GGAGAAATGGCGGGTAAGGT T-3′ (forward) and 3′- GCCTCTTTACCGCCCATTCCA AGATC-5′ (reverse), respectively, where the added nucleotides for the *Nhe*I site are underlined. HEK293 or HeLa cells were cultured in growth medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in an atmosphere of 5% CO_2. Subconfluent cells cultured in 12-well plates were transiently co-transfected with 0.5 μg pGL3-Basic vector DNA or each reporter construct (pGL3/−1993T or pGL3/−1993C) and 10 ng pRL-TK vector DNA (Promega) as an internal control for transfection efficiency, by using 1.5 μl FuGENE six transfection reagent (Roche Diagnostics, Basel, Switzerland). After 24 h, we then lysed the cells and measured firefly and *Renilla* luciferase activities in a luminometer by using the Dual-Luciferase Reporter Assay System (Promega).

Electrophoretic mobility shift assay

Nuclear extracts were prepared from HEK293 and HeLa cells as described previously (Dignam et al. 1983). Double-stranded oligonucleotides −1993T and −1993C were obtained by annealing three concatenated copies of 5′-GAAATGGTGGGTAAG-3′ and 5′-GAAATGGC-GGGTAAG-3′ with their respective complementary oligonucleotides. Electrophoretic mobility shift assay (EMSA) analysis was performed by using DIG gel shift kit (Roche). We prepared digoxigenin (DIG)-labeled double-stranded oligonucleotides corresponding to the sequence at position −2000 to −1986 of the *TBX21* promoter containing the −1993 polymorphism. For each binding reaction, we incubated DIG-labeled probes with nuclear extract (2–5 μg) in 1× binding buffer (20 mM HEPES, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM dithiothreitole, 30 mM KCl), 1 μg poly (dI-dC), and 0.1 μg poly L-lysine for 30 min on ice. For competition studies, we incubated unlabeled double-stranded oligonucleotide (100-fold molar excess) during preincubation. Reaction products were separated on 6% non-denaturing polyacrylamide gels in 0.3× TBE buffer (1× TBE buffer = 0.09 M TRIS-borate, 0.002 M EDTA, pH 8.3) and visualized by chemiluminescent detection. We scanned results into an LAS-3000 CCD camera system (Fuji Photo Film, Tokyo, Japan) and quantified each band intensity by using image analysis software Image Gauge Version 2.0 (Fuji Photo Film).

Statistical analysis

We calculated allele frequencies and tested agreement with Hardy–Weinberg equilibrium by using a χ^2 goodness of fit test at each locus. We then compared differences in allele frequencies and genotype distribution of each polymorphism between case and control subjects by using a 2×2 contingency χ^2 test with one degree of freedom or Fisher exact test and calculated odds ratios (ORs) with 95% confident intervals (95% CI). For multiple comparisons, *P*-values were corrected by the Bonferroni method. The linkage disequilibrium (LD) statistic *D′* was calculated by using the SNP Alyze statistical package (Dynacom, Chiba, Japan) as described elsewhere (Nakajima et al. 2002). Comparisons in reporter assays and EMSA experiments were performed with the Student's *t* test. A *P*-value of less than 0.05 was considered statistically significant.

Results

Screening for common polymorphisms in TBX21

Direct DNA sequencing of the indicated regions in 12 asthmatic and 12 healthy subjects (total 24 subjects) identified 24 biallelic SNPs in *TBX21*: three in the 5′ flanking region, three in the coding region (one non-synonymous and two synonymous), three in the 3′ untranslated region, and 15 in the intron (Table 2, Fig. 1). Five of these 24 SNPs (532G → C, 729G → T, 2839G → A, 9408C → A, and 10143C → A) are novel, and another 14 have been reported recently in Korean (Chung et al. 2003) and Finnish (Ylikoski et al. 2004) populations. Nucleotide position one (+1) is the first adenine of the initiation codon (ATG), and the positions for other SNPs are relative to the ATG on genome contig AC003665. A graphical overview of 24 SNPs identified in relation to the exon/intron structure of the human *TBX21* gene is given in Fig. 1. Since most of the SNPs were of relatively low frequency and in view of their location and LD with other sites, further genotyping and association studies in our asthma population focused on four SNPs: −1993T → C, 99C → G, 1298T → C, and 7725T → C. The distributions of all four SNPs were in Hardy–Weinberg equilibrium in the control group ($P > 0.05$). We calculated both *D′* and r^2 as statistical values for LD pair-wise between each SNP (Fig. 2). One of the three promoter SNPs (−1993T → C) and one synonymous coding SNP (390A → G, G130G) in exon one, were shown to be in strong LD.

TBX21 genotyping and association studies in asthma and related phenotypes

Initially, the association study was carried out on four clinical groups: child patients with asthma ($n = 361$), adult patients with atopic asthma ($n = 313$), adult patients with non-atopic asthma ($n = 88$), and adult patients with AIA ($n = 72$). Adult asthmatics, except AIA patients, had a negative reaction to the aspirin challenge or no past history of aspirin hypersensitivity. Allele frequencies of each selected SNP were compared between the patients and the normal controls by using a χ^2 test with 1 d.f. (Table 3). After correction for the number of SNPs investigated (Bonferroni correction), we found a significant association between the promoter SNP at −1993 and AIA in our Japanese cohort ($P = 0.004$;

Table 2 Locations and allele frequencies of *TBX21* SNPs in Japanese (*UTR* untranslated region)

Number	SNP	Location	Amino acid substitution	Minor allele frequency (%)	Primers
1	−1993T → C[a]	Promoter	–	8.3	F3R3
2	−1514T → C	Promoter	–	7.1	F4R4
3	− 1499G → A	Promoter	–	2.4	F4R4
4	99C → G[a]	Exon 1	H33Q	6.3	F6R6
5	390A → G	Exon 1	GI30G	8.3	F6R6
6	532G → C	Intron 1	–	2.2	F7R7
7	729G → T	Intron 1	–	4.2	F7R7
8	1298T → C[a]	Intron 1	–	16.7	F8R8
9	1662C → A	Intron 1	–	2.1	F8R8
10	2608G → A	Intron 1	–	2.1	F9R9
11	2839G → A	Intron 1	–	4.2	F9R9
12	7725T → C[a]	Intron 1	–	18.8	F11R11
13	8381A → T	Intron 1	–	16.7	F12R12
14	8762G → C	Intron 1	–	4.2	F12R12
15	9408C → A	Intron 2	–	2.3	F13R13
16	9882C → T	Intron 3	–	4.2	F14R14
17	9898C → T	Intron 3	–	4.2	F14R14
18	10143C → A	Intron 3	–	2.1	F14R14
19	10150T → C	Intron 3	–	4.2	F14R14
20	11268C → T	Intron 5	–	4.2	F16R16
21	11755G → A	Exon 6	P485P	2.1	F16R16
22	12077T → C	3′UTR	–	4.2	F17R17
23	12211G → A	3′UTR	–	2.1	F17R17
24	12403A → C	3′UTR	–	4.2	F17R17

[a]These SNPs were genotyped in a larger population

corrected P, $P_c = 0.016$). There was an increased risk for AIA associated with a C allele (OR = 1.93; 95% CI 1.22–3.06). Association analysis also demonstrated that a significant difference in allele frequency of −1993 SNP between AIA and other adult asthmatics who had no past history of aspirin hypersensitivity ($P = 0.001$). No other statistically significant association between disease status and genotype or any specific allele was detected from any of the other three case-control disease-association comparisons. We further analyzed the genotype and allele frequencies of the SNP at position 390, which was found to be in LD with SNP at −1993, in the AIA and control groups and then calculated the LD coefficient D' and r^2 between the −1993T → C and 390A → G SNPs ($D' = 0.92$; $r^2 = 0.85$). ORs of developing AIA at −1993T → C and 390A → G were 2.15 (95% CI 1.26–3.64) and 2.19 (95% CI 1.27–3.77), respectively, when the TC and TT (AG and GG) genotypes were compared with the wild-type TT (AA) genotype (Table 4). To determine whether these two SNPs in *TBX21* could also be associated with another AIA-related phenotype, we analyzed −1993T → C and 390A → G SNPs in 42 samples from independent adult AS/NP who either had a negative reaction to the aspirin challenge or no past history of aspirin hypersensitivity. Interestingly, comparison of the genotype and allele frequencies also revealed significant differences between the AS/NP group and the normal control group ($P = 0.008$ and 0.012, respectively). Furthermore, in the AIA case group, the C-allele frequency in AIA patients with NP tended to be much higher than that in AIA patients without NP (data not shown). Thus, although the sample size was small, we confirmed the *TBX21* SNP effect by using independent samples of AS/NP, regardless of aspirin hypersensitivity. We further analyzed two-loci haplotype distributions constituting the −1993T → C and 390A → G SNPs in the control, AIA, and AS/NP samples. Haplotype −1993T-390A was the

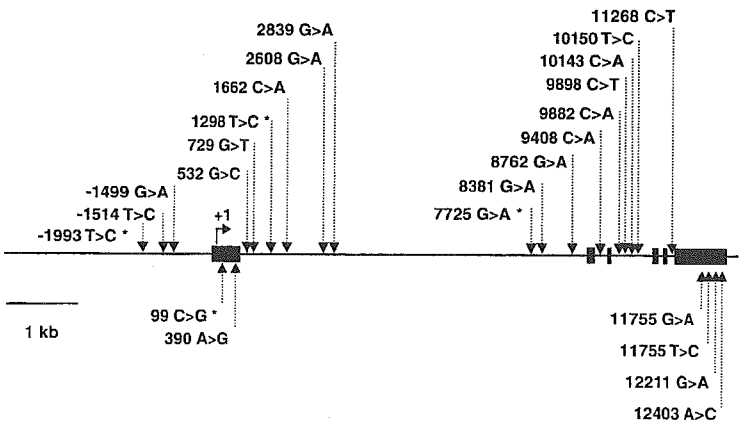

Fig. 1 Graphical overview of 24 SNPs identified in relation to the exon/intron structure of the human *TBX21* gene (*black boxes* five coding exons, *asterisks* SNPs genotyped in a larger population). Positions for SNPs are relative to the translation start site (+1)

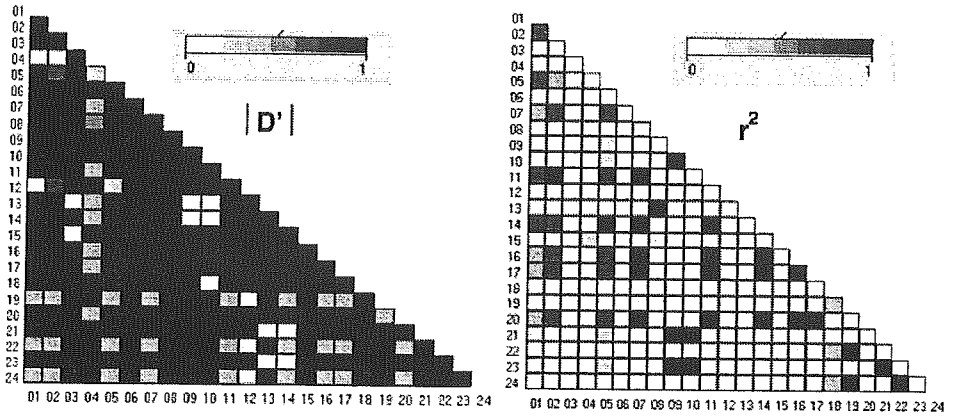

Fig. 2 Pair-wise linkage disequilibrium (*LD*) was measured by $|D'|$ and r^2 among the all of the SNPs identified in 24 sequenced samples. The blocks are *shaded* corresponding to the values obtained from the LD analysis program, SNP Alyze

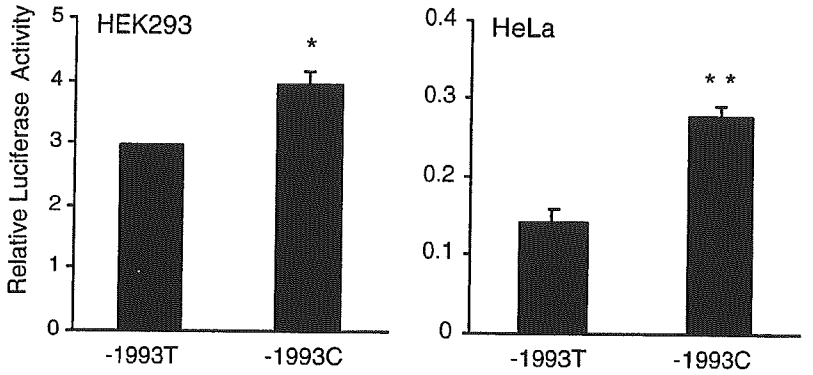

Fig. 3 Effect of the −1993T → C SNP on the transcription activity of the human *TBX21* promoter. HEK293 cells or HeLa cells were transiently cotransfected with pGL/−1993T or pGL/−1993C and pRL-TK vector. The relative luciferase activity of the *TBX21* reporter constructs is represented as the ratio of the firefly luciferase activity to that of *Renilla*. Each experiment was conducted in triplicate for each sample, and the results are expressed as mean ± SD for three independent experiments. $*P < 0.001$; $**P < 0.005$, as determined by the Student's *t* test

most common, followed by −1993C-390G and −1993C-390A. The two major haplotypes −1993T-390A and −1993C-390G were named haplotypes 1 and 2, respectively. The overall distribution of two loci haplotypes was not different between cases and controls (3 d.f.; $P = 0.10$ for AIA, and $P = 0.09$ for AS/NP), although the frequencies of two major haplotypes, haplotypes 1 and 2, were significantly different in a χ^2 test (1 d.f.; $P = 0.014$ for AIA, and $P = 0.016$ for AS/NP) (Table 4).

