c-Fos/activator protein-1 transactivates *wee1* kinase at G₁/S to inhibit premature mitosis in antigen-specific Th1 cells

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M-phase promoting factor is a complex of cdc2 and cyclin B that is regulated positively by cdc25 phosphatase and negatively by wee1 kinase. We isolated the *wee1* gene promoter and found that it contains one AP-1 binding motif and is directly activated by the immediate early gene product c-Fos at cellular G₁/S phase. In antigen-specific Th1 cells stimulated by antigen, transactivation of the *c-fos* and *wee1* kinase genes occurred sequentially at G₁/S, and the substrate of wee1 kinase, cdc2-Tyr15, was subsequently phosphorylated at late G₁/S. Under prolonged expression of the *c-fos* gene, however, the amount of wee1 kinase was increased and its target cdc2 molecule was constitutively phosphorylated on its tyrosine residue, where Th1 cells went into aberrant mitosis. Thus, an immediate early gene product, c-Fos/AP-1, directly transactivates the *wee1* kinase gene at G₁/S. The transient increase in *c-fos* and *wee1* kinase genes is likely to be responsible for preventing premature mitosis while the cells remain in the G₁/S phase of the cell cycle.

Keywords: activator protein-1/c-Fos/G₁/S phase/mitotic cell division/wee1 kinase

Introduction

c-fos is an immediate early gene that is expressed transiently in the G₁ phase of the cell cycle after antigen stimulation (Shipp and Reinherz, 1987; Ullman *et al.*, 1990). The gene product, c-Fos, interacts with members of the Jun family to form an activator protein-1 (AP-1) heterodimer (Angel and Karin, 1991). This heterodimer transactivates genes containing the consensus AP-1 motif TGA(G/C)TCA in their promoter region (Halazonetis *et al.*, 1988; Nakabeppu *et al.*, 1988), and plays an important role in inducing gene expression required for cell proliferation (Muegge *et al.*, 1989; Kim *et al.*, 1990). However, while progression of the cell cycle is known to be coordinated by a family of cyclin-dependent protein kinases (Morgan, 1995), the molecular target of the immediate early gene *c-fos* in cell cycle regulation remains unclear.

The role of c-Fos/AP-1 in the regulation of inflammatory cytokine gene expression, including that of several cytokines important in arthritis, such as interleukin (IL)-1, IL-6 and tumor necrosis factor- α , is better understood (Rhoades *et al.*, 1992; Serkkola and Hurme, 1993; Dendorfer *et al.*, 1994). We have previously examined the role of c-Fos/AP-1 in tumor-like synovial overgrowth and rheumatoid joint destruction, and found that mutation of *c-fos* causes not only cellular overgrowth, but also a skewed immune response (Shiozawa *et al.*, 1992, 1997; Kawasaki *et al.*, 1999). In the present work, we show that expression of wee1 kinase, a kinase important for mitotic cell division, is increased in cells overexpressing the *c-fos* gene.

wee1 kinase inhibits mitotic cell division by inactivating the M-phase promoting factor (MPF). MPF is composed of cdc2 kinase and cyclin B, and specifically regulates the cellular G₂/M transition (Nurse, 1990). In mammalian cells, cdc2 maintains more or less constant levels throughout the cell cycle (Draetta and Beach, 1988; Dalton, 1992), and gradually increases toward G₂/M in T cells (Furukawa *et al.*, 1990; Kim *et al.*, 1992; Lucas *et al.*, 1992). cdc2 forms a complex with cyclin B at the late S and G₂ phases of the cell cycle (Pines and Hunter, 1989). Following its association with cyclin B, cdc2 undergoes a series of phosphorylation events that modulate its kinase activity (Solomon, 1993). As mammalian cells progress towards mitosis, the concentration of cdc2–cyclin B complexes gradually increases. cdc2–cyclin B complexes can be rapidly activated by dephosphorylation of cdc2 at Thr14 and Tyr15 (Norbury *et al.*, 1991), and negatively regulated by phosphorylation at Tyr15 (Gould and Nurse, 1989). Dephosphorylation of Thr 14 and Tyr 15 is catalyzed by cdc25 (Strausfeld *et al.*, 1991), while phosphorylation of Tyr15 is catalyzed by wee1 kinase (Russell and Nurse, 1987; Parker *et al.*, 1992).

