adaptations to different kinds of stress, the liver stimulates expression of individual Gadd45 protein in various responses to injury, inflammation, chemicals, and drugs. Liver function is diverse and includes essential processes like xenobiotic detoxification, bile metabolism, adipogenesis, carbohydrate metabolism, and serum protein synthesis. Each process must modulate in response to metabolic resources and specific stimuli, persist during liver injury and inflammation, and coordinate with cell growth and proliferation.

Because they are so similar, the distinct biological effect of each Gadd45 protein probably reflects the context of specific inducing signals, or the specific cell type that expresses the protein. Indeed, the liver consists of hepatocytes, Kupffer cells (fixed macrophages), stellate cells, biliary epithelium, and a variable number of inflammatory cells. It is often unclear which of these cells expresses a particular Gadd45 protein, so this review will highlight the main liver cell, i.e., the hepatocyte. Gadd45 proteins are also associated with hepatocytic neoplasia, both carcinogenesis and established hepatocellular carcinoma (HCC). Nevertheless, the most striking changes occur in normal hepatocytes. These cells induce an exceptional increase Gadd45β in response to either xenobiotic compounds or cell loss, with critical effects on cell survival and liver growth (Su et al. 2002; Locker et al. 2003; Papa et al. 2008; Tian et al. 2011). The survival effects act through MKK4 and MKK7, which activate the p38 and JNK pathways, in response to stress and cytokines like TNFα (Papa et al. 2009). The growth effects act through a different process, transcriptional coactivation, an important activity of Gadd45 proteins. Because these transcriptional functions have received limited attention in the liver or any other tissue, the last section reviews these mechanisms in detail.

5.2 Gadd45α and Gadd45γ

Normal liver expresses moderate levels of Gadd45α, with four- to sevenfold induction by ischemia, partial hepatectomy, chemical induction of hyperplasia with TCPOBOP (1,4-bis[2-(3,5)-dichoropyridyloxy] benzene), or treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a "nongenotoxic" carcinogen (Su et al. 2002; Locker et al. 2003; Fallsehr et al. 2005; Fletcher et al. 2005). In contrast, hepatic Gadd45α is induced >20-fold by dimethylbenzanthracene (DMBA). The liver activates this carcinogen to produce DNA adducts that require nucleotide excision repair, and DMBA produces three times more mutations in the *Gadd45a*-null mouse. (Hollander et al. 2001) Thus, as in other tissues, Gadd45α responds to DNA damage and facilitates DNA repair.

Transcriptional regulation of *Gadd45a* links to DNA damage through factors expressed in the liver and most other tissues, including p53 (Zhan et al. 1998; Kastan and Bartek 2004), BRCA1 (Jin et al. 2000; Campanero et al. 2008), nuclear receptor TR4/NR2C2 (Yan et al. 2012), and Myc (Amente et al. 2011). Gadd45α is also regulated by the stress-responsive ATF/CREB family of transcription factors (Maekawa et al. 2008). Among these, CREBH and ATF5 are abundant latent factors in the liver, CREBH activated by unfolded proteins and ER stress (Luebke-Wheeler et al. 2008), ATF5 by fasting, ER stress, and oxidative stress (Zhou et al. 2008; Shimizu et al. 2009).

Foxo3a, a transcriptional mediator of oxidative stress, also regulates *Gadd45a*, via a pathway characterized in HCC cells. Binding of APRIL, a TNF superfamily ligand, to its receptor, BCMA, leads to JNK2 activation, which then phosphorylates and activates Foxo3a (Amente et al. 2011; Notas et al. 2012). These findings also suggest feedback inhibition among Gadd45 proteins. Their prior expression might block activation of Foxo3a, since they are strong inhibitors of JNK2 (Papa et al. 2008) (see below).

Gadd45γ has received little attention in the liver, although expression profile studies have confirmed low-level constitutive expression with modulation in response to growth stimulators or injury (Su et al. 2002; Jee et al. 2007). However, the main transcriptional regulators attributed to *Gadd45g*, Oct1 and NF-Y (Campanero et al. 2008), have little relationship to phenotypic expression in the liver. The most notable liver effect was a 22-fold increase in mice observed 180 days after infection with a helminthic parasite, but in this case, expression was localized to infiltrating lymphocytes (Zhang et al. 2012).