Transcriptional effect of TBX21 −1993T → C polymorphism

In functional assays, since the 390A → G polymorphism is a synonymous substitution, which is less likely to be directly associated with disease in general, we focused on the promoter SNP at position −1993, viz., −1993T → C. To understand the role of the T/C polymorphism at −1993 in the transcriptional regulation of the human *TBX21* gene, we performed transient expression of the −1993T and −1993C luciferase reporter constructs, pGL3/−1993T and pGL3/−1993C, in HEK293 and HeLa cells. Luciferase activity in cell extracts was analyzed after 24 h of transfection and was standardized against the internal control (*Renilla* activity). The results of this experiment showed that the −1993C construct had significantly higher luciferase reporter activity compared with the wild-type −1993T construct (33%–98% increase; $P < 0.005$). These results suggest that the −1993C allele may be associated with the increased transcriptional activity of the *TBX21* gene in human lungs.

EMSA analysis

To examine whether −1993T → C affected interaction of a nuclear factor(s) with the *TBX21* sequence around −1993, we then performed EMSAs. We prepared 2000/−1986 double-stranded oligonucleotide probes containing either the T or the C allele at −1993 bp. HEK293 nuclear extract contained nuclear proteins binding specifically to this region of the *TBX21* promoter, resulting in the formation of one major and one minor complex. Competition with 100-fold to 200-fold excess of unlabeled −1993T of −1993C probes resulted in complete inhibition of complex formation. The single band corresponding to the −1993C allele was significantly more intense than that corresponding to the −1993T allele (21% increase; $P = 0.02$ by Student's *t* test), suggesting the two different alleles had different affinities for a

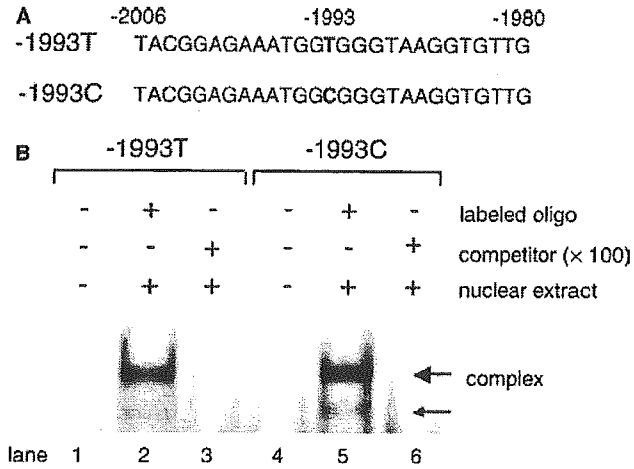

Fig. 4 EMSAs with nuclear extracts prepared from HEK293 cells. Extracts were incubated with DIG-labeled 27-bp double-stranded oligonucleotides corresponding to the −1993T or −1993C alleles of *TBX21*. Competition studies were performed by preincubating with a 100-fold excess of the unlabeled −1993T or −1993C double-stranded competitor oligonucleotides. **a** Oligonucleotide sequences containing T or C at −1993 bp (*bold*) and that were used as a probe or a competitor are shown. **b** Unknown nuclear protein of HEK293 nuclear extracts formed a much stronger complex with the −1993C oligonucleotide compared with the −1993T oligonucleotide (compare *lane 2* vs. *lane 5*; $P = 0.02$ by Student's *t* test). Binding complex was specifically competed by excess of unlabeled −1993T or C oligonucleotide (*lanes 3, 6*). Band intensity was quantified by using the LAS-3000 camera system and image analysis software Multi Gauge Version 2.0 (Fuji Photo Film). A representative result of three independent experiments is shown

particular nuclear factor. The same trend was also observed in HeLa cells (data not shown). Computer analysis of sequences covering −1993 bp, by using NSITE, available at http://www.softberry.com/berry.phtml?topic=nsite&group=programs&subgroup=promoter, indicated that the −1993T → C SNP is situated on a putative binding site for the E2F-1 transcription factor. To identify whether these putative consensus sites were involved in the transcriptional regulation of the *TBX21* gene, we performed a gel shift assay in the presence of specific anti-E2F-1 antibody (C-20; Santa Cruz Biotechnology, Calif., USA). However, preincubation with anti-E2F-1 antibody did not result in a supershift of the DNA-protein complexes (data not shown), suggesting that this protein (family) was not present in the complex binding to this region under these conditions. Together, these data indicate that the −1993T → C SNP in the human *TBX21* gene increases the affinity of an unknown nuclear protein to the binding site around −1993, leading to increased transcriptional activity and a higher expression of the T-bet protein.

Discussion

In the adaptive immune system, $CD4^+$ Th cells differentiate into at least two classes of effector cells, Th1 and Th2, in response to different pathogen-derived antigens. Th1 cells mediate cellular immunity and provide protection against intracellular pathogens and viruses, whereas Th2 cells produce IL-4, IL-5, and IL-13 and eradicate helminthes and other extracellular parasites (Mosmann and Coffman 1989). T-bet, a T box expressed in T cells, has recently been described as a master transcriptional regulator specific to IFN-γ-expressing lineages and is sufficient to induce IFN-γ and IL-12 receptor β2 expression, even under Th2-polarizing conditions (Afkarian et al. 2002; Szabo et al. 2000). Recent experiments have found that, without any allergic sensitization or challenge, the bronchi in mice lacking the T-bet gene, *tbx21*, are infiltrated with eosinophils and lymphocytes and exhibit signs of the airway remodeling and AHR to methacholine that are typical of allergic asthma (Finotto et al. 2002).

In order to examine whether polymorphisms in the candidate gene *TBX21* are related to the risk of human asthma phenotypes, we have characterized sites of

Table 3 Allele frequencies of *TBX21* SNPs in Japanese patients from different asthma groups and controls. Values are the number (%) of successfully genotyped chromosomes

Allele	Healthy controls ($n=640$)	Child patients with asthma ($n=361$)	P^a	Adult patients with					
				Atopic asthma ($n=313$)	P^a	Non-atopic asthma ($n=88$)	P^a	AIA ($n=72$)	P^a
−1993T → C									
T	1149 (89.8)	624 (89.1)		565 (90.3)		161 (93.6)		118 (81.9)	
C	131 (10.2)	76 (10.9)	0.67	61 (9.7)	0.74	11 (6.4)	0.11	26 (18.1)	0.004[b]
99C → G									
C	1127 (88.5)	617 (88.6)		509 (85.1)		143 (88.3)		116 (85.3)	
G	147 (11.5)	79 (11.4)	0.9	89 (14.9)	0.04	19 (11.7)	0.94	20. (14.7)	0.28
1298T → C									
T	1073 (83.8)	603 (83.5)		541 (87.0)		149 (84.7)		125 (86.8)	
C	207 (16.2)	119 (16.5)	0.86	81 (13.0)	0.07	27 (15.3)	0.78	19 (13.2)	0.35
7725G → A									
G	1062 (83.4)	577 (79.9)		502 (82.6)		139 (81.8)		122 (84.7)	
A	212 (16.6)	145 (20.1)	0.05	106 (17.4)	0.67	31 (18.2)	0.6	22 (15.3)	0.68

[a] *P*-value for the comparison with controls
[b] *P*-value statistically significant after Bonferroni correction (corrected $P = 0.016$)

Table 4 Genotype, allele, and haplotype frequencies in Japanese AIA, AS/NP cases, and controls for the *TBX21* SNPs at—1993 and 390

Locus	Haplotype number		Controls ($n=640$)	AIA ($n=72$)	Uncorrected P	Odds ratio (95% CI)	AS/NP ($n=42$)	Uncorrected P	Odds ratio (95%)
−1993T → C		Genotype TT	519 (81.1)	48 (66.7)		1.0	27 (64.3)		1.0
		Genotype TC+CC	121 (18.9)	24 (33.3)	0.004	2.15 (1.26–3.64)	15 (35.7)	0.008	2.38(1.23–4.62)
		Allele T	1149 (89.8)	118 (81.9)		1.0	68 (81.0)		1.0
		Allele C	131 (10.2)	26 (18.1)	0.004	1.93 (1.22–3.06)	16 (19.0)	0.012	2.06(1.16–3.66)
390A → G		Genotpye AA	533 (83.3)	50 (69.4)		1.0	29 (69.0)		1.0
		Genotype AG+GG	107 (16.7)	22 (30.6)	0.004	2.19 (1.27–3.77)	13 (31.0)	0.019	2.23(1.12–4.44)
		Allele A	1165 (91.0)	120 (83.3)		1.0	70 (83.3)		1.0
		Allele G	116 (9.0)	24 (16.7)	0.004	2.01 (1.25–3.24)	14 (16.7)	0.021	2.01 (1.10–3.68)
[−1993]-[390]	1	T-A	1148 (89.7)	120 (83.3)			68 (80.9)		
	2	C-G	114 (8.9)	22 (15.3)	0.014[a]		14 (16.7)	0.016[a]	
	3	C-A	17 (1.3)	2 (1.4)			2 (2.4)		
	4	T-G	1 (0.1)	0 (0.0)	0.10[b]		0 (0.0)	0.09[b]	

[a] *P*-value for the comparison of the frequencies of haplotype 1 and 2
[b] *P*-value for the overall distribution of two loci haplotypes

genetic variation in selected genomic regions of *TBX21*. Among 24 SNPs identified (five are novel), four polymorphic sites were selected for further analysis. All SNPs fulfilled Hardy–Weinberg expectations in both asthmatic and non-asthmatic subjects, and our study showed a significant association between AIA and a SNP in the regulatory region −1993T → C of the human *TBX21* gene ($P_c=0.016$); this was found to be in strong LD with a synonymous coding SNP, 390A → G, located in exon 1 ($D'=0.92$; $r^2=0.85$). Consistent with recent data (Chung et al. 2003; Ylikoski et al. 2004), these four *TBX21* SNPs lack association with any other asthma phenotype in Japanese subjects.

The percent of the −1993C or 390G allele was much higher in AIA patients than normal controls. In an attempt to extend and support these findings, we further genotyped the −1993T → C and 390A → G SNPs in independent adult AS/NP patients and also found a significant association between these SNPs and AS/NP for the allele and genotype frequencies ($P=0.008$). Furthermore, our data indicated that the single base substitution corresponding to the −1993 *TBX21* polymorphic site produced differences in the transcriptional activity of the *TBX21* gene. Unexpectedly, the *TBX21*/−1993C reporter construct was transcriptionally more active than the wild-type −1993T construct in HEK293 and HeLa cells. In addition, EMSA analysis demonstrated that the −1993T → C substitution increased the affinity of a particular nuclear protein to the binding site of *TBX21* covering the −1993 position.

AIA refers to the development of bronchoconstriction following the ingestion of aspirin and other NSAIDs. This clinically distinct syndrome is characterized by aspirin hypersensitivity, bronchial asthma, and chronic rhinosinusitis with nasal polyposis, commonly called the "aspirin triad". AIA affects 5%–20% (about 10%) of adult asthmatics with a higher prevalence in women and is infrequently found in asthmatic children (Babu and Salvi 2000; Szczeklik and Stevenson 1999). Chronic persistent inflammation is the hallmark of patients with AIA. Recently, the importance of arachidonic acid metabolites in the pathogenesis of AIA has become apparent. The cyclo-oxygenase (COX) theory is widely accepted: AIA attacks are triggered by the specific inhibition of COX in the respiratory tract, which is followed by a reduction of prostaglandin E2 (a brake on leukotriene synthesis) and an overproduction of cysteinyl leukotrienes. Thus, cysteinyl leukotrienes have been recognized as the key mediators of AIA, but the precise molecular mechanism involved in AIA remains unclear.