Wee1 kinase activity is controlled by three mechanisms (Watanabe *et al.*, 1995). First, as demonstrated in *Schizosaccharomyces pombe*, wee1 kinase activity is down-regulated during the M phase as a result of phosphorylation in its catalytic domain by nim1/cdr1 (Coleman *et al.*, 1993; Parker *et al.*, 1993; Wu and Russell, 1993). Secondly, wee1 levels decrease in M phase as a result of both decreased synthesis and increased degradation, the latter of which is mediated by the ubiquitin-conjugating enzyme cdc34 in *Xenopus* egg extracts (Michael and Newport, 1998). Finally, the synthesis of wee1 kinase has been shown to increase during S phase (Watanabe *et al.*, 1995), although the mechanisms responsible for this increase have been unclear.

We show that there is one AP-1 binding motif in the *wee1* promoter which is directly bound by c-Fos/AP-1 to transactivate the *wee1* gene. Following receptor ligation in Th1 cells, upregulation of the *c-fos* and *wee1* kinase genes

occurs sequentially during the G₁/S phase. When the *c-fos* gene was expressed for an abnormally prolonged period, Th1 cells shifted to a state with tetraploid DNA content (4C), a state in which *wee1* kinase activity increases, leading to increased Tyr15 phosphorylation of its target *cdc2* and consequent inhibition of mitosis. This suggests that c-Fos/AP-1 causes increased *wee1* synthesis in the G₁/S phase of the cell cycle. We discuss this result in the context of checkpoint controls for cellular DNA synthesis and mitosis.

Results

Identification of an AP-1 binding site in the mouse *wee1* promoter

We isolated the 5'-flanking region of the mouse *wee1* gene from genomic DNA by nested PCR using primers complementary to the sense strand of the published *wee1* cDNA sequence (Honda *et al.*, 1995). The resulting 1069 bp fragment was confirmed to be the 5'-flanking region of the murine *wee1* gene by PCR with a sense primer that annealed to the newly isolated sequence and an antisense primer that annealed to a known exon of the *wee1* gene (Figure 1A). A transcription start site was found 78 bp upstream of the ATG initiation codon (+1) using the oligonucleotide-capping method (Figure 1B). One AP-1 binding motif was found in the promoter region (−418/−412, GGAGTCA; Figure 1A), as well as one NF-κB (−286/−277), two Sp-1 (−457/−448, −95/−86) and two c-Myc (−948/−943, −304/−299) binding motifs. We tested which of these transcription motifs are involved in the transactivation of *wee1* kinase gene by monitoring gene activation following anti-CD3 stimulation (Figure 1C). The 28-4 Th1 clone is a T-cell clone that is specifically activated by keyhole limpet hemocyanin (KLH). This clone was stimulated by anti-CD3 after transfection with one of several reporter constructs: one consisting of 5× AP-1 sites from the mouse *wee1* promoter (GGAGTCA) fused to luciferase, 7× consensus AP-1 (TGAGTAA)–luciferase, 5× consensus NF-κB (GGGGACTTTCC)–luciferase, 4× consensus Sp1 (GGGGCGGGG)–luciferase, or 6× consensus c-Myc (CACGTG)–luciferase. Luciferase reporter assays showed that both the AP-1 motif of mouse *wee1* promoter and the consensus AP-1 motif were sufficient to mediate transactivation following anti-CD3 stimulation, with maximum activation observed 7 h after stimulation (Figure 1C). None of the other reporter constructs responded to anti-CD3 stimulation.

