A novel HSF1-mediated death pathway that is suppressed by heat shock proteins

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Heat shock response is an adoptive response to proteotoxic stress, and a major heat shock transcription factor 1 (HSF1) has been believed by to protect cells from cell death by inducing heat shock proteins (Hsps) that assist protein folding and prevent protein denaturation. However, it is revealed recently that HSF1 also promotes cell death of male germ cells. Here, we found a proapoptotic Tdag51 (T-cell death associated gene 51) gene as a direct target gene of HSF1. Heat shock and other stresses induced different levels of Hsps and Tdag51, which depend on cell types. Hsps bound directly to the N-terminal pleckstrin-homology like (PHL) domain of Tdag51, and suppressed death activity of the C-terminal proline/glutamine/histidine-rich domain. Tdag51, but not major Hsps, were induced in male germ cells exposed to high temperatures. Analysis of Tdag51-null testes showed that Tdag51 played substantial roles in promoting heat shock-induced cell death in vivo. These data suggest that cell fate on proteotoxic condition is determined at least by balance between Hsp and Tdag51 levels, which are differently regulated by HSF1.

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Introduction

The ability to adapt to changes in temperatures is essential for the viability of all living organisms. One of major detrimental effects of the exposure to high temperatures is to denature cellular proteins. To prevent protein denaturation, cells express a set of proteins called heat shock proteins (Hsps) that assist protein folding and inhibit protein denaturation (Parsell and Lindquist, 1993; Young et al., 2004). This response is known as heat shock response, and is regulated mainly at the level of transcription by heat shock transcription factor 1 (HSF1) in mammals (Morimoto, 1998). HSF1 protects from cell death against various kinds of stresses and these effects are mediated by regulation of Hsp expression (McMillan et al., 1998; Tanabe et al., 1998; Nakai and Ishikawa, 2001; Zhang et al., 2002) and other unidentified genes (Inouye et al., 2003; Fujimoto et al., 2005). HSF1 also plays critical functions in physiology (Pirkkala et al., 2001) and developmental processes such as gametogenesis, neurogenesis, and lens formation (Christians et al., 2000; Bu et al., 2002; Kallio et al., 2002; Wang et al., 2003, 2004; Fujimoto et al., 2004; Min et al., 2004; Santos and Saraiva, 2004; Chang et al., 2006; Takaki et al., 2006), as well as pathophysiological status such as ischemia (Yan et al., 2002; Zou et al., 2003) and protein folding diseases (Hsu et al., 2003; Morley and Morimoto, 2004; Fujimoto et al., 2005) by regulating Hsp and cytokine gene expression (Singh et al., 2002; Fujimoto et al., 2004; Inouye et al., 2004; Takaki et al., 2006).

Paradoxically, HSF1 also promotes apoptotic cell death of male germ cells. Mice expressing an active HSF1 in the testis are infertile due to a block in spermatogenesis (Nakai et al., 2000), and heat-induced apoptosis of spermatocytes are inhibited in mice lacking HSF1 (Izu et al., 2004). Furthermore, Fas-mediated apoptosis is enhanced in HeLa cells overexpressing an active HSF1 (Xia et al., 2000). Although comprehensive chromatin immunoprecipitation (ChIP) analysis revealed that HSF1 binds to a lot of genes in vivo in human cells (Trinklein et al., 2004), we did not know how HSF1 promotes cell death. Here, we identified a proapoptotic Tdag51 (T-cell death associated gene 51) gene as a target gene of HSF1, and demonstrate a novel mechanism as to how HSF1 determines cell death or life.

Results

HSF1 activates a proapoptotic gene

Microarray analysis of the profiles of gene expression in primary cultures of mouse embryo fibroblasts (MEFs) treated with or without heat shock at 42°C for 1 h identified eight HSF1-mediated heat shock-inducible genes (Figure 1A) (Inouye et al., 2004). Seven genes were heat shock genes and act as molecular chaperones or co-chaperones. In marked contrast, another Tdag51 gene induces apoptosis of culture cells such as T lymphocytes and neuronal cells (Park et al., 1996; Gomes et al., 1999; Neef et al., 2002), and is required for the induction of a proapoptotic Fas gene in T-cell hybridoma cells in vitro although its requirement was not confirmed in vivo (Park et al., 1996; Wang et al., 1998; Rho et al., 2001). Therefore, we analyzed regulation and biological significance of Tdag51 expression on stress condition.