Surprisingly, our results have shown a significant increase in the −1993C allele, the putative higher expression of T-bet, among patients with AIA or AS/NP, compared with controls in our Japanese cohort. An inappropriate or excess Th2-biased immune response to environmental antigens has generally been considered to play a crucial role in the development of asthma. Whereas Th2 cells promote asthmatic inflammation, Th1 cells, which secret IFN-γ, have been proposed to protect against asthma by dampening the Th2 response. However, the evidence from many studies of asthma in human and animal models conflicts with this interpretation (Busse and Lemanske 2001; Salvi et al. 2001). For example, IFN-γ production is elevated in the serum of patients with asthma (Corrigan and Kay 1990), in supernatants of bronchoalveolar lavage (BAL) cells (Cembrzynska-Nowak et al. 1993), in T cells themselves in BAL (Krug et al. 1996), and in whole blood culture (Magnan et al. 2000). By using an adaptive transfer system in mice, previous reports have shown that antigen-specific Th1 cells cause considerable airway inflammation instead of attenuating Th2-mediated lung disease (Hansen et al. 1999; Li et al. 1998; Randolph et al. 1999). IFN-γ has been demonstrated to activate eosinophils in vitro, not only with an increased expression of Fcγ receptors, CD69, HLA-DR, and intercellular adhesion

molecule-1, but also with increased viability (Busse and Lemanske 2001; Krug et al. 1996). Furthermore, therapy with IL-12, a Th1-inducing cytokine, fails to reduce AHR or the late asthmatic reaction (Bryan et al. 2000). These and other recent data (Ford et al. 2001; Sugimoto et al. 2004) suggest that IFN-γ contributes to the augmentation of allergic lung inflammation partly through the activation of eosinophils, highlighting the importance of both Th1 and Th2 cytokines in the development of asthma. Thus, the classification of allergic inflammation in asthma as a Th2-mediated disease is too simplistic (Busse and Lemanske 2001), and, as pointed out by recent work (Sugimoto et al. 2004), we propose that asthma may be classified roughly into at least two subgroups, Th2-type asthma and Th1/Th2 mixed-type asthma, including AIA.

Previous reports have suggested that Th1 cells can actually cooperate with Th2 cells in vivo and enhance Th2, eosinophil, and neutrophil recruitment by increasing the expression of TNF-α, chemokines, and adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) in the lungs (Randolph et al. 1999; Takaoka et al. 2001). Actually, aspirin sensitivity and AS/NP often coexist with severe asthma, and the airways of AIA patients with NP show signs of persistent inflammation with marked eosinophilia and enhanced VCAM-1 expression (Hamilos et al. 1996). The continuous airway inflammation in AIA could result from a non-IgE-mediated reaction to specific endogenous or exogenous antigens such as a virus (Szczeklik 1988; Szczeklik and Stevenson 1999). A latent or chronic viral infection has been shown to alter the expression of many cellular genes, including several constituents of the arachidonic acid pathway (Zhu et al. 1998), and virally infected cells are more prone to drug and drug-metabolite-related toxicity (Levy 1997; Nakagawa et al. 2001). Moreover, an antiviral drug, acyclovir, is reported to inhibit analgesic-induced bronchoconstriction and decrease the urinary levels of leukotrieneE4 in patients with AIA (Yoshida et al. 1998). Based upon these observations of AIA, virus-specific Th1 cells responding to a respiratory tract infection could alter the local lung environment sufficiently to increase Th2 and eosinophil recruitment, leading to strong Th2 responses to inhaled antigens induced by IL-4, 5, 13, and other mediators. We postulate that the $-1993T \rightarrow C$ SNP in the *TBX21* promoter causes a functional difference in T-bet expression, resulting in increased T-bet production and (viral-induced) an excessive Th1 inflammatory reaction in the lungs.

Asthma is a phenotypically heterogeneous disorder with many etiologic factors and clinical characteristics. Although we find no associations of *TBX21* SNPs with other asthma groups except for AIA, our data also indicate that the presence of the $-1993C$ allele increases the risk of AS/NP, regardless of aspirin sensitivity. Thus, in asthma phenotypes, the *TBX21* SNPs are probably not strictly associated with aspirin sensitivity itself. NP is a chronic inflammatory disease of the paranasal sinus mucosa, leading to the protrusion of edematous polyps into the nasal cavities (Mygind 1990). NP is commonly found in association with non-atopic asthma and aspirin sensitivity, and this association of NP with asthma might reflect the shared pathophysiology of these disorders of the upper and lower airways, respectively. Furthermore, previous studies have shown that NP-infiltrating T cells expressed a mixed Th1/Th2 pattern of cytokines (Hamilos et al. 1995; Sanchez-Segura et al. 1998). Together, our present data suggested that, in a variety of asthma-related conditions, the amplification of either side of the Th1/Th2 pathway, or both, could be adverse to the host. Churg-Strauss syndrome (CSS), also known as allergic granulomatosis and angiitis, is another asthma-related disorder characterized by systemic small vessel vasculitis. Indeed, analysis of the cytokine profile of T cell lines from patients with CSS has shown both type-1 cytokine and type-2 cytokine responses (Kiene et al. 2001). Of note, clinical signs of autoimmunity such as vasculitis have been observed in some patients with AIA (Szczeklik et al. 1995, 1997).

The human *TBX21* gene is located on chromosome 17q21.32, which has previously been linked with asthma and skin tests (Dizier et al. 2000). Moreover, the region on mouse chromosome 11, a region that has been linked to AHR, is syntenically homologous to human chromosome 17q12-q22 (Zhang et al. 1999). *TBX21* is likely to be a novel candidate gene in this region, in addition to other candidate genes such as eotaxin (*CCL11*). However, our data cannot exclude the possibility that $-1993T \rightarrow C$ is in LD with another polymorphism in *TBX21* or a neighboring gene. Further studies in larger or other populations will be required to confirm the effect of the *TBX21* polymorphism. To date, several candidate genes of the enzymes in the arachidonic pathway, such as *LTC4S* and *ALOX5*, have been proposed to increase the susceptibility to AIA (Choi et al. 2004; Kawagishi et al. 2002; Sanak et al. 1997); indeed many other genes, in addition to *TBX21*, probably contribute to the pathogenesis of AIA. Genetic epidemiology on larger numbers of AIA patients is an important future requirement in order to clarify the importance of our findings.

In conclusion, we have identified 24 SNPs (five novel) in the *TBX21* gene, and our studies demonstrate that the $-1993T \rightarrow C$ SNP in the *TBX21* promoter is likely to be associated with an increased risk for AIA in Japanese. This is the first report demonstrating a relationship between the *TBX21* SNPs and clinical features of human asthma. Furthermore, we have shown that the $-1993T \rightarrow C$ polymorphism affects the transcriptional activity of the gene and may contribute to an increase in T-bet expression. In certain asthma subgroups, such as AIA and AS/NP, this promoter SNP may cause inappropriate Th1 responses in the airway, leading to severe airway inflammation, in combination with antigen-specific Th2 responses. Our present data shed light on an important area of further study regarding the precise phenotype classification of asthma by using genotypes and also focus on the Th1 response in the pathogenesis of AIA.

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Simple Fibroblast-Based Assay To Test the Pyrazinamide Susceptibility of *Mycobacterium tuberculosis*

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A simple fibroblast-based assay (SFA) was found to be efficient in evaluating the susceptibilities of clinical isolates of *Mycobacterium tuberculosis* to pyrazinamide (PZA). Forty-five clinical isolates were examined. The MICs of PZA for susceptible strains in an SFA were between 3.13 and 12.5 μg/ml, and the MICs of PZA for resistant strains were more than 100 μg/ml.

The incidence of tuberculosis is still increasing in some countries, and control of the disease is threatened by the emergence of drug-resistant strains (23). At this time, pyrazinamide (PZA) is one of the first-line drugs in the standard treatment regimen used for tuberculosis patients, and it is especially indispensable to DOTS (directly observed treatment, short course) (5, 11, 12). In the past few years, considerable progress has been made in understanding the mechanism of action of PZA and the genetic basis of resistance to the compound (11, 24). Most of the resistance to PZA has been shown to be accompanied by the loss of pyrazinamidase (PZase) activity in *Mycobacterium tuberculosis* (9). Mutations in the *pncA* gene have been identified as the cause for acquired PZA resistance in *M. tuberculosis*, and the sequence alteration of this gene has also been reported in naturally resistant *Mycobacterium bovis* strains (16, 17, 18). However, the mutation sites in the *pncA* gene are diverse (1), and in addition, mechanisms other than mutation of *pncA* are operative. In order to detect PZA-resistant strains, in vitro testing of the susceptibility of *M. tuberculosis* to PZA is highly recommended. Unfortunately, conventional agar-based testing for PZA susceptibility often leads to ambiguous results because of insufficient growth in the acidified medium (21). Therefore, a new method for PZA susceptibility testing is necessary. We have previously reported that viable *M. tuberculosis* $H_{37}Rv$ exhibits cytotoxicity to a human lung fibroblast cell line (19, 20). As the cytotoxicity was counteracted by drugs, including isoniazide, rifampin, ethambutol, streptomycin, and PZA, a simple fibroblast cell-based assay (SFA) was developed to screen antimycobacterial drugs (20). In this study, we applied SFA to test the susceptibilities of clinical isolates of *M. tuberculosis* against PZA.

Clinical isolates. Forty-five clinical isolates of *M. tuberculosis* from Asian countries and Canada, which were characterized for PZase activity and susceptibility to PZA in acidic agar medium, were used for susceptibility testing by an SFA. For some isolates, mutation analysis of the *pncA* gene was also performed by sequencing a 561-bp region (8). In addition, three PZA-resistant mutants (KK-117, KK-118, and KK-123) obtained from *M. tuberculosis* clinical isolates by serial passage in Middlebrook 7H9 liquid medium (pH 5.5) with twofold increasing concentrations of PZA ranging from 50 to 3,200 μg/ml were also examined. These three mutants had no mutations in the *pncA* gene.

PZA susceptibility testing using acidic agar medium. The MIC (drug concentration required to inhibit more than 99% of colony formation) of PZA was determined on Middlebrook 7H11 agar at a pH value of 6.0 containing twofold concentrations of drug ranging from 25 to 800 μg/ml. The inoculum consisted of 0.1 ml of a 10^{-3} dilution of a 2-week-old liquid culture (10^7 to 10^8 CFU/ml) of mycobacteria. Cultures were incubated at 37°C with 5% CO_2 for 3 weeks before the MIC was determined. The threshold concentration for the evaluation of PZA resistance in the acidic medium was defined as 200 μg of PZA per ml.

Susceptibility testing of *M. tuberculosis* by SFA. The human lung fibroblast cell line MRC-5 has been shown to be very sensitive to the cytotoxicity of live virulent bacilli of *M. tuberculosis* $H_{37}Rv$, and the host cell viability was reflected by the state of the bacilli inside the host cells (19, 20). As MRC-5 is a healthy diploid cell line, the cells are difficult to propagate for long periods. Accordingly, in this study, we used the transformed strain MRC-5 SV TG1 (Cell Bank, Osaka, Japan), which is immortal. The cell line was susceptible to the cytotoxicity of live bacilli in the same manner as the parent MRC-5 cells (data not shown).

MRC-5 SV TG1 cells were plated as previously described (20), and the bacterial suspensions (up to 50 μl) of clinical isolates of *M. tuberculosis* were added. After incubation for 16 to 18 h, the host cells were washed with phosphate-buffered saline (PBS), and then 200 μl of fresh tissue culture medium with or without PZA (at twofold concentrations from 0.78 to 100 μg/ml) was added. After 3 days of culture, the host cells were stained with crystal violet, and the optical density at 595 nm was measured to determine the host cell viability.