5.3 Induction of Gadd45β in the Liver

Two types of hepatocyte proliferative responses induce striking immediate-early expression of Gadd45β: regeneration, the compensatory proliferation after the loss of hepatocytes, and hyperplasia, the de novo proliferation caused by drugs and

xenobiotic activators of the constitutive androstane receptor (CAR). The induction is exceptionally high—150-fold following CAR stimulation by the hydrocarbon TCPOBOP and 70-fold after partial hepatectomy (PH)—making Gadd45β one of the most strongly induced genes in either process (Su et al. 2002; Locker et al. 2003; Tian et al. 2011). Gadd45β is also induced by agents that activate another nuclear receptor of xenobiotics, the pregnane X receptor (PXR) (Kodama and Negishi 2011), that is not associated with proliferation.

Regeneration and hyperplasia stimulate Gadd45β through separate transcriptional mechanisms. PH, or liver damage from toxic agents like CCl_4, activates two known signaling pathways that stimulate Gadd45β transcription, TNFα-NFκB, and TGFβ-SMAD. This leads to rapid activation of NFκB (Ohmura et al. 1996), which specifically binds to upstream sites near the Gadd45β promoter and strongly activates transcription (Jin et al. 2002). The impairment of Gadd45β induction by PH in the *Tnfr1−/−* mouse confirms this relationship (Papa et al. 2008). NFκB also accounts for the induction of Gadd45β by *S*-adenosylmethionine (Seewoo et al. 2012).

TGFβ, another inducer of Gadd45β transcription (Yoo et al. 2003), is an important mediator of early liver regeneration that is released into the local circulation within 1 h after PH (Michalopoulos 2007). TGFβ activates Smad3 and Smad4, which stimulate *Gadd45b* transcription through a downstream enhancer (Major and Jones 2004). Three other agents induce hepatic transcription of *Gadd45b* but through undefined transcriptional regulators and binding sites. The multikinase inhibitor sorafenib strongly induces Gadd45β in sensitive, but not resistant, HCC cell lines, acting through a 72-bp upstream regulatory region (Ou et al. 2010). Oxaliplatin, a DNA-damaging drug, and insulin also induce Gadd45β in HCC cell lines, by unknown mechanisms (Bortoff et al. 2010; Seewoo et al. 2012).

The induction of Gadd45β by CAR is independent of the TNFα and TGFβ pathways, since TCPOBOP treatment activates neither (Columbano et al. 2005). The full induction of by TCPOBOP in *Tnfr1−/−* and *Tnfr1−/− Tnfr2−/−* knockout mice confirms this independence. In contrast, the *Car−/−* genotype prevents induction by TCPOBOP. CAR binds to a specific site near the Gadd45β promoter and activates reporter genes through this site (unpublished results). PXR also directly stimulates *Gadd45b*, in this case through a specific upstream binding site (Kodama and Negishi 2011).

Chronic hepatitis C virus (HCV) infection causes inflammation, hepatocyte loss, compensatory proliferation, and strong TNFα signaling. Surprisingly, these processes do not induce Gadd45β expression in HCV-infected liver, HCV transgenic mice, or HCV-induced liver cancer (Higgs et al. 2010). This suppression of Gadd45β occurs via promoter methylation. Thus, hepatocytes from HCV transgenic mice fail to arrest their cell cycles after UV-C treatment. However, treatment with the DNA demethylating agent, 5-azacytidine, restores Gadd45β expression and UV-induced cell cycle arrest. Although the mechanism that induces the methylation is unclear, these studies do confirm a relationship between hepatocyte DNA repair and Gadd45β.

5.4 Gadd45 Proteins in HCC

The Gadd45β promoter is hypermethylated in many HCC (Qiu et al. 2003, 2004; Higgs et al. 2010), a change that correlates with low or absent expression. Treatment with 5-azacytidine induces reexpression of Gadd45β and growth inhibition, suggesting a suppressive impact on cancer cells, the opposite of its growth effects in nonneoplastic liver. However, the mechanism of tumor suppression is unresolved. In contrast to Gadd45β, studies of Gadd45γ in HCC do not indicate suppressive effects. One paper reported that increased Gadd45γ expression is part of a profile that correlates with the most aggressive HCC in rats and humans (Frau et al. 2012). In another study, increased Gadd45γ mRNA was part of a liver profile that discriminated the responses to genotoxic and nongenotoxic carcinogens (Suenaga et al. 2013). The association of Gadd45γ expression with the response to genotoxic carcinogens indicates DNA repair functions similar to Gadd45α (Hollander et al. 2001).