We found that a profile of time-dependent accumulation of Tdag51 mRNA was similar to those of Hsp70 when MEF cells were heat-shocked at 42°C (Figure 1B). A profile of Tdag51 protein accumulation was also similar to those of major Hsps,
whereas Tdag51 protein accumulated earlier than the major Hsps (Figure 1C). In contrast to c-Jun expression that was high in both wild-type and HSF1-null cells, no induction of Tdag51 was observed in HSF1-null cells (Figure 1B). As previous report showed that Tdag51 expression is induced by various kinds of stresses (Hossain et al., 2003; Nagata et al., 2004; Schneider et al., 2004), we examined Tdag51 expression in various stress conditions. We found that an induced level of Tdag51 mRNA was low, but was still observed even in HSF1-null cells treated with sodium arsenite and a proline analog azetidine, which activate HSF1 (Figure 1D). These results indicate that Tdag51 gene is activated through different pathways, however HSF1 is required for its activation in response to heat shock.

To find enhancer elements responsible for HSF1-mediated gene expression, we cloned an upstream region of the Tdag51 gene and performed reporter analysis. Analysis of nucleotide sequences revealed a putative heat shock element (HSE) located at -4 to -33 from a transcription start site (Figure 1E) (Park et al., 1996). Reporter activity was induced in the presence of the HSE element, but was never induced when the HSE element was deleted or mutated. Furthermore, gel shift assay showed that an activated HSF1 binds specifically to the HSE element, but not to a mutated HSE (Figure 1F). Moreover, ChIP analysis showed that HSF1 binds directly to the Tdag51 gene as well as Hsp70-1 gene in vivo (Figure 1G). These results indicate that HSF1 directly binds to and activates Tdag51 gene in response to heat shock.

**Expressions of Tdag51 and Hsps are differentially regulated**

Because Tdag51 and Hsps exhibit opposing effect on cell fate upon stress condition, their expression could be differentially regulated. Therefore, we examined their expression in various cell types. In MEF cells, profiles of Tdag51 accumulation were similar to that of Hsp70 regardless of severity of heat shock (Figures 1A and B). Inversely, Tdag51 was markedly induced (Figure 2A). These results indicate that Tdag51 gene is activated through different pathways, however HSF1 is required for its activation in response to heat shock.

**Microarray analysis identified eight heat shock genes.** Genes undergoing more than three-fold change in MEF cells are clustered analyzed, and class c genes, whose expression increased after heat shock in wild-type cells but did not increase in HSF1-null cells (Inouye et al., 2004), are listed. (B) Expression of Tdag51 mRNA in wild-type (+/+ and HSF1-null (−/−) MEF cells after heat shock. Northern blot analysis was performed using total RNAs isolated from cells heat-shocked at 42 °C for the indicated periods. (C) Western blot analysis of expression of Tdag51 and major Hsps using extracts isolated from wild-type cells that were heat-shocked at 42 °C for 1 h and then recovered until 24 h, and from HSF1-null cells that were heat-shocked and recovered for 6 h (−/−). (D) Northern blot analysis using RNAs isolated from cells treated without (C) or with heat shock at 42 °C for 1 h (HS), 20 μM sodium arsenite for 6 h (As), or 5 mM azetidine for 6 h (AzC). (E) Reporter analysis of Tdag51 promoter. Numbers indicate positions of the Tdag51 promoter, which was inserted upstream of HSV-TK promoter (tk) relative to the transcription start site as +1. COS7 cells transiently transfected with the reporter plasmid were untreated (gray bars), or incubated at 42 °C for 1 h and recovered for 6 h (black bars), and harvested to estimate luciferase activities. Error bars indicate s.d. (n = 3). Sequences of wild-type putative HSE and mutated HSE (mHSE) are shown below. Stars indicate essential G and C nucleotides. (F) Gel shift assay was performed using an HSE oligonucleotide corresponding to the sequence of mouse Tdag51 gene (−40 to −1) (HSE) or mutated HSE (mHSE) as probes (left). Each 32P-labelled probe was mixed with extract prepared from cells treated with (+) or without (−) heat shock at 42 °C for 1 h. Specificity of the binding was examined by adding each specific antisense or each oligonucleotide into binding mixture. (G) ChIP-enriched DNAs using preimmune serum (pi) or anti-HSF1 serum (shSF1) as well as input DNAs were prepared, and DNA fragments of the Tdag51 gene (−259 to +124) and Hsp70-1 gene (−272 to +47) were amplified by PCR.
HSF1 bound to Hsp70 gene, but not to Tdag51 gene in heat-shocked F9 cells, whereas it bound to Tdag51 gene, but not to Hsp70 gene in heat-shocked Neuro-2a cells. These data indicate that differential expression of Tdag51 and major Hsps depends on the accessibility of HSF1 to each chromatin.