In the SFA, MIC was defined as the lowest concentration of drug exhibiting a statistically significant inhibitory effect on the bacterial cytotoxicity as determined by examining cell viability (20). In this study, the MICs of PZA for sensitive clinical isolates of *M. tuberculosis* were between 3.13 and 12.5 μg/ml,

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TABLE 1. Comparison of SFA and three methods in determining the susceptibilities of 45 clinical isolates of *M. tuberculosis* to PZA

Isolate or strain	Method used to determine the susceptibility to PZA			
	PZase activity[a]	Acidic agar test[b]	Mutation in *pncA* gene[c]	SFA[d]
PZA-susceptible clinical isolates				
A.1.1	Positive	S (100)	ND	S (6.25)
A.1.2	Positive	S (100)	ND	S (6.25)
A.1.3	Positive	S (50)	ND	S (3.13)
A.2.1	Positive	S (50)	ND	S (6.25)
A.2.3	Positive	S (100)	ND	S (6.25)
A.2.4	Positive	S (100)	ND	S (12.5)
A.3.5	Positive	S (100)	ND	R (>100)
A.3.9	Positive	S (100)	ND	S (3.13)
A.3.11	Positive	S (100)	ND	S (3.13)
A.3.12	Positive	S (200)	ND	S (12.5)
A.3.13	Positive	S (100)	ND	S (3.13)
A.3.16	Positive	S (50)	ND	S (3.13)
A.3.20	Positive	S (100)	ND	S (3.13)
A.3.22	Positive	S (100)	ND	S (6.25)
A.3.24	Positive	S (100)	ND	S (6.25)
A.3.47	Positive	S (100)	ND	S (3.13)
A.3.92	Positive	S (100)	ND	S (6.25)
A.4.25	Positive	S (100)	ND	S (6.25)
A.4.71	Positive	S (50)	ND	S (6.25)
A.4.74	Positive	S (50)	ND	S (6.25)
PZA-resistant clinical isolates				
A.2.6	Negative	R (>800)	ND	R (>100)
A.4.30	Negative	R (800)	ND	S (3.13)
Can III-283	Negative	R (>800)	+	R (>100)
Can III-291	Negative	R (>800)	+	R (>100)
D-2-74	Negative	R (>800)	ND	R (>100)
Ind-2	Negative	R (>800)	+	R (>100)
Ind-10	Negative	R (>800)	+	R (>100)
Mal-87	Negative	R (>800)	+	R (>100)
Mya-20	Negative	R (>800)	+	R (>100)
Mya-26	Negative	R (>800)	+	R (>100)
Mya-50	Negative	R (>800)	+	R (>100)
Mya-54	Negative	R (>800)	+	R (>100)
Thai S2	Negative	R (>800)	+	R (>100)
Thai S6	Negative	R (>800)	+	R (>100)
Thai S13	Negative	R (>800)	+	R (>100)
Thai R10	Negative	R (>800)	+	R (>100)
Thai-88-2	Negative	R (>800)	+	R (>100)
V.3.20	Negative	R (>800)	ND	R (>100)
V.3.28	Negative	R (800)	ND	R (>100)
YE-7	Negative	R (>800)	+	R (>100)
YE-14	Negative	R (>800)	+	R (>100)
YE-43	Negative	R (>800)	+	R (>100)
YE-67	Negative	R (>800)	+	R (>100)
YEY-31	Negative	R (>800)	+	R (>100)
YEY-36	Negative	R (>800)	+	R (>100)
Laboratory-derived PZA-resistant clinical isolates without mutations in *pncA*				
KK-117	Positive	R (>800)	−	R (>100)
KK-118	Positive	R (>800)	−	R (>100)
KK-123	Positive	R (>800)	−	R (>100)
Laboratory strain				
$H_{37}Rv$	Positive	S (100)	−	S (3.13)

[a] PZase activity was assayed by the method of Wayne (22).

[b] MIC (drug concentration required to inhibit more than 99% of CFU) of PZA was determined on Middlebrook 7H11 agar at a pH value of 6.0. The threshold value for PZA resistance in acidic agar test was defined as 200 μg/ml. S, sensitive; R, resistant. Values in parentheses are MICs (in micrograms per milliliter).

[c] ND, not done; +, mutated; −, not mutated.

[d] In SFA, MIC was defined as the minimal dose of drugs exhibiting a statistically significant ($P < 0.05$) inhibitory effect on bacterial cytotoxicity compared to host cells without bacteria. The threshold value for PZA resistance in SFA was defined as 100 μg/ml. Data are means of three results in duplicated wells of a 96-well plate. S, sensitive; R, resistant. Values in parentheses are MICs (in micrograms per milliliter). Some test results are from our other article, reference 8.

TABLE 2. Antimycobacterial activity of PZA to the PZA-susceptible and -resistant clinical isolates of *M. tuberculosis* in MRC-5 SV TG1 host cells

Isolate	No. of colonies (CFU/ml) of the bacilli in host cells[a]		*P* value[b]
	Without PZA	With PZA	
PZA-susceptible clinical isolates			
A.1.2	$(3.2 \pm 1.3) \times 10^5$	$(1.7 \pm 1.0) \times 10^5$	<0.05
A.1.3	$(2.8 \pm 1.0) \times 10^5$	$(1.5 \pm 0.1) \times 10^5$	<0.05
A.2.1	$(4.7 \pm 0.2) \times 10^5$	$(1.8 \pm 1.1) \times 10^5$	<0.05
PZA-resistant clinical isolates			
Ind-10	$(1.1 \pm 0.7) \times 10^5$	$(1.5 \pm 1.5) \times 10^5$	
Thai S6	$(2.2 \pm 1.1) \times 10^5$	$(2.0 \pm 0.7) \times 10^5$	
V.3.20	$(0.6 \pm 0.3) \times 10^5$	$(1.6 \pm 0.8) \times 10^5$	

[a] MRC-5 SV1 TG1 host cells were cultured with medium containing the bacilli for 16 to 18 h. The host cells were then washed with PBS, and fresh tissue culture medium with or without PZA (100 μg/ml) was added. After 3 days of culture, the cells were washed with PBS and then lysed with sterilized water. The lysates were inoculated on Middlebrook 7H11 agar plates after dilution. The colonies on the plate were counted after 2 weeks of incubation at 37°C. The results are means ± standard deviations of three wells in a 96-well plate. Experiments were performed two times, and representative data are shown.

[b] *P* values found by Student's *t* test comparing the values without PZA to the value with PZA.

and the MICs of PZA for the resistant isolates were more than 100 μg/ml. The PZA-sensitive and -resistant isolates could be clearly distinguished at the PZA concentration of 100 μg/ml. Therefore, the threshold value for PZA resistance in the SFA was defined as 100 μg/ml.

Forty-five clinical isolates of *M. tuberculosis* were used to determine the susceptibilities of the isolates to PZA in a SFA. Twenty isolates were susceptible to PZA, and 25 isolates were resistant (Table 1). The results obtained by SFA are based on the viability of the bacilli inside host cells. Using clinical isolates of *M. tuberculosis*, we attempted to confirm this fact (Table 2). The data show that PZA reduced the number of PZA-susceptible bacilli inside host cells, but not PZA-resistant bacilli. These results clearly indicate that the viability of clinical isolates inside host cells also correlates with the cytotoxicity against host cells. The concordance ratio between SFA and the PZase assay and/or growth test in acidic medium was 95.0 and 96.0% for susceptible and resistant strains, respectively. These ratios are comparable to or higher than those of other methods on the basis of PZase activity, drug susceptibility test in acidic agar, BACTEC system, and sequence analysis of the *pncA* gene (2, 3, 7, 10). All the resistant strains with mutations in *pncA* appeared to be resistant by SFA as well. Three PZA-resistant mutants (KK-117, KK-118, and KK-123), which do not have mutations in *pncA*, could also be defined as resistant strains by SFA. There was a discrepancy between the result obtained by PZase activity or acid agar test and SFA in two clinical isolates A.3.5 and A 4.30. No phenotypic abnormality was observed in these two strains, and the reason for this discrepancy is not clear.

It is interesting that the PZA MICs for PZA-susceptible isolates were between 3.13 and 12.5 μg/ml by modified SFA (Table 1), which is close to the clinical concentration of PZA reported in the lungs of the patients (about 20 μg/ml) (15). On the other hand, the MICs of PZA for the PZA-resistant strains were more than 100 μg/ml. These results strongly indicate that a SFA could determine the susceptibility of clinical isolates to PZA and also provide us with the critical information of the drug dose that is effective in vivo.

Host cell-based methods using macrophages of either human or animal origin are documented (4, 6, 14, 15), but the drawback of the use of macrophages derived from experimental animals is the heterogeneity of different batches of animal cells and consequent problems of reproducibility (13). Also, it takes more than 2 weeks to obtain results by the conventional method, because the number of live bacilli has to be counted after growth on agar plates. The SFA yields results within 3 to 4 days, which is highly advantageous compared to other host cell-based assays. In our experience, about half of the clinical isolates cannot grow in the acidic conditions at pH 5.5. Therefore, in addition to the speed with which the assay can be conducted, SFA can evaluate the clinical strains that are difficult to grow in acidic medium. Also, the strains with no mutations in the *pncA* gene can be defined as resistant strains by the SFA.

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TRB3, a novel ER stress-inducible gene, is induced via ATF4–CHOP pathway and is involved in cell death

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C/EBP homologous protein (CHOP) is a stress-inducible nuclear protein that is crucial for the development of programmed cell death and regeneration; however, the regulation of its function has not been well characterized. Slbo, a *Drosophila* homolog of C/EBP (CCAAT/enhancer binding protein), was shown to be unstabilized by tribbles. Here, we identified *TRB3* as a *tribbles* ortholog in humans, which associated with CHOP to suppress the CHOP-dependent transactivation. TRB3 is induced by various forms endoplasmic reticulum (ER) stress later than CHOP. Tunicamycin treatment enhanced the *TRB3* promoter activity, while dominant-negative forms of CHOP suppressed the tunicamycin-induced activation. In addition, the tunicamycin response region in the *TRB3* promoter contains amino-acid response elements overlapping the CHOP-binding site, and CHOP and ATF4 cooperated to activate this promoter activity. Knockdown of endogenous ATF4 or CHOP expression dramatically repressed tunicamycin-induced TRB3 induction. Furthermore, knockdown of TRB3 expression decreased ER stress-dependent cell death. These results indicate that TRB3 is a novel target of CHOP/ATF4 and downregulates its own induction by repression of CHOP/ATF4 functions, and that it is involved in CHOP-dependent cell death during ER stress.

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Introduction

CCAAT/enhancer binding proteins (C/EBPs) are a family of leucine zipper transcription factors that are critical for the regulation of various aspects of cellular differentiation and function in multiple tissues. This family consists of six members: C/EBPα, β (NF-IL6), γ (Ig/EBP), δ, ε and C/EBP homologous protein (CHOP)/growth arrest-DNA damage inducible 153 (GADD153) (Lekstrom-Himes and Xanthopoulos, 1998). The prototypic C/EBP consists of a transcriptional activation domain and a bZIP region for DNA binding and dimerization. All family members share strong homology in the carboxyl-terminal domain, which carries a basic DNA-binding domain and a leucine zipper motif.

CHOP was originally isolated as the gene induced in response to DNA-damaging agents; subsequently, it has been revealed that CHOP is induced by extracellular and endoplasmic reticulum (ER) stress (Fornace *et al*, 1989; Zinszner *et al*, 1998). From experiments on the overexpression of its protein and knockout mice, CHOP has been shown to act as an inducer of cell cycle arrest and apoptosis during ER stress (Barone *et al*, 1994; Matsumoto *et al*, 1996; Zinszner *et al*, 1998). At first, because the basic region (BR) of CHOP is less conserved than that of other C/EBP family proteins, CHOP was thought to lack DNA-binding activity. Actually, by forming heterodimers with other C/EBP proteins such as NF-IL6, CHOP inhibits their ability to bind DNA and their transcriptional activity (Ron and Habener, 1992). Interestingly, however, a CHOP-C/EBP heterodimer has been reported to bind to a unique DNA sequence different from classical C/EBP-binding sites and to act as a positive transactivator (Ubeda *et al*, 1996). In recent studies, several CHOP-inducible genes have been induced during ER stress via this CHOP-binding sequence (Wang *et al*, 1998).

ER stress responses are alterations in homeostasis following cellular stress, which prevent protein folding and cause misfolding or malfolding proteins to accumulate in the ER (Kaufman, 1999; Mori, 2000). Under such conditions, the homeostasis of protein folding in the ER is maintained by inter-organelle signaling from the ER to the nucleus, a process known as unfolded protein response (UPR). Thus, from yeast to humans, the transcription of genes encoding molecular chaperones and folding enzymes in the ER is induced in the nucleus in response to unfolding in the ER, and excessive or long-term accumulations of unfolding proteins in the ER result in the apoptosis of cells.