5.5 Contradictory Effects on Hepatocyte Proliferation

The *Gadd45b−/−* mouse had significantly reduced proliferation during liver regeneration, showing that the protein is essential for the full adaptive response to loss of liver mass (Papa et al. 2008). In contrast, the *Gadd45b−/−* genotype caused a moderate increase in proliferation following treatment with TCPOBOP along with doubling of cyclin D1 expression (Tian et al. 2011). Proliferation in these two models has numerous differences, so it remains possible that Gadd45β functions in a pathway that activates the compensatory proliferation. Nevertheless, an opposite effect on the proliferation of hyperplasia, however, indicates that Gadd45β does not have in intrinsic role in direct cell cycle or replication processes. In addition, PXR stimulation activates expression of Gadd45β, but not proliferation (Kodama and Negishi 2011). The effects of Gadd45β on hepatocyte proliferation are therefore variable and facultative. They depend on the specific inducing process and the context of concurrent changes.

5.6 Gadd45β Mutation Impairs Liver Regeneration

Following PH, the absence of Gadd45β causes liver injury with significant mortality due to the unopposed activation of JNK (Papa et al. 2008). Notably, the knockout mouse is unable to compensate for Gadd45β deficiency via increased stimulation of Gadd45α or Gadd45γ. This confirms their separate transcriptional regulation and demonstrates that the effects are entirely dependent on Gadd45β deficiency.

By binding and inhibiting the Jun kinase kinase MKK7/JNKK2, Gadd45β prevents activation of JNK and thus ameliorates the potential damage mediated by TNFα signaling (De Smaele et al. 2001; Papa et al. 2004). This is a critical pathway because TNFα initiates liver regeneration and the *Tnfr1*−/− mouse fails to regenerate its liver following PH (Yamada et al. 1998). The *Gadd45b*−/− genotype does not completely abolish regeneration, but 56 % of these mice die after PH because of severe cell injury and inflammation (Papa et al. 2008). Compensatory proliferation is also lower than normal. PH in wild-type mice causes rapid phosphorylation of JNK2 and MKK7 with significant reduction by 8 h, while the *Gadd45b*−/− mouse has much greater JNK2 and MKK7 phosphorylation with persistent high levels through 72 h. The effect is JNK specific because two other MAPK pathways—ERK and p38—showed no differences between wild type and *Gadd45b*−/− so the effect is JNK specific. A further experiment confirmed this relationship, because JNK2 knockout introduced into the *Gadd45b*−/− background fully restored liver regeneration (Papa et al. 2008). The experiments also confirm an antiproliferative effect of JNK2 observed in isolated hepatocytes (Sabapathy et al. 2004). The effects also contrast the functions of JNK1 of JNK2. JNK1 activates proliferation via phosphorylation of Jun. JNK2 instead reduces cellular levels of Jun and reduces its activation of cell proliferation (Sabapathy and Wagner 2004). The critical function of Gadd45β liver regeneration is therefore to moderate the damaging effects of TNFα signaling, because dampening the activation of JNK2 shifts the balance towards protective growth-stimulatory responses.

5.7 Gadd45β Impairs Rapid Growth During Hyperplasia

Because inflammatory mediators initiate and guide liver regeneration, similar effects seemed likely for hyperplasia. Indeed, several papers have shown that CAR activation—and CAR-induced Gadd45β—can protect liver cells from apoptosis (Baskin-Bey et al. 2006, 2007; Yamamoto et al. 2010). These studies combined a CAR inducer with liver damage via Fas ligand, TNFα, or a methionine–choline-deficient diet. Pure hyperplasia, however, is the response of an intact liver without inflammation, cell injury, or activation of TNFα signaling (Columbano and Shinozuka 1996; Ledda-Columbano et al. 2000; Tian et al. 2011). Nevertheless, CAR activation produces extremely rapid liver growth (Tian et al. 2011).