**Hsps inhibit Tdag51-mediated cell death**

It was shown previously that overexpression of Tdag51 or GFP-Tdag51 fusion protein induces apoptosis of various cell types, which is associated with detachment from culture dishes (Park et al., 1996; Gomes et al., 1999; Neef et al., 2002; Hossain et al., 2003). We found that overexpression of GFP-Tdag51 or HA-tagged Tdag51 into HeLa cells induces nuclear condensation and DNA fragmentation, indicating apoptotic cell death (Figure 3A, upper and lower left). More than 70% of cells expressing GFP-Tdag51 were apoptotic cells with condensed nuclei at 36 h after transfection (Figure 3A, lower middle), and the rest attached onto dishes with normal morphology and were alive. We estimated percentage of these alive cells as a cell survival rate (Figure 3B, left), and it was well correlated with cell viability measured by MTT assay (Figure 3B, right).

As HSF1 activates Tdag51 genes as well as major Hsp genes, we firstly examined effects of Hsp40 on Tdag51-mediated cell death. We transiently expressed different amounts of Tdag51 in HeLa cells and cells stably overexpressing Hsp40 (HeLa/Hsp40). We found that HeLa/Hsp40 cells were more resistant to low levels of Tdag51 than control HeLa cells (Figure 3C). Furthermore, we found that cells expressing high levels of Hsp40 were more resistant to Tdag51 than cells expressing low levels of Hsp40. These gene-dosage effects clearly indicate that Hsp40 inhibits Tdag51-mediated cell death (Figure 3C and D). HeLa cells overexpressing Hsp70 and Hsp110 were also resistant to Tdag51, but cells overexpressing Hsp27 were not (Figure 3D). These observations suggest that cell death is determined at least by balance between levels of antiapoptotic major Hsps and proapoptotic Tdag51.

**Hsps bind directly to a PHL domain of Tdag51**

We next examined how Hsps inhibit Tdag51-mediated cell death. In addition to suppressing aggregate formation, Hsp70 and Hsp27 inhibit cell death by preventing cytochrome c-mediated interaction of Apaf-1 with procaspase-9 (Beere et al., 2000; Bruet et al., 2000; Saleh et al., 2000). However, caspase-3 was not activated, and a pan-caspase inhibitor z-VAD did not inhibit Tdag51-mediated cell death in HeLa cells (Figure 3A and B) (Hossain et al., 2003), and Hsp27 had no effect on Tdag51-mediated cell death (Figure 3D), indicating that caspase-mediated death pathways should not be direct targets of Tdag51 in this cell line. We therefore examined direct interaction of Hsps with Tdag51. HEK293 cells were transiently transfected with an expression vector for GFP or GFP-Tdag51. Immunoprecipitation was performed using a specific antiserum for each Hsp and an antiserum...
Major Hsps inhibit Tdag51-mediated cell death. (A) Apoptotic cell death was induced by overexpression of Tdag51. HeLa cells were transiently transfected with expression vector for GFP or GFP-Tdag51 fusion protein. GFP fluorescence and DNA staining of boxed cells are shown (upper). Arrows indicate apoptotic cells. Bar; 10 μm. DNA fragmentation was analyzed in cells overexpressing GFP, GFP-Tdag51, and HA-Tdag51 as well as UV-irradiated (UVC 1000 J/m² and 4 h recovery) cells (lower left). We estimated percentages of apoptotic cells by counting cells with condensed nuclei (lower middle). Western blot analysis was performed using antibodies for procaspase-3, GFP, and β-actin (lower right). (B) Percentages of cell survival. After transfection, cells were incubated for 24 or 36 h in the absence or presence of 20 μM z-VAD. Percentages of cell survival (left) and MTT assay (right) are shown. (C) Hsp40 inhibits Tdag51-mediated cell death. HeLa cells and HeLa/Hsp40 cells were transfected with increasing amounts (0.3–1.9 μg) of an expression vector for GFP or GFP-Tdag51, and percentages of cell survival was estimated (left). Western blot analysis was performed using antibodies for GFP, Hsp40, and β-actin. HeLa/Hsp40 clones (clones 1–4) expressing different amounts of Hsp40 were transfected with an expression vector for GFP or GFP-Tdag51, and percentages of cell survival was estimated (right). Error bars indicate s.d. (n = 3). (D) HeLa cells stably expressing Hsp27, Hsp70, and Hsp10, as well as Hsp40 were transfected for 24 and 48 h with an expression vector for GFP (HeLa-GFP) or GFP-Tdag51, and percentages of cell survival was estimated (left). Error bars indicate s.d. (n = 3). Western blot analysis was performed using each specific antibody (right).