A large number of transcription factors undergo degradation via a ubiquitin-proteasome-dependent pathway (Hochstrasser, 1995; Pahl and Baeuerle, 1996). A genetic study on *Drosophila* revealed that Slbo, a *Drosophila* homolog of C/EBP, is specifically degraded by the ubiquitin-proteasome pathway (Rorth *et al*, 2000). As the degradation of Slbo is augmented by expression of tribbles, tribbles may be involved in the ubiquitin-proteasome pathway. In humans, we have previously reported that C/EBP family transcription factors, CHOP and Ig/EBP (C/EBPγ), are multiubiquitinated and subsequently degraded by proteasomes, but the molecular mechanism involved is still unclear (Hattori *et al*, 2003a). In an effort to clarify the mechanism by which C/EBP is degraded, we searched a database for the *tribbles* ortholog in humans, and identified *TRB3* (*tribbles*-related protein 3),

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its gene having been cloned from HepG2 cells with function unknown. In this study, TRB3 was revealed to be a novel target of CHOP/ATF4 and downregulates its own induction by repression of CHOP/ATF4 functions, and also seemed to be involved in CHOP-dependent cell death as a second messenger during ER stress.

Results

Identification of a mammalian homolog of tribbles

To clarify the regulation of C/EBP degradation, we searched the GenBank database to determine the human ortholog of *Drosophila tribbles*. This search identified several mammalian genes displaying high levels of identity to *tribbles* and we determined the sequence of a full-length cDNA clone, a gene of unknown function that had been cloned from the human hepatoma cell line HepG2 (GenBank accession #AK026945). It was identical to human *SKIP3* (GenBank accession #AK0250311), and very recently, Du *et al* (2003) identified its product (termed TRB3, tribbles-related protein 3) as a novel Akt-binding and -regulating protein (Du *et al*, 2003). A search of the GenBank database with the full coding sequence revealed that *TRB3* is related to *TRB1* or *TRB2*.

ER stress induces TRB3 expression

First, we analyzed the expression level of *TRB3* mRNA by RT–PCR and Northern blotting. *TRB3* mRNA was not expressed in steady-state 293 cells, human embryonic kidney cells, but was induced during ER stress by treatment with tunicamycin for 4–6 h (Figure 1A). In HepG2 cells, *TRB3* mRNA was expressed in normal conditions and its expression was augmented 6 h after treatment (Figure 1B). *TRB3* induction was also observed in tunicamycin-treated A375, HeLa and SH-SY5Y cells (see Supplementary Figure S1). The induction of *TRB3* mRNA was late compared to that of *CHOP* mRNA. In addition, cycloheximide treatment blocked the expression of *TRB3* mRNA, indicating that its induction required *de novo* protein synthesis (Figure 1A, lane 7). *TRB3* mRNA was also induced by treatment with other ER stress inducers, such as MG132, a proteasome inhibitor, methanesulfonic acid methyl ester (MMS), a DNA alkylating agent and A23187, an ER Ca^{2+}-ATPase inhibitor (Figure 1C). As shown in Figure 1D, this induction of *TRB3* was observed at the protein level as well. In HepG2 cells, a small amount of TRB3 protein was detected, and its expression was upregulated by tunicamycin 8 h after treatment. TRB3 protein was not detected in steady-state 293 and A375 cells, but was detected 4–6 h after tunicamycin treatment (Figure 1D). At the protein level as well, CHOP induction usually preceded TRB3 induction.

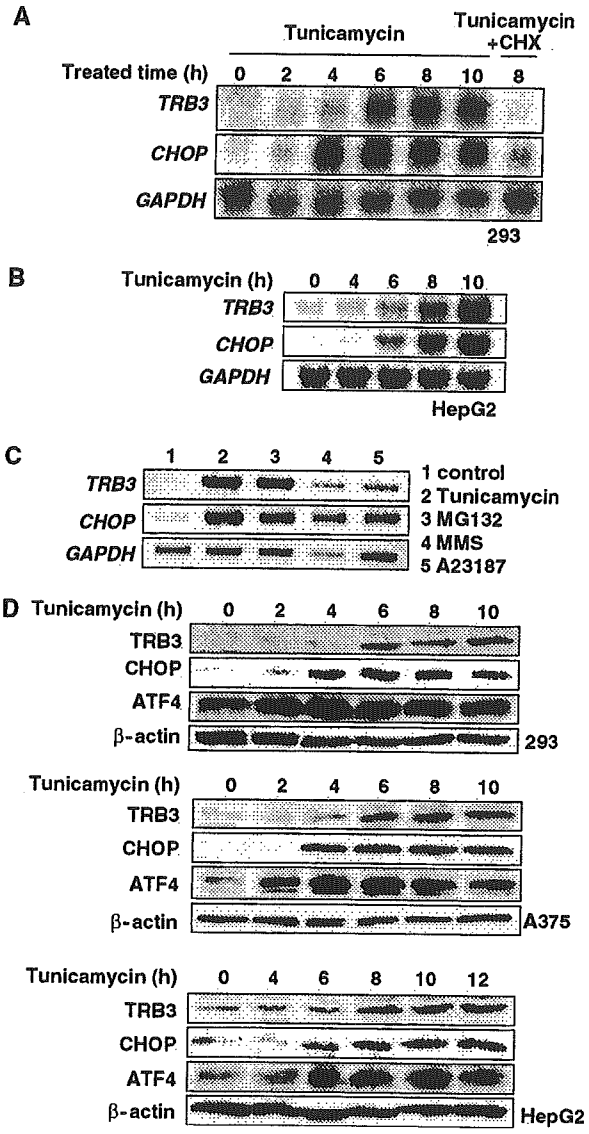

Figure 1 TRB3 is induced during ER stress. (**A**, **B**) 293 (A) or HepG2 cells (B) were treated with 2 μg/ml of tunicamycin in the presence or absence of 10 μg/ml of cycloheximide (CHX) for the indicated periods. Total RNA was prepared and analyzed by Northern blotting using each specific probe. (**C**) 293 cells were treated with 2 μg/ml of tunicamycin, 10 μM MG132, 100 μg/ml of MMS or 2 μM A23187 for 6 h. Each mRNA level in the cells was analyzed by RT–PCR using specific primers. *TRB3*, 32 cycles ; *CHOP*, 30 cycles ; *GAPDH*, 24 cycles. (**D**) 293 (top), A375 (middle) and HepG2 (bottom) cells were treated with 2 μg/ml of tunicamycin for the indicated periods. Cell lysate was prepared and equal amounts of protein were subjected to SDS–PAGE. Western blotting was performed with anti-human TRB3, anti-CHOP, anti-ATF4 or anti-β-actin antibodies.

TRB3 interacts with CHOP but does not promote CHOP degradation

In *Drosophila*, Slbo was reported to interact with tribbles to inhibit its function via protein degradation (Rorth *et al*, 2000). We first examined the physical interaction between TRB3 and CHOP. CHOP was co-immunoprecipitated with TRB3 in transfected 293 cells (Figure 2B, lane 4). To identify the domain of CHOP required for its interaction with TRB3, we analyzed the ability of several CHOP deletion mutants as shown in Figure 2A to co-immunoprecipitate with TRB3. CHOP interaction with TRB3 does not require either the BR or potential phosphorylation sites ($Ser^{79,82}$), which are critical for DNA-binding and transactivation activity, respectively. Leucine zipper (LZ) domain, responsible for dimer formation, was not necessary for the binding with TRB3 either (see Supplementary Figure S2A). Full-length TRB3 strongly interacted with CHOP, CHOPΔN9 (amino acids (aa) 10–169) and CHOPΔ19–26 (aa 1–18, 27–169); in contrast, further truncation in the N-terminus of CHOP (CHOPΔN18 (aa 19–169) and

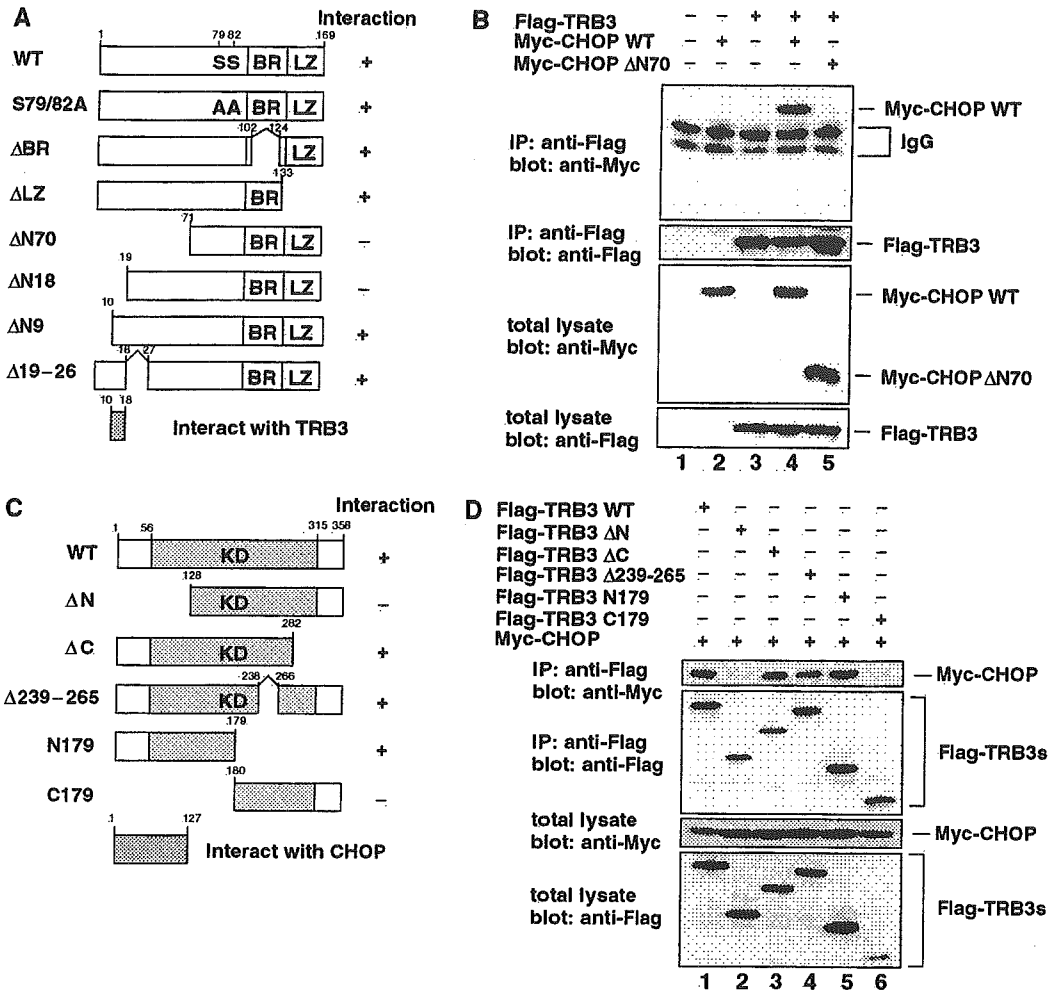

Figure 2 TRB3 interacts with CHOP *in vivo*. (**A**) Constructs of CHOP mutants. (**B, D**) 293 cells were transiently transfected with the indicated constructs. After 36 h, cells were treated with 10 μM MG132 for 12 h. The cell lysates were immunoprecipitated with anti-Flag antibody and immunoblotted with anti-Myc antibody. The expression level of each protein was assessed by the immunoblotting of cell lysates with anti-Flag or anti-Myc antibodies. (**C**) Deletion mutants of TRB3. KD: kinase-like domain.

CHOPΔN70 (aa 71–169)) abolished the interaction with TRB3 (Figure 2B, lane 5; see Supplementary Figure S2B). These results indicate that the region between aa 10 and 18 of CHOP is responsible for the interaction with TRB3 (Figure 2A). Next, to identify the domain of TRB3 required for its interaction with CHOP, we analyzed the possibility of CHOP coimmunoprecipitating with several TRB3 deletion mutants as shown in Figure 2C. TRB3ΔC (aa 1–282), TRB3Δ239–265 (aa 1–238, 266–358) and TRB3N179 (aa 1–179) interacted with CHOP as well as full-length TRB3, but TRB3ΔN (aa 128–358) and TRB3C179 (aa 180–358) have no or only faint interaction with CHOP (Figure 2D). These results indicate that the region between aa 1 and 127 of TRB3 is responsible for the interaction with CHOP (Figure 2C).

Next, we examined whether TRB3 promotes the protein degradation of CHOP. Unexpectedly, the CHOP expression level and degradation rate were unchanged by coexpression with TRB3 or knockdown of endogenous TRB3 by expression of *TRB3* siRNA (see Supplementary Figure S3). These results suggest that TRB3 interacts with CHOP; however, it does not promote degradation of CHOP.