c-Fos/AP-1 binds to the *wee1* promoter in vivo and in vitro

To examine protein binding to the AP-1 motif (−418/−412, GGAGTCA) of the mouse *wee1* promoter *in vivo*, we performed an *in vivo* dimethyl sulfate (DMS) footprinting assay. DMS treatment of T cells caused cleavage of three G residues around the region of the AP-1 motif in the *wee1* promoter: −442, −411 and −401 (Figure 2A, lane 1, solid arrowheads). These peaks were absent in the control DMS-untreated T cells (Figure 2A, lane 8). Following anti-CD3 treatment, the peak corresponding to −411 G diminished at 6 and 7 h (Figure 2A, lanes 4 and 5, open arrowheads), suggesting that this region was protected by binding of a protein, possibly AP-1, at the time when the transactiv-

Fig. 1. Nucleotide sequence of the mouse *wee1* gene promoter region. (A) Binding motifs for transcription factors are indicated by arrowheads and closed squares. These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession No. AB024614. Nucleotide numbering is relative to +1 at the initiating ATG codon. (B) The transcription start site (TSS) of the *wee1* kinase gene is indicated by an arrowhead. Nested adaptor primer 2RC is indicated by the horizontal line. (C) Trans-reporting assay. The 28-4 Th1 clone was transfected with the pGL3-Enhancer vector containing binding motifs in a promoter driving expression of the luciferase reporter gene: 5× AP-1 of mouse *wee1* promoter (GGAGTCA)–luciferase (mAP-1); 7× consensus AP-1 (TGAGTAA)–luciferase (AP-1); 5× consensus NF-κB (GGGGACTTTCC)–luciferase (NF-κB); 4× consensus Sp1 (GGGGCGGGG)–luciferase (Sp-1); or 6× consensus c-Myc (CACGTG)–luciferase (c-Myc). The 28-4 Th1 clone was stimulated with anti-CD3 mAb for 0, 7 and 24 h. Cell lysates were assayed for luciferase activity (firefly/*Renilla* Luc) in a dual-luciferase reporter assay in which luciferase activity at 0 h was taken as 1. Each bar represents the mean $\pm$ SD of three experiments.

ation through the AP-1 binding motif was maximum (Figure 1C). Binding to this motif (GGAGTCA) was examined *in vitro* by using an electrophoretic mobility shift assay (EMSA). Binding to the wild-type AP-1 motif, but not a mutant motif (GGTGTG), was observed in nuclear extracts from the 28-4 Th1 clone obtained 7 h after anti-CD3 stimulation (Figure 2B, lanes 1 and 3). The band obtained with the wild-type AP-1 motif was supershifted by anti-c-Fos antibody (Ab) (Figure 2B, lanes 4 and 8). It was found that c-Fos bound c-Jun to make an AP-1 heterodimer to bind the AP-1 motif of *wee1* promoter (Figure 2B, lanes 5–7).

c-Fos/AP-1 directly transactivates *wee1* kinase

To test whether or not binding of c-Fos/AP-1 to the promoter region of *wee1* gene plays a major role in transactivation of the *wee1* gene, we introduced deletion

Fig. 2. c-Fos/AP-1 binding to *weel* promoter *in vivo* and *in vitro*. (A) *In vivo* DMS footprinting assay. The 28-4 Th1 clone stimulated with anti-CD3 mAb was treated with 0.1% DMS. Extracted DNA was cleaved by 1 M piperidine, and processed for linker-mediated PCR. 4,7,2',7'-tetrachloro-6-carboxyfluorescein (TET)-labeled DNA was electrophoresed with TAMRA-labeled size standards on a 36 cm well-to-read gel (6% polyacrylamide–6 M urea) plate in an ABI377 at 3000 V for 2 h. Three peaks (arrowheads) indicated the G residues methylated by DMS. The open arrowheads indicate the disappearance of the –411 G peak. (B) EMSA for c-Fos/AP-1. The 28-4 Th1 clone was stimulated with anti-CD3 mAb. Nuclear extracts were obtained 7 h after stimulation, and assayed in EMSA using DIG-labeled double-stranded oligonucleotides (–434/–400) containing wild-type (WT) or mutated (Mu) AP-1 binding motifs. The competition assay was performed by adding unlabeled oligonucleotides. Abs reactive against c-Fos, c-Jun, JunB or JunD were added for supershift experiments.