**PHL domain is required for inhibiting cell death activity of the C-terminal regions**

We determined which regions are responsible for its ability to induce cell death. Each domain fused to GFP was transiently expressed into cells. We found that cells expressing C-terminal proline/glutamine-rich and proline/histidine-rich tracts died, whereas cells expressing the PHL domain or any regions of its domain were alive (Figure 4E, data not shown). Interestingly, the C-terminal proline/glutamine-rich region induced cell death much stronger than a full-length Tdag51 (Figure 4E and F). Furthermore, cell death induced by the two C-terminal regions was not inhibited by overexpression of Hsp40, Hsp70, and Hsp10 (Figure 4F). These data indicate that the PHL domain negatively regulates cell death activity of the C-terminal regions through interaction with Hsps.

To concisely physiological relevance of HSF1-mediated Tdag51 induction, we next examined survival of Tdag51-null MEF cells. We found that induction of major Hsps was similarly observed in both wild-type and Tdag51-null cells in

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**Figure 3**

Major Hsps inhibit Tdag51-mediated cell death. (A) Apoptotic cell death was induced by overexpression of Tdag51. HeLa cells were transiently transfected with expression vector for GFP or GFP-Tdag51 fusion protein. GFP fluorescence and DNA staining of boxed cells are shown (upper). Arrows indicate apoptotic cells. Bar; 10 μm. DNA fragmentation was analyzed in cells overexpressing GFP, GFP-Tdag51, and HA-Tdag51 as well as UV-irradiated (UVC 1000 J/m² and 4 h recovery) cells (lower left). We estimated percentages of apoptotic cells by counting cells with condensed nuclei (lower middle). Western blot analysis was performed using antibodies for procaspase-3, GFP, and β-actin (lower right). (B) Percentages of cell survival. After transfection, cells were incubated for 24 or 36 h in the absence or presence of 20 μM z-VAD. Percentages of cell survival (left) and MTT assay (right) are shown. (C) Hsp40 inhibits Tdag51-mediated cell death. HeLa cells and HeLa/Hsp40 cells were transfected with increasing amounts (0.3–1.9 μg) of an expression vector for GFP or GFP-Tdag51, and percentages of cell survival was estimated (left). Western blot analysis was performed using antibodies for GFP, Hsp40, and β-actin. HeLa/Hsp40 clones (clones 1–4) expressing different amounts of Hsp40 were transfected with an expression vector for GFP or GFP-Tdag51, and percentages of cell survival was estimated (right). Error bars indicate s.d. (n = 3). (D) HeLa cells stably expressing Hsp27, Hsp70, and Hsp10, as well as Hsp40 were transfected for 24 and 48 h with an expression vector for GFP (HeLa-GFP) or GFP-Tdag51, and percentages of cell survival was estimated (left). Error bars indicate s.d. (n = 3). Western blot analysis was performed using each specific antibody (right).
response to heat shock or arsenite treatment (Figure 5A) and that Tdag51-null cells were more resistant to the stresses than wild-type cells examined by attached cell numbers and MTT cell viability assay (Figure 5B). We further generated adenovirus expressing full-length Tdag51 and its C-terminal region, and infected them into Tdag51-null MEF cells (Figure 5C). As MEF cells did not die just by infecting these viruses until 12 h, we treated the infected cells with heat shock at 42°C. Re-expression of wild-type Tdag51 in Tdag51-null cells restored cell survival at the level in wild-type cells (Figure 5D). Consistent with the role of its C-terminal region, survival rate of cells expressing the C-terminal region was much lower than that of cells expressing full-length of Tdag51. These observations support the idea of interplay between the C-terminal region and its negative regulator, the N-terminal PHL domain.