TRB3 downregulates CHOP-dependent transcriptional activity

It has been shown that several CHOP-inducible genes have been induced during ER stress or mitochondrial stress via novel CHOP-binding sequence (Wang *et al*, 1998; Zhao *et al*, 2002) (Figure 3A). The region between aa 10 and 18 in CHOP is critical for the binding with TRB3 and also crucial for CHOP transcriptional activity (Figure 3B). Therefore, to explore the possibility that TRB3 affects the transcriptional activity of CHOP, we constructed a luciferase reporter gene containing four tandem repeats of the CHOP-binding site and performed a luciferase reporter assay. When CHOP alone or a low level of NF-IL6 alone was transiently expressed in A375 cells, the transcriptional activity was unaffected (Figure 3C). However, this activity was upregulated on coexpression of NF-IL6 and CHOP. Overexpression of TRB3 repressed the transcriptional activity stimulated by CHOP/NF-IL6 in a dose-dependent manner (Figure 3D). In addition, a high level of NF-IL6 expression alone also stimulated the transcriptional activity for these sites; however, TRB3 failed to suppress this activation (Figure 3C). These results

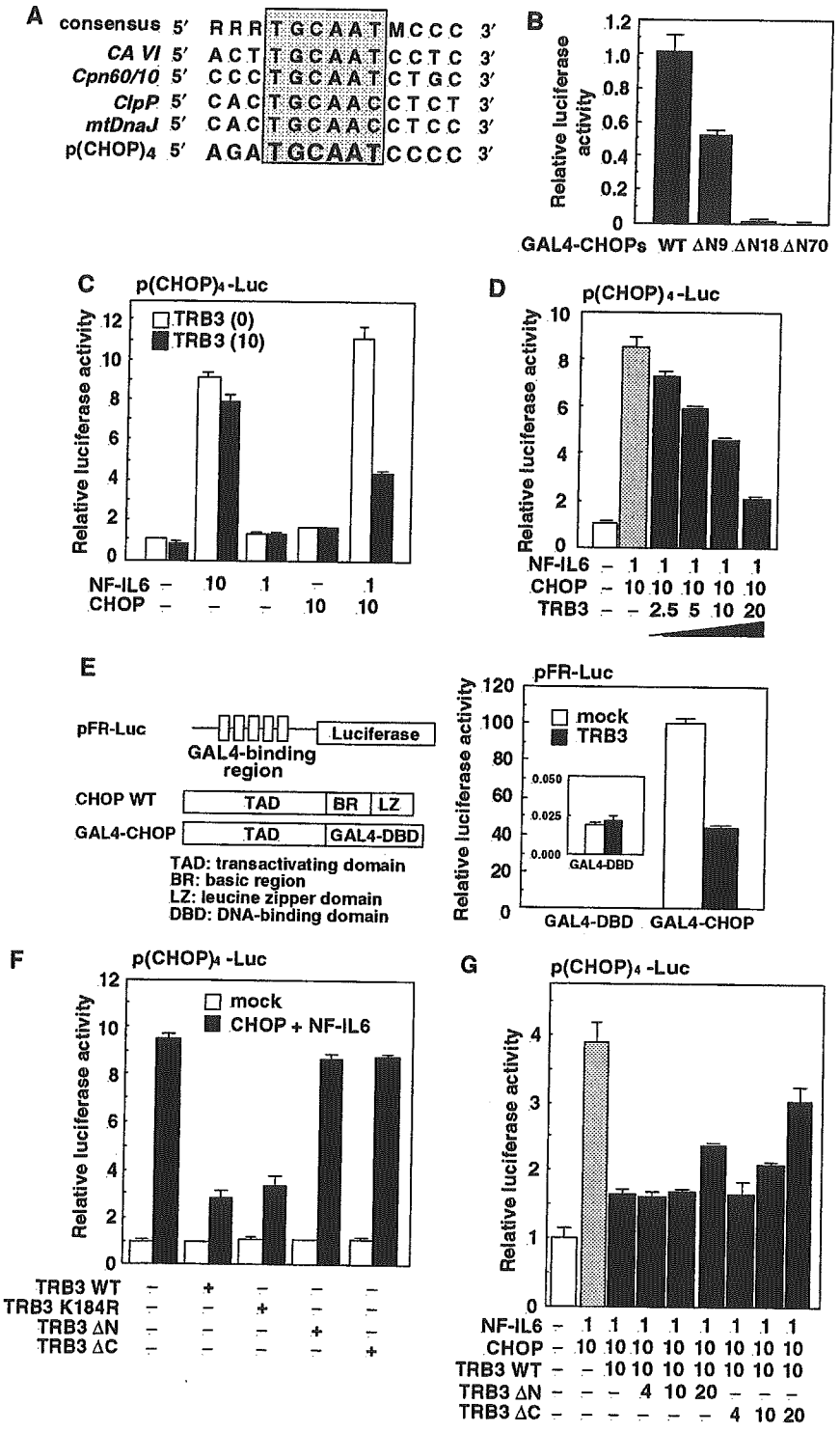

Figure 3 TRB3 represses CHOP transactivation. (**A**) Alignment of CHOP-responsive element in various genes. (**B**) 293 cells were transiently transfected with the expression plasmids for wild-type or deletion mutants of CHOP fused with GAL4-dbd, pCMV5-Gal4-CHOPs, pFR-luc and pCMV-β-gal. After 48 h, the luciferase activity in cell lysates was measured and was normalized with β-galactosidase activity. (**C, D**) A375 cells were transiently transfected with $p(CHOP)_4$-Luc, pCMV-β-gal and various combinations of the expression vectors for CHOP and NF-IL6 with or without TRB3. After 48 h, the luciferase activity in cell lysates was measured and was normalized with β-galactosidase activity. (**E**) 293 cells were transiently transfected with the expression plasmids for Gal4-CHOP(WT) and/or TRB3, pFR-luc and pCMV-β-gal. After 48 h, the luciferase activity in cell lysates was measured and was normalized with β-galactosidase activity. The inset depicts the enlarged figure for the data of GAL4-dbd. (**F, G**) A375 cells were transiently transfected with $p(CHOP)_4$-Luc, pCMV-β-gal and various combinations of the expression vectors for CHOP and NF-IL6 with or without wild type or deletion mutants of TRB3. After 48 h, the luciferase activity in cell lysates was measured and was normalized with β-galactosidase activity. Similar results were obtained in three independent experiments.

suggest that TRB3 selectively inhibits the transcriptional activity of CHOP.

TRB3 interacted with CHOP via its transactivation domain (Figures 2A and 3B) but not DNA-binding or leucine zipper domains, and inhibited its transactivation activity. Consistent with the function of TRB3-binding region on CHOP, TRB3 did not interfere with the dimerization of CHOP with NF-IL6 nor with DNA binding of CHOP (see Supplementary Figure S4). In addition, TRB3 repressed even the transactivation activity of GAL4 fusion protein of CHOP (Figure 3E). These results suggest that TRB3 primarily inhibits CHOP transactivation, probably by inhibiting the modification of CHOP required for its transactivation or by interfering with the association of coactivator(s) or recruiting corepressor(s) to DNA.

To investigate the functional domain of TRB3 inhibiting the transactivating activity of CHOP, we examined the effect of several deletion and point mutants of TRB3 on this activity. Deletion of the N-terminal (Δ1–127, TRB3ΔN) or C-terminal region of TRB3 (Δ283–358, TRB3ΔC) abolished the inhibitory effect on the CHOP-dependent transactivation (Figure 3F). These results suggest that the functional domain of TRB3 for this inhibitory action exists in both the N-terminal region, necessary for the association with CHOP, and the C-terminal region. In addition, TRB3ΔC mutants strongly, and TRB3ΔN mutants slightly, interfered with CHOP-dependent transactivation in a dominant-negative manner (Figure 3G). Substitution of Lys^{184}, a potential catalytic core in the kinase-like domain, with Arg (TRB3 $K^{184}R$) resulted in almost the same inhibitory activity as wild-type TRB3, indicating that this region is not critical for the inhibitory action (Figure 3F).

CHOP overexpression activates TRB3 promoter activity

To study whether *TRB3* mRNA expression induced by ER stress is regulated at the transcriptional level, we cloned the promoter region of human *TRB3* (−1265 to +609), and constructed a luciferase reporter plasmid (pTRB3-Luc). As shown in Figure 4A, transient transfection experiments in 293 cells using this reporter gene confirmed that ER stress induced by tunicamycin caused the promoter activation. This activation was also observed in tunicamycin-treated HepG2 cells, and other ER stressors, thapsigargin and A23187, also stimulated the promoter (see Supplementary Figure S5). These results suggest that TRB3 expression is induced at the transcriptional level during ER stress.

The expression of *TRB3* mRNA during ER stress was blocked by cycloheximide treatment, and the induction of *TRB3* was late compared to that of *CHOP* (Figure 1). In

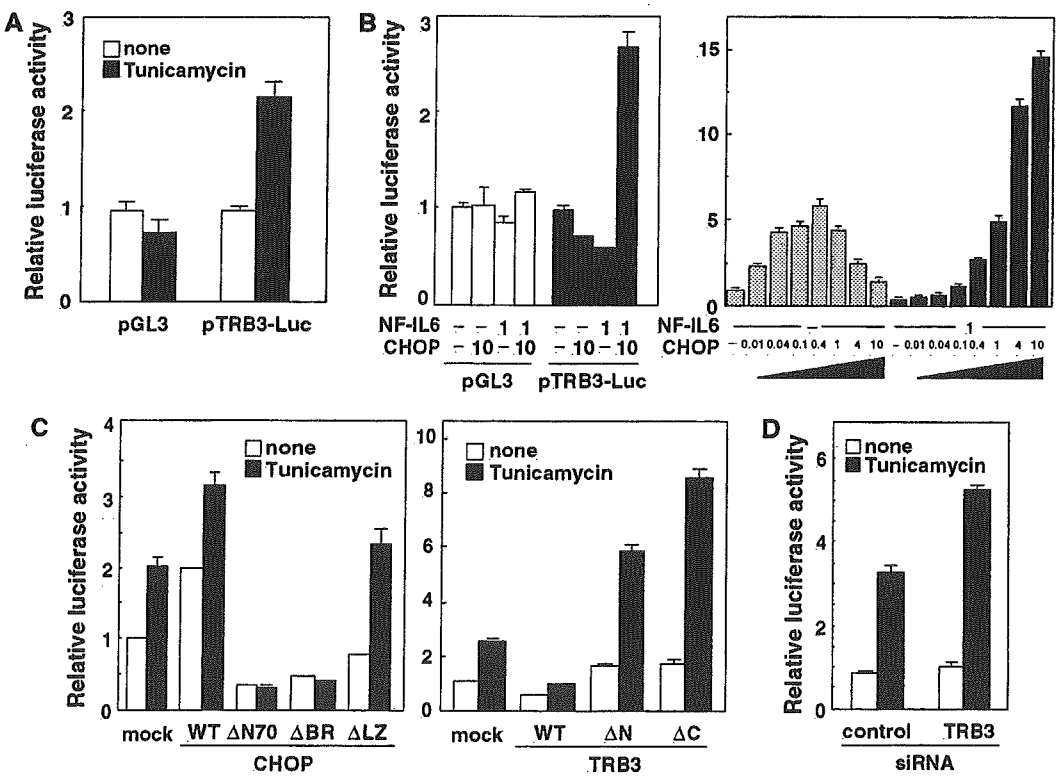

Figure 4 Overexpression of CHOP causes TRB3 induction. (**A**) 293 cells were transiently transfected with pTRB3-Luc and pCMV-β-gal. After 24 h, cells were left untreated or treated with 2 μg/ml of tunicamycin for 16 h. The luciferase activity in cell lysates was measured and was normalized with β-galactosidase activity. (**B**) 293 cells were transiently transfected with pTRB3-Luc and pCMV-β-gal combination with expression vectors for CHOP and/or NF-IL6 for 48 h. The luciferase activity in cell lysates was measured and was normalized with β-galactosidase activity. (**C**) 293 cells were transiently transfected with pTRB3-Luc and pCMV-β-gal in the presence of expression vectors for wild type or mutants of CHOP or TRB3. After 24 h, cells were left untreated or treated with 2 μg/ml of tunicamycin for 16 h. The luciferase activity in cell lysates was measured and was normalized with β-galactosidase activity. (**D**) 293 cells were transiently transfected with pTRB3-Luc, pCMV-β-gal, control siRNA and/or TRB3 siRNA. After 36 h, cells were left untreated or treated with 2 μg/ml of tunicamycin for 16 h. The luciferase activity in cell lysates was measured and was normalized with β-galactosidase activity. Similar results were obtained in three independent experiments.

addition, TRB3 interacts with CHOP to downregulate its transactivation activity (Figures 2 and 3). We next investigated the effect of CHOP overexpression on *TRB3* promoter activity, to explore the possibility that TRB3 expression is induced via CHOP expression. By cotransfection with CHOP and NF-IL6 expression plasmids, *TRB3* promoter activity was enhanced (Figure 4B, left). This result was confirmed by Northern blotting experiments using cells transiently transfected with CHOP and NF-IL6 (see Supplementary Figure S6). In addition, a low to middle level of CHOP expression alone effectively activated *TRB3* promoter activity; however, a high level of expression failed to activate it (Figure 4B, right). On the other hand, a high level of CHOP was able to activate *TRB3* promoter activity when coexpressed with NF-IL6 (Figure 4B, right). These results suggest that ectopically expressed CHOP can activate *TRB3* promoter activity via heterodimerization with endogenous proteins such as NF-IL6, but overexpressed CHOP cannot, due to a shortage of dimerization partners.