Following treatment with TCPOBOP, liver mass increases 30 % in 3 h and doubles by 18 h. This anabolic response occurs the G1 cell cycle phase. Growth then pauses during S phase—beginning at 24 h—but resumes at 40 h after cell division. Hyperplasia is part of an adaptive response to xenobiotic and toxic exposure, and rapid growth results from synthesis of inactivating and conjugating enzymes, transport molecules, and membrane scaffolds for these proteins. Cell division presumably makes hepatocyte more efficient in detoxification by increasing the surface to volume ratio. In the *Gadd45b*−/− mouse, the proliferation was increased but rapid liver growth was impaired. This inhibition reflected blunting of early mRNA

synthesis induced by TCPOBOP—less upregulation of induced transcripts, more downregulation of inhibited transcripts, and de novo inhibition of other transcripts. By 48 h, however, the growth of the mutant liver was equivalent to wild type. The effect is most apparent at early time points because the essential function of Gadd45β is to enable rapid adaptation through transcription.

5.8 Gadd45β Is a Transcriptional Coactivator

The general effect on early mRNA synthesis suggested a transcriptional mechanism, but such effects could be the indirect consequence of a signal transduction pathway. However, an earlier study by Yi et al. showed that Gadd45 proteins act as direct transcriptional coactivators of nuclear receptors (Yi et al. 2000). These authors noted the characteristic coactivator sequence motifs, LXXLL, in all three Gadd45 proteins and then demonstrated critical properties of coactivators. (1) Each Gadd45 protein bound directly to nuclear receptor RXRα in yeast two-hybrid assays. (2) GST-fusions of each Gadd45 protein, synthesized in vitro, bound directly to nuclear receptors RXRα, RARα, ERα, PPARα, PPARβ, and PPARγ2 in pull-down assays. (3) Gadd45α and -γ coactivated nuclear receptors RXRα, PPARα, and PPARγ2 in assays of transfected reporter plasmids. Similarly, a recent study characterized direct binding of Gadd45β to CAR (Yamamoto et al. 2010).

Essential coactivator functions are intrinsic to different domains of Gadd45β (Fig. 5.1) (Tian et al. 2011). First, the Gadd45β coactivation of CAR is strong, comparable to coactivation by the p160 coactivator, Src1/Ncoa1. Second, Gadd45β contains an intrinsic domain that has direct activation function when bound to a reporter gene via fusion to a heterologous DNA-binding domain. Activation localized to the C-terminal region from aa 125–160. Third, Gadd45β bound directly to CAR, demonstrated with cell-free translated protein and with native protein synthesized in 293T cells. The latter analysis localized the CAR-binding domain to a central region from aa 69–92. Fourth, the two LXXLL domains—in a region between the binding and activation domains—are essential for coactivation, because mutation of either converts Gadd45β from a coactivator to a dominant-negative inhibitor of CAR. Fifth, chromatin immunoprecipitation assays of TCPOBOP-treated liver show that Gadd45β and CAR bind together at a characteristic response element upstream of a major regulatory target, the cytochrome P450 2b10 gene (*Cyp2b10*).

Gadd45β has strong coactivator function comparable to 160-kDa Src1, and there is significant homology between their α-helical LXXLL-containing segments (Fig. 5.1). In Src1, mutation of these motifs blocks transcriptional activation (Heery et al. 1997). In Gadd45β, similar mutations convert the protein to a dominant-negative inhibitor, although they do not block binding by a more proximal domain. LXXLL mutations have a similar effect on another coactivator, nuclear receptor binding factor 2 (Nrbf2) (Yasumo et al. 2000; Flores et al. 2004). When bound to nuclear receptors, an LXXLL domain of Src1 simultaneously aligns with Helix 3 and Helix 12 of the ligand-binding domain (Shiau et al. 1998; Pike 2006).