**Tdag51 is uniquely induced and promotes heat-induced cell death in vivo**

To reveal functional relevance of HSF1-Tdag51 pathway in vivo, we next focused on male germ cells that are susceptible to high temperatures (Print and Loveland, 2000). When the testicle was exposed to hot water at 43°C for 15 min, Tdag51 mRNA as well as Hsp70-1 mRNA was induced in unstressed testes, and were induced only in the interstitial Leydig cells, but not in germ cells (Figure 6Be and f) (Izu et al, 1997). We further generated adenovirus expressing full-length Tdag51 and its C-terminal region, and infected them into Tdag51-null MEF cells (Figure 5C). As MEF cells did not die just by infecting these viruses until 12 h, we treated the infected cells with heat shock at 42°C. Re-expression of wild-type Tdag51 in Tdag51-null cells restored cell survival at the level in wild-type cells (Figure 5D). Consistent with the role of its C-terminal region, survival rate of cells expressing the C-terminal region was much lower than that of cells expressing full-length of Tdag51. These observations support the idea of interplay between the C-terminal region and its negative regulator, the N-terminal PHL domain.
In contrast, Tdag51 mRNA was expressed at substantial levels in unstressed germ cells, especially pachytene spermatocytes (Figure 6Ba). Surprisingly, we found marked induction of Tdag51 mRNA in wild-type germ cells including pachytene spermatocytes in response to heat shock (Figure 6Bb). In HSF1-null mice, however, constitutive expression of Tdag51 mRNA was decreased, and its heat shock-induced expression was significantly impaired although we still detected induction at a faint level (Figure 6Bc and d). Consistent with reduced apoptotic cell death in heat-shocked HSF1-null testis (Izu et al., 2004), Fas expression was hardly induced (Figure 6A). These data demonstrate unique induction of Tdag51 by HSF1 in male germ cells, and implicate the relationship between apoptotic cells death and Tdag51 induction in vivo.

We next examined impact of Tdag51 induction on germ cell death. Tdag51-null mice were fertile and the testis was morphologically normal (Rho et al., 2001) (data not shown). We examined apoptotic cells when the testicles were heat-shocked at 43°C for 15 min, and found less numbers of apoptotic germ cells in Tdag51-null mice than that in wild-type mice (Figure 6C and D). These data demonstrated an important role of HSF1-Tdag51 pathway on germ cell death in vivo, and suggest that a proapoptotic Tdag51 functionally dominates antiapoptotic gene products such as Hsps in heat-shocked germ cells.

Discussion

It has been postulated that HSF1 may activate cell death pathways (Nakai et al., 2000; Xia et al., 2000; Izu et al., 2004). Here, we firstly identified a heat shock-inducible Tdag51 gene that promotes cell death (Park et al., 1996; Gomes et al., 1999; Neef et al., 2002). Although it was also reported that Tdag51 has no role in human T-cell death or promotes antiapoptotic effects of insulin-like growth factor-I (Oberg et al., 2004; Toyoshima et al., 2004), we showed that overexpression of Tdag51 induced cell death or caused cells sensitive to stres-
ses, and Tdag51-null cells were more resistant to stresses. These observations revealed a novel mechanism as to how cell fates are determined against proteotoxic stress. HSF1 not only protects from cell death by activating Hsp genes but also promotes it by activating a proapoptotic Tdag51 gene.

How does HSF1 decide cell to be dead or alive? In other adoptive cellular responses, some proapoptotic factors are similarly induced in addition to survival factors. In response to the accumulation of unfolded protein in the endoplasmic reticulum, cells, for example, induces CHOP that is involved in cell death (Zinszner et al., 1998; Rutkowski and Kaufman, 2004). Furthermore, proapoptotic Bcl-2 family proteins are induced in response to hypoxia (Bruick, 2000; Kim et al., 2004). In the later case, a proapoptotic Nip3 protein accumulates after exposure to prolonged hypoxia, indicating that it may play roles in eliminating irreversibly injured cells. In contrast, induction profiles of Tdag51 and Hsp70 are similar in MEF cells even after severe heat shock. Rather, we found that Tdag51 expression is uniquely regulated compared with expression of major Hsps in some cell lines including teratocarcinoma cells and neuroblastoma cells. Furthermore, male germ cells induced only Tdag51 after heat shock in vivo. Although sequence-specific co-factor should modulate HSF1-mediated expression of Tdag51 and Hsps, there is also the case that inability of heat activation of Tdag51 or Hsp genes was due to the lack of HSF1 accessibility on the genes. These results suggest that cell fate determined by HSF1-mediated gene expression on stress condition may be originally programmed in chromatin.