Overexpression of dominant-negative forms of CHOP, CHOPΔN70 or CHOPΔBR diminished the tunicamycin-induced *TRB3* promoter activation, while that of CHOPΔLZ, which has no transactivation activity or a dominant-negative effect, did not affect or only slightly potentiated the activity (Figure 4C, left). Ectopic expression of TRB3 caused an inhibition of transcription, while dominant-negative forms of TRB3 (TRB3ΔN and TRB3ΔC) and *TRB3* siRNA upregulated the tunicamycin-stimulated TRB3 expression (Figure 4C and D). These results strongly suggest that TRB3 is induced by ER stress via CHOP expression and suppresses CHOP transactivational activity via negative feedback.

Identification of ER stress response elements in TRB3 promoter

To clarify the regulation of TRB3 induction via CHOP expression, we identified the ER stress response region in the *TRB3* promoter using luciferase reporter plasmids of its promoter in various lengths as shown in Figure 5A. The reporter genes containing the region +201 to +312 (pTRB3-Luc1, 2, 5, 6), but not the others, were activated by tunicamycin (Figure 5B, left). This result revealed that the tunicamycin-induced proteins activate the *TRB3* promoter via this region.

As *TRB3* mRNA expression induced by ER stress required *de novo* protein synthesis, we next examined the effects of overexpression of three transcription factors induced during ER stress, CHOP, ATF4 and XBP1, on *TRB3* promoter activity. Not only CHOP/NF-IL6 but also ATF4 overexpression activated the *TRB3* promoter via the region +201 to +312, which is similar to tunicamycin treatment; however, XBP1 overexpression had no effect (Figure 5B; see Supplementary Figure S7). These results suggest that ATF4 is able to activate the *TRB3* promoter as well.

The ER stress response region in the *TRB3* promoter, +201 to +312, contains three identical tandem repeats each consisting of 33 bp (Figure 5C). In addition, this region contains an amino-acid response element (AARE) overlapping a CHOP-binding site at the center, a CCAAT-like box on the left and a palindromic sequence on the right (Figure 5D). AARE has been discovered between nucleotides −313 and −295 in the *CHOP* promoter. The minimum core sequence (5′-ATTGCATCA-3′) is related to C/EBP- and ATF/CRE-binding sites and was described to bind *in vitro* ATF2/NF-IL6 or ATF4/NF-IL6 (Averous *et al*, 2003). This element is involved in the response to amino-acid starvation but not ER stress. On the other hand, ER stress response element (ERSE) was identified as a sequence of 19 nucleotides, the consensus of which is CCAAT-N_9-CCAAG, in the *CHOP* and ER chaperone promoters (Yoshida *et al*, 2000). A mature form of ATF6 directly binds to the CCACG part of the ERSE in a manner dependent on the binding of NF-Y to the CCAAT part. To clarify whether these elements are involved in the tunicamycin-induced *TRB3* promoter activation, we constructed the reporter gene containing one repeat of three tandem repeats sequence (pTRB3-RS) and its deletion and point mutants as shown in Figure 5D. As shown in Figure 5E (left), tunicamycin-induced ER stress did not cause the transactivation of the reporter gene containing mutations in the CHOP-binding site (pTRB3-RS mut1) or AARE not overlapping a CHOP-binding site (pTRB3-RS mut4, 6). Interestingly, the transactivation of mutants of the CCAAT-like box (pTRB3-RS mut3, 5) was not caused either. The mutation to a palindromic sequence (pTRB3-RS mut2) did not cause a significant decrease in tunicamycin-induced transactivation as compared with the wild type. These results revealed that the CHOP-binding sites, the AAREs and the CCAAT-like boxes, but not the palindromic sequences, are involved in the *TRB3* promoter activation by tunicamycin-induced ER stress.

Next, we examined the effects of CHOP/NF-IL6 and ATF4 overexpression on these promoters (Figure 5E, right). CHOP/NF-IL6 overexpression demonstrated almost the same activation profile as that of tunicamycin treatment. On the other hand, ATF4 overexpression caused the transactivation of not only pTRB3-RS wt and mut2 but also pTRB3-RS mut3 and 5. Therefore, the *TRB3* promoter activation by ATF4 overexpression is independent of the CCAAT-like box and not correlated with tunicamycin-induced activation. These results suggest that ATF4 expression alone is not enough for physiological *TRB3* promoter activation and additional factors, such as CHOP, are necessary for this induction.

CHOP and ATF4 cooperate to activate TRB3 promoter activity

A consensus C/EBP-binding site consists of the palindromic decanucleotide ATTGCGCAAT and a consensus ATF/CRE box consists of the palindromic octanucleotide TGACGTCA (Montminy *et al*, 1986; Osada *et al*, 1996). Usually, the homodimers of members of the C/EBP family or those of the ATF/CREB family bind to the C/EBP-binding site or the ATF/CRE box, respectively (Bruhat *et al*, 2002). The AARE in the *CHOP* promoter and nutrient-sensing response element (NRSE) 1 (an AARE-related element) in the asparagine synthetase (*AS*) promoter (also termed C/EBP-ATF site) consist of one-half of each of the palindromic sequences that comprise an optimal C/EBP-binding site and an ATF/CRE box (Fawcett *et al*, 1999) (Figure 6A), and these sites were shown to bind C/EBP homodimers, ATF/CREB homodimers or C/EBP-ATF heterodimers. CHOP/NF-IL6 (heterodimers of the C/EBP family) or ATF4 (homodimers of the ATF/CREB family) caused transcriptional activation of the *TRB3* promoter containing an AARE- and a CHOP-binding site. Therefore, we next examined whether C/EBP-ATF heterodimers can activate the *TRB3* promoter as well. As ATF4 overexpression caused *TRB3* promoter transcriptional activation, ATF4 is the strongest candidate for a component of the ATF part. As a

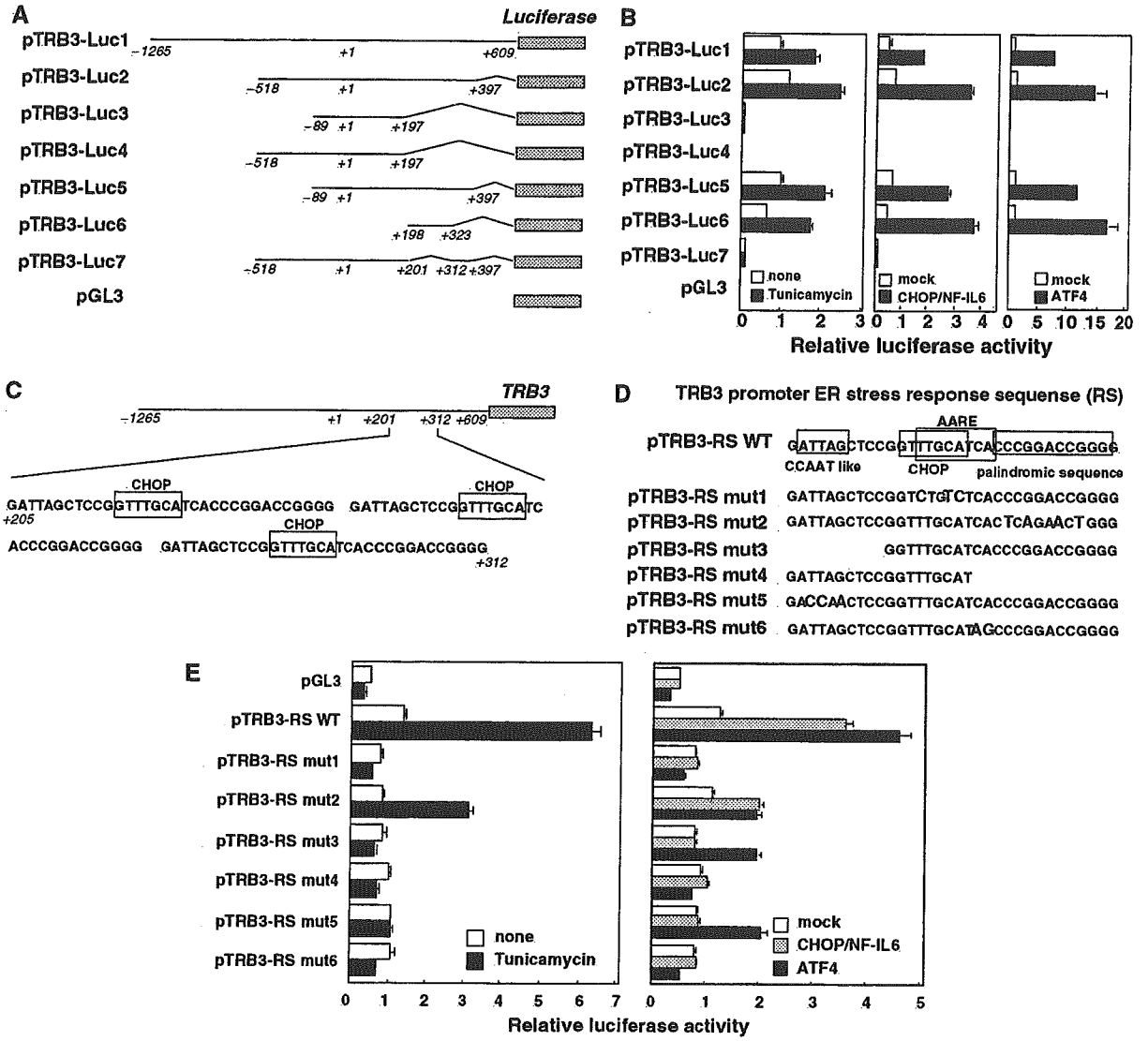

Figure 5 Mutagenesis analysis of the *TRB3* promoter. (**A**) Constructs of *TRB3* promoter reporter genes. Position +1 demonstrates the initiation site for the TRB3 transcription. (**B**) 293 cells were transiently transfected with pTRB3-Luc, pCMV-β-gal and the expression vectors for CHOP/NF-IL6 or ATF4. After 24 h, cells were left untreated or treated with 2 μg/ml of tunicamycin for 16 h. The luciferase activity in cell lysates was measured and was normalized with β-galactosidase activity. (**C**) The sequence of the ERSE in the *TRB3* promoter. (**D**) Constructs of *TRB3* promoter reporter genes. (**E**) HepG2 cells were transiently transfected with pTRB3-Luc and pCMV-β-gal, and after 24 h, treated with 2 μg/ml of tunicamycin for 16 h (left). 293 cells were transiently transfected with pTRB3-Luc, pCMV-β-gal and the indicated expression vectors for CHOP/NF-IL6 or ATF4. The luciferase activity in cell lysates was measured and normalized with β-galactosidase activity (right). The results in HepG2 cells were shown because the tunicamycin-induced activation level was higher than in 293 cells. Similar results were obtained in 293 cells as well. Similar results were obtained in three independent experiments.

component of C/EBP part, NF-IL6 is the partner of ATF4 for activation of the *TRB3* promoter as well as the *CHOP* and *AS* promoters, but overexpression of NF-IL6 caused the potent repression of basal or ATF4-stimulated *TRB3* promoter (Figure 6B, right). On the other hand, the coexpression of CHOP with ATF4 dramatically increased *TRB3* promoter activation compared with the expression of ATF4 alone or CHOP/NF-IL6 (Figure 6B, left lane 5). In addition, this activation was stimulated by both CHOP and ATF4 in a dose-dependent manner (Figure 6C). This cooperative activation was not caused by the coexpression of a dominant-negative form of CHOP, CHOPΔBR or a dysfunctional CHOP, CHOPΔLZ, with ATF4 (Figure 6D). These results suggest that CHOP and ATF4 bind to the *TRB3* promoter dependent on those DNA-binding regions and a precedent heterodimerization via the leucine zipper domains, and cooperate to activate this promoter.