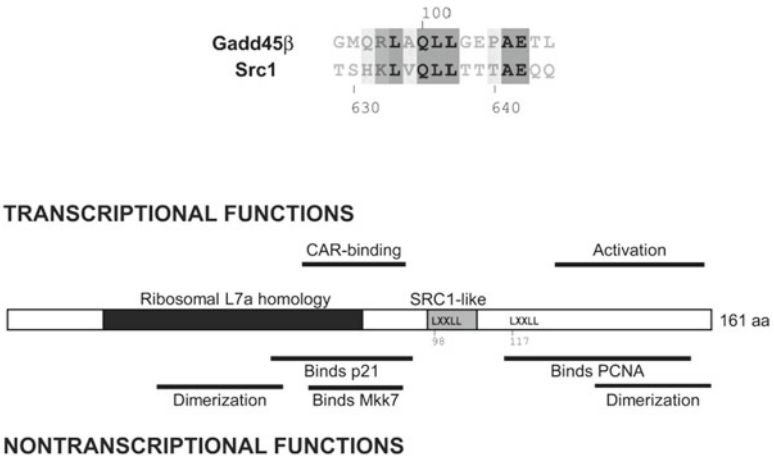

Fig. 5.1 Gadd45β domain structure. Above, alignment of LXXLL regions of Gadd45β and Src1/Ncoa1. There is strong sequence homology between the LXXLL motifs of Gadd45β at aa 98 and Src1 at aa 633, and each is within a short amphipathic α-helical segment, an essential property for binding and activation of nuclear receptors (Torchia et al. 1997; Tornatore et al. 2008). *Below*, mapped domains of Gadd45β. Gadd45 proteins contain a domain homologous to several RNA-binding proteins, including ribosomal protein L7a. RNA binding has been demonstrated in vivo and in vitro for Gadd45α, although the binding function has not been mapped (Sytnikova et al. 2011). Mapped transcriptional functions include binding to CAR, independent transcriptional activation, and mutation of either LXXLL motif converts Gadd45β to a dominant-negative inhibitor of CAR (Tian et al. 2011). Study of peptide functions has also localized domains that bind Mkk7 (Papa et al. 2007), p21 (Zhao et al. 2000), and PCNA (Vairapandi et al. 2000). Gadd45β dimerizes by interaction of proximal and C-terminal domains. There is significant overlap of function. CAR, p21, and Mkk7 all bind to the same region, which includes the distal part of the L7a homology domain. PCNA binding, transcriptional activation, and one of dimerization domains overlap at the C-terminal. The proximal dimerization domain overlaps with the proximal part of the L7a homology domain

Ketoconazole—an agent that binds this region of CAR and blocks binding of Src1—has the same effect on Gadd45β binding (Huang et al. 2007; Tian et al. 2011). The mutant forms of Gadd45β presumably bind to the same region but cannot align correctly with Helices 3 and 12. Thus, mutated Gadd45β not only fails as a coactivator, it acts as a dominant negative by blocking access of other coactivators.

The baseline levels of Gadd45β are comparable to many other LXXLL-containing coactivators in liver cells, but 150-fold induction more than doubles the total of all LXXLL-containing coactivators, an extraordinary change in the transcriptional resources of the hepatocyte. CAR activation stimulates a detoxification response, and the rapid growth of the liver is a vigorous adaptation to potentially toxic agents. The early induction of Gadd45β by CAR is a feed-forward mechanism that enables the high-level transcription necessary for adaptation. Although CAR is exclusively an activating transcription factor, treatment with TCPOBOP also causes CAR-dependent downregulation of many genes without a clear relationship to CAR, growth, or xenobiotic metabolism (Columbano et al. 2005). The increased

downregulation in the *Gadd45b−/−* mouse may explain the puzzling transcriptional mechanism. The findings suggest an indirect effect, downregulation by unsuccessful competition for limiting levels of coactivators.

5.9 Conclusions

The three Gadd45 proteins are so similar that they are likely to share almost all functions. The critical features of their different biological roles in the liver are the processes that induce them, which strongly favor Gadd45β in the liver. In various liver models, Gadd45β has contradictory roles. Its positive effects promote proliferation, growth, and cell survival. Its negative effects inhibit proliferation and stimulate apoptosis. The positive effects dominate in hepatocytes, and the negative functions are most apparent in HCC. All of these roles clearly depend on context and interacting partners.

The studies of Gadd45 proteins in hepatocytes exemplify the diverse mechanisms attributed to these proteins, ranging from signal transduction to transcriptional coactivation. Surprisingly, the domains that mediate transcriptional functions also mediate binding to Mkk7, p21, and PCNA (Fig. 5.1). This functional dichotomy seems to reflect two biological circumstances in the liver. During injury, especially with inflammation, the signal transduction mechanisms are dominant. In the absence of injury, the transcriptional mechanisms dominate. Perhaps the striking high-level induction of Gadd45β reflects simultaneous participation in multiple processes. Alternatively, it is possible that some effects attributed to direct interaction in signal transduction pathways are actually mediated by transcription, an indirect mechanism in which upregulation of transcriptional targets stimulates or inhibits responses. Despite the functional dichotomy, Gadd45β responses in hepatocytes do have a unifying feature. The robust early synthesis reflects the importance of Gadd45β for rapid adaptations.

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