HSF1 regulates constitutive and inducible expression of Hsps, and this regulation is essential for thermotolerance (McMillan et al., 1998; Tanabe et al., 1998) and determining temperatures at which cells can survive (Nakai and Ishikawa, 2001). Hsps prevent protein denaturation and aggregation that are detrimental to cells (Parsell and Lindquist, 1993), and promote degradation of irreversibly denatured toxic proteins (Friant et al., 2003; Massey et al., 2006). More specifically, Hsps directly inhibit activity of death-related proteins such as cytochrome c and Apaf-1 (Beere et al., 2000; Bruey et al., 2000; Saleh et al., 2000). This study further demonstrated a novel

Figure 6 Induction of Tdag51 promotes cell death in vivo. (A) Induction of Tdag51 mRNA in vivo. Testicles of anesthetized wild-type (+/+) and HSF1-null (−/−) mice were submerged in water at 22 or 43 °C for 15 min and were allowed to recover for 5 h. RNAs were isolated and Northern blot analysis was performed using cDNA probes for Tdag51, Hsp70, and some death-related genes. (B) Unique induction of Tdag51 expression in male germ cells. In situ hybridization was performed on the untreated (a, c, e) and heat-treated (b, d, f) testes as described above using probes specific for Tdag51 (a–d) and Hsp70-1 (e, f). (C) Decreased apoptotic cells in Tdag51-null male germ cells. Testicles were heat-shocked at 43 °C for 15 min and were allowed to recover for 8 h. Sections were stained with PAS and were counterstained with methyl green (a, b). Seminiferous tubules at stage XI–XII are shown. Apoptotic cells were detected by TUNEL staining (brown) and were counterstained with methyl green (c, d). (D) Total numbers of apoptotic cells were counted in 200 tubes per testis treated as described in (C). Error bar indicate s.d. (n = 4). A star indicates that it is significant (P = 0.0194).
survival pathway conducted by Hsps, in which Hsps inhibit Tdag51-mediated cell death by binding to it directly.

Tdag51 is a member of the pleckstrin homology-related (PHL) domain family that consists of three members including Ipl/Tssc and Tih (Frank et al., 1999). Consistent with the fact that the PHL domain of Ipl/Tssc and Tih binds to phosphatidylinositol phosphate (Saxena et al., 2002), Tdag51 localizes in the cytoplasm including cytoplasmic and vesicular membranes (Hossain et al., 2003). We showed that Hsp70, Hsp40, and Hsp110 bind to the PHL domain consisting of seven β-sheet structures. Among three members, only Tdag51 possesses the C-terminal proline/glutamine/histidine-rich domain, which, we showed, has a strong ability to induce cell death. It was reported that Hsc70, a member of Hsp70 family, binds to the pleckstrin homology (PH) domain of Dbl oncogene product, and modulates guanidine nucleotide exchange activity of an adjacent domain (Kauppinen et al., 2005). Likewise, the binding of Hsps to the PHL domain might affect conformation of the C-terminal death domain to reduce its activity, or might inhibit death-related complex formation of Tdag51.

How does Tdag51 promote apoptosis? Tdag51 is necessary for upregulation of Fas expression in a T-cell hybridoma cells stimulated with anti-T-cell receptor antibody (Park et al., 1996), and expression of Tdag51 is also required for protein kinase C-mediated Fas expression in the same T-cell hybridoma cells (Wang et al., 1998). We here showed that the loss of Tdag51 induction is associated with the reduced induction of Fas in male germ cells in response to heat shock, which is important for germ cell death (Lee et al., 1997). Taken together, Tdag51 may act upstream of the Fas-mediated cell death pathway. In contrast to T cells and germ cells that are susceptible to cell death (Print and Loveland, 2000), expression of Fas is not induced by heat shock or overexpression of Tdag51 in MEF and HeLa cells. Furthermore, normal levels of Fas expression are observed in various tissues in Tdag51-null mice (Rho et al., 2001). Moreover, Tdag51-mediated HeLa cell death is independent from caspase-3 activation. Thus, Tdag51-mediated cell death is not always correlated with the induction of Fas expression. These observations suggest that Tdag51 may promote apoptosis through both Fas-mediated and Fas-independent mechanisms depending on cell types.