and site-directed mutations into the promoter. We transfected 28-4 Th1 cells with reporter gene constructs driven by the normal mouse *weel* promoter (WT, –1069/–158), a truncated promoter containing the AP-1 motif (AP-1⁺ del, –520/–158), a truncated promoter lacking the AP-1 motif (AP-1[–] del, –382/–158) or a promoter containing a mutated AP-1 motif (mut, –1069/–158; –418/–412, GGAGTTG) (Figure 3A). Both the wild-type (WT) and the truncated promoter containing the AP-1 motif (AP-1⁺ del) were activated in 28-4 Th1 clones after anti-CD3 stimulation. In contrast, activation of the promoter lacking the AP-1 motif (AP-1[–] del) or the promoter containing the mutated AP-1 motif (mut) was poor (Figure 3B), while activation of AP-1[–] del was almost completely inhibited, activation of mut was inhibited ~50%, probably because mut contains binding motifs other than AP-1, especially Sp-1 (Pugh and Tjian, 1991). To study whether or not

Fig. 3. Luciferase reporter assay for mouse *weel* promoter activity. (A) Plasmid constructs of the *weel* promoter driving the luciferase reporter gene in pGL3-Enhancer vector. (B) Luciferase assay. Reporter plasmids were introduced into the 28-4 Th1 clone. After overnight culture, cells were stimulated by anti-CD3 mAb for 0, 5, 10 and 24 h. Lysates were assayed in a similar way as described in Figure 1. Each symbol represents the mean $\pm$ SD of three experiments. (C) Antisense oligonucleotide treatment. Luciferase reporter plasmid containing wild-type *weel* promoter (–1069/–158) was electroporated into the 28-4 Th1 clone with or without *c-fos* sense or antisense oligonucleotides. After overnight culture, cells were stimulated with anti-CD3 mAb for 24 h in the case of luciferase assay (upper panel) and for 7 h in the case of western blotting (lower panel). Each bar represents the mean $\pm$ SD of three experiments.

expression of the *weel* kinase gene required induction of c-Fos expression, *c-fos* antisense oligonucleotides were co-transfected with the wild-type *weel* promoter–luciferase reporter gene construct (WT) and the transfected cells were stimulated with anti-CD3. We found that transactivation of the *weel* kinase gene was specifically inhibited by *c-fos* antisense oligonucleotides (Figure 3C, upper panel). Western blot analysis also confirmed that expression of c-Fos protein was significantly decreased in cells transfected with *c-fos* antisense oligonucleotides (Figure 3C, lower panel). Furthermore, transactivation of 5 $\times$ AP-1 (GGTGTCA)–luciferase was enhanced when the *c-fos* and *c-jun* genes were transiently co-expressed in COS-7 cells (data not shown).

Fig. 4. Time course of the expression of *c-fos* and *wee1* kinase genes. (A) Western blot analyses. The extracts of 28-4 Th1 clone were obtained after anti-CD3 mAb treatment. SDS-PAGE (7.5–10%) and western blot visualization with Abs reactive against c-Fos, *wee1*, *cdc2*, phospho (p) Tyr15-*cdc2*, *cdc25B*, *cdc25C* or cyclin B (left panel). Schematic representation of gene expression (right panel). (B) Quantitative RT-PCR by ABI7700 of 28-4 Th1 clone mRNA after anti-CD3 stimulation. The amount of *c-fos* and *wee1* mRNAs and 18S *rRNA* was quantified. The levels of *c-fos* (closed circles) and *wee1* (open circles) were divided by that of 18S *rRNA* for normalization. Each circle represents the mean $\pm$ SD of three experiments. (C) Nuclear run-on assay. Nuclei prepared from the 28-4 Th1 clone after anti-CD3 stimulation were incubated with [α - ^{32} P]UTP. Run-on transcripts were hybridized to 10 μ g of mouse 18S ribosomal RNA (*rRNA*), mouse *c-fos* cDNA (*c-fos*), mouse *wee1* cDNA (*wee1*) or control vector. (D) DNase I HS assay of the mouse *wee1* promoter region. Nuclei of the 28-4 Th1 clones obtained 0 h (lanes 1–5) or 4 h (lanes 6–10) after anti-CD3 stimulation were incubated with DNase I (