Moreover, coexpression with TRB3 strongly suppressed the *TRB3* promoter activation by ATF4 as well as CHOP (Figure 6E), indicating that TRB3 regulates its own expression via the repression of both CHOP and ATF4 transactivation activity in a double negative feedback loop.

Shutdown of ATF4–CHOP pathway represses TRB3 induction

The expression of DOCs (downstream of CHOP) during ER stress was not induced in CHOP knockout mouse embryonic fibroblasts (MEFs), and this suppression occurred in NF-IL6

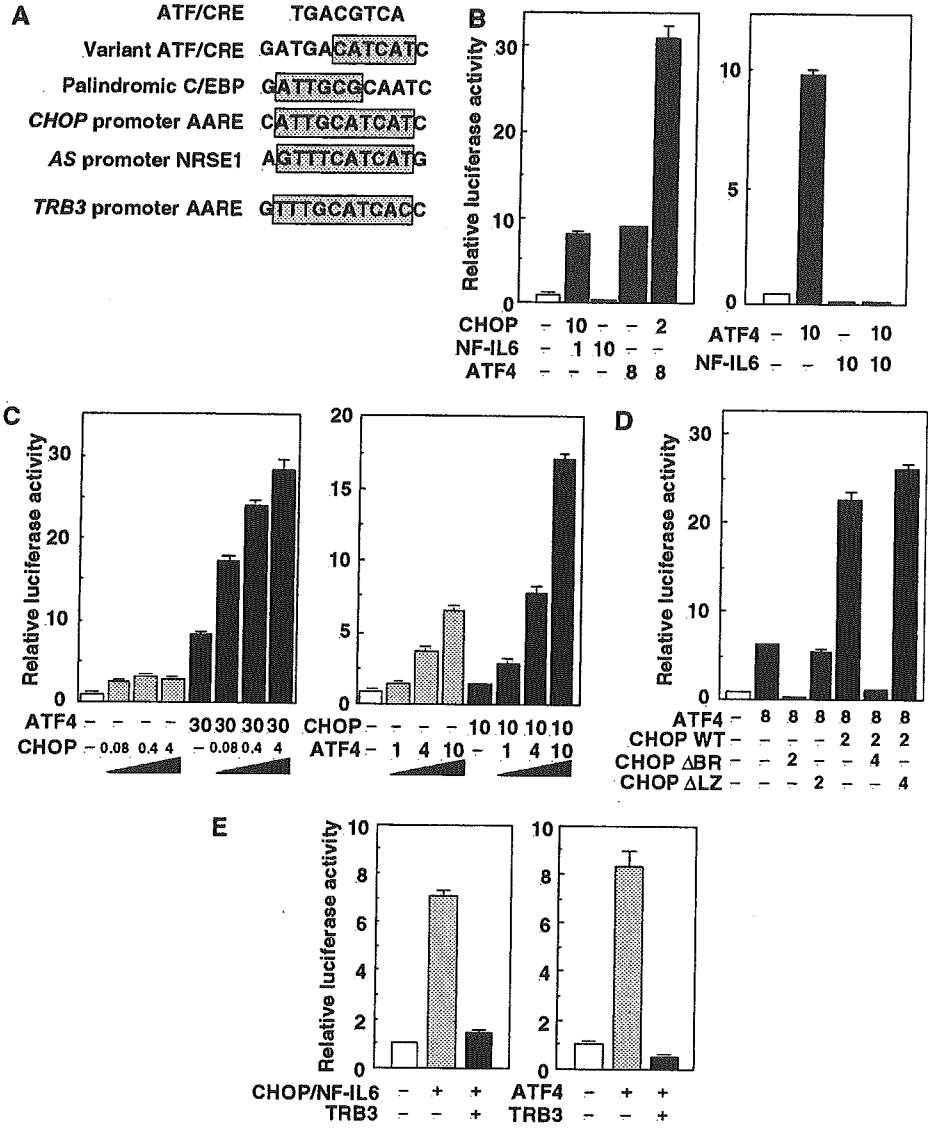

Figure 6 CHOP and ATF4 cooperate to activate the *TRB3* promoter. (**A**) Alignment of the AARE in the *TRB3* promoter with its related elements. (**B–E**) 293 cells were transiently transfected with pTRB3-Luc and pCMV-β-gal in combination with the indicated expression vectors for 48 h. The luciferase activity in cell lysates was measured and normalized with β-galactosidase activity. Similar results were obtained in three independent experiments.

knockout MEFs as well (Wang *et al*, 1998). From this observation, it is believed that NF-IL6 is a critical partner of CHOP for DOC induction during ER stress. In the expression of TRB3, CHOP/NF-IL6 and CHOP/ATF4 caused the critical activation of this promoter (Figures 3 and 6), and therefore we examined the effect of knockout/down of these genes on TRB3 induction. In NF-IL6 knockout MEFs, the expression of *TRB3* mRNA was induced by tunicamycin treatment, comparable to normal MEFs (Figure 7A), while knockdown of ATF4 by the transfection of siRNA dramatically repressed TRB3 expression along with CHOP expression (Figure 7B). Furthermore, the knockdown of CHOP strongly repressed TRB3 expression 8 h after tunicamycin treatment (Figure 7C). These results suggest that CHOP causes TRB3 induction by cooperating with ATF4 at the early stage and is essential for maintenance of the induction at the later stage.

These results also suggest that NF-IL6 is not essential for the induction of TRB3 as the partner of CHOP different from the induction of DOCs and the ATF4–CHOP pathway is critical for the TRB3 induction.

TRB3 is involved in cell death during ER stress

As CHOP-deficient mice are partially resistant to ER stress-dependent cell death (Zinszner *et al*, 1998; Oyadomari *et al*, 2002), CHOP can be related to the ER stress-induced apoptosis; however, the function of its target genes (DOCs) does not sufficiently account for the cell death. TRB3 negatively regulated CHOP-dependent transactivation, while *TRB3* was also a target gene of CHOP. We speculated that TRB3 causes the ER stress-dependent apoptosis by a function different from the suppression of CHOP transactivation and accounts for CHOP-dependent cell death by a novel mechanism via

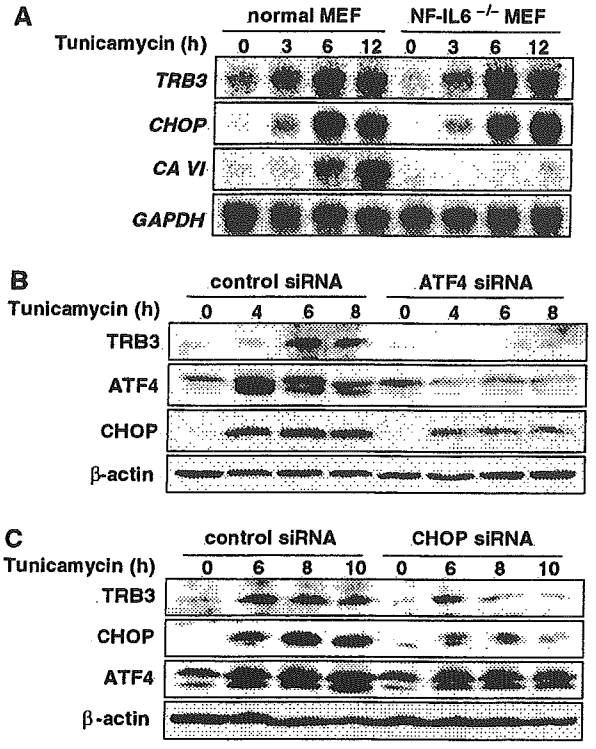

Figure 7 Knockdown of either ATF4 or CHOP repressed TRB3 induction. (**A**) Wild-type and NF-IL6$^{-/-}$ MEFs were treated with 2 μg/ml of tunicamycin for the indicated periods. Total RNA was prepared and analyzed by Northern blotting using indicated mouse cDNAs as probes. (**B, C**) 293 cells were transiently transfected with control siRNA, ATF4 siRNA or CHOP siRNA. After 48 h, cells were treated with 2 μg/ml of tunicamycin for the indicated periods. The cell lysates were analyzed by immunoblotting using anti-TRB3, anti-ATF4, anti-CHOP and anti-β-actin (bottom). Similar results were obtained in three independent experiments.

TRB3 expression. As shown in Figure 8A, knockdown of TRB3 in 293 cells significantly caused resistance to the decrease in adherent cells by tunicamycin treatment. The TRB3-dependent cell death induced by tunicamycin was also observed in the HeLa cells (Figure 8B). In addition, ectopic TRB3 expression in 293 cells increased tunicamycin-dependent cell death, and this augmented cell death was inhibited by zVAD, a caspase inhibitor, indicating that it was apoptosis (Figure 8C). Condensation and fragmentation of nuclei in HeLa cells induced by tunicamycin were significantly decreased by knockdown of TRB3 (Figure 8D). These results suggest that TRB3 mediates ER stress-dependent apoptosis and could be a link between ER stress-induced CHOP and cell death.

Discussion

CHOP, a member of the C/EBP family, has a dual role in the regulation of cellular gene expression: as an inhibitor of the binding of C/EBP to classical C/EBP target genes and as an activator of genes that have CHOP-C/EBP-binding sites. In this study, we identified a novel stress-inducible gene, *TRB3*, downstream of CHOP that is regulated by the latter part of CHOP. A luciferase assay using a reporter gene with the 5′-flanking region of *TRB3* demonstrated that ER stress-dependent expression of TRB3 occurred at the transcriptional level. This region is approximately 1.9 kbp in length and consists of several putative CHOP/NF-IL6-binding elements. Consistent with this, ectopic expression of both CHOP and NF-IL6 stimulated promoter activation. In addition, a low to moderate level of CHOP expression alone activated *TRB3* promoter activity. This activation would be caused via heterodimerization of CHOP with endogenous dimerization partners, probably ATF4 (a low level of ATF4 was detected in steady-state 293 cells). In this paper, we defined a novel CHOP-binding site consisting of two crucial sequences, an AARE and a CCAAT-like box, similar to NRSE1 and NRSE2. The authentic AARE is involved in the response to amino-acid starvation but not ER stress; however, NRSE1, which is an AARE-related element, is involved not only in the response to amino-acid starvation but also in the response to ER stress in the presence of NRSE2. In the *TRB3* promoter, the presence of the second element, the CCAAT-like box, may be involved in the response to ER stress as well. Ectopic expression of CHOP/NF-IL6 promoted the transcriptional activity of a reporter gene containing four tandem repeats of the consensus CHOP-binding site (p(CHOP)$_4$-Luc); however, the activation of this reporter gene was not caused by either a CHOP expression alone or treatment with tunicamycin (data not shown), indicating that an additional *cis*-regulatory element, such as CCAAT-like box, is required for the CHOP-dependent activation of *TRB3* promoter and other promoters. NF-Y is one of the candidates to bind to the CCAAT-like box; however, the study using dominant-negative forms of NF-Y (Mantovani *et al*, 1994) suggested that NF-Y would not be related to this box and affect the transcriptional activity of the *TRB3* promoter (see Supplementary Figure S8). In this paper, the proteins associated to the CCAAT-like boxes in the *TRB3* promoter have not been identified. Identification of the proteins involved in the response to ER stress and amino-acid starvation and adaptor protein(s) is important for the elucidation of these stress responses.

In humans and mice, three orthologs of *tribbles* (*TRB1, 2, 3*) have so far been identified. Aspects of their expression are different. The expression levels of *TRB3* mRNA and protein were quite low in untreated cells, and were induced by ER stress in several cells. In contrast, *TRB1* and *TRB2* mRNA was expressed in normal conditions and the expression level was not changed by treatment with tunicamycin in these cells (data not shown).

Here we demonstrate that TRB3 functions as a novel negative regulator of CHOP. The mechanism of the inhibitory action of TRB3 against CHOP is still unclear. The *Drosophila* tribbles has been shown to promote the degradation of slbo (Rorth *et al*, 2000); however, TRB3 did not promote the degradation of CHOP protein, and the degradation does not account for the inhibitory effect of TRB3 on transactivation activity. The TRB3-binding region in CHOP exists in the transactivation domain, indicating that binding may cause a block of the transactivation activity of CHOP.

The CHOP-binding region on TRB3 was in its N-terminal portion, and the deletion of this region sufficiently decreased its repression activity for CHOP (Figure 3F and Supplementary Figure S9). In addition, the deletion of carboxyl-terminal region or Akt-binding site (239–265) (Du *et al*, 2003) on TRB3 did not affect the binding with CHOP; however, these mutants lost CHOP repression ability like TRB3 ΔN