The decision between life and death of cells depends on the balance between survival signals and death signals. We revealed interplay between a proapoptotic Tdag51 and anti-apoptotic Hsps, both of which are direct target genes of HSF1. Roles of HSF1 may be analogous to those of a transcription factor p53. In response to genotoxic stress, p53 induces cell cycle arrest to allow cells to repair DNA before replication or mitosis. Alternatively, it induces apoptotic cell death to eliminate irreparably damaged cells (Amundson et al., 1998; Sionov and Haupt, 1999). The choice between growth arrest (survival) and apoptosis is determined by many factors depending on cell types. For example, T cells undergo extensive apoptosis in response to DNA damage, whereas fibroblasts enter cell cycle arrest (Bates and Vousden, 1999). Analogous to p53, HSF1 is a major factor that senses proapoptotic stress and transduces it to the expression of proapoptotic and antiapoptotic genes depending on cell types. Thus, the HSF1-mediated pathway may be one of quality control mechanisms not only in male germ cells but also in other somatic cells.
mouse Tdag51 (amino acids 62–261) fused to GST (a product of pGEX-Tdag3 expression vector).

**Reporter analysis**

A DNA fragment (~1013 to +129 relative to the transcription start site) was isolated by the calcium phosphate method. At 4 h after the transfection, cells were incubated in 96-well plates. After transfection, 10 μl of MTT (5 mg/ml in H2O) (Sigma) was introduced, and cells were incubated for 4 h followed by addition of 100 μl of acid-isopropanol (0.04 N HCl). After the reduced MTT crystals were dissolved, the plate was immediately scanned by an ELISA reader with a 595 nm filter.

**Primary cultures of MEF cells from wild-type and Tdag51** (Rho et al, 2001) 15-day-old mouse embryos were prepared as described previously (Inouye et al, 2003), and were maintained in DMEM containing 10% FBS at 37°C. Cells were incubated in medium containing adenosvir (10^5 PFU/ml) for 24 h, and then maintained in 2% CO2 for the indicated periods. The numbers of adherent cells were counted, and MTT assay was performed.

**Immunoprecipitation**

HEK293 cells were plated in 100-mm dishes for 16 h, and were transfected with an expression vector pEGFP2 or pEGFP2-Tdag51 (20 μg) by the calcium phosphate method. At 4 h after the transfection, cells were washed with PBS and incubated further for 44 h in normal medium. Cells were then washed with PBS, harvested, suspended in five packed cell pellet volumes of RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris (pH 7.5), 0.5 mM phenylmethylsulfon fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin) for 10 min on ice. The supernatants were collected after centrifugation at 15,000 r.p.m., for 10 min, with 1 μl of antisera for each Hsp (Fujimoto et al, 2004) at 4°C overnight, and mixed with 40 μg of protein A-Sepharose beads (1:1 suspension in PBS) (Amersham Biosciences) by rotating at 4°C for 1 h. The complexes were washed five times with RIPA buffer, suspended in SDS-sample buffer, and boiled for 3 min. The samples were loaded on SDS-PAGE, and transferred onto nitrocellulose membranes. The membranes were immunoblotted using mouse monoclonal antibody for GFP (GF200). Alternately, immunoprecipitation was performed using antisera for Tdag51 (2mTdag51-3), and immunoblot analysis was performed using antisera for each Hsp or mouse monoclonal antibody for Hsp70 (W27, Santa Cruz).

**In situ hybridization and in situ detection of apoptotic cells**

Wild-type, HSF1-null (Inouye et al, 2003), and Tdag51-null (Rho et al, 2001) mouse testes were treated with heat shock at 43°C for 15 min. The Committee for Ethics on Animal Experiments of Yamaguchi University School of Medicine reviewed all experimental protocols. In situ hybridization was performed essentially as described previously using the Tdag51 and Hsp70-1 cDNA fragments (see above) as probes (Izu et al, 2004). In situ detection of apoptotic cells was performed using In Situ Cell Detection Kit (Roche).

**Statistical analysis**

Significant values were determined by analyzing data with the Mann–Whitney’s U-test using StatView version 4.5J for Macintosh (Abacus Concepts, Berkeley, CA). A level of P<0.05 was considered significant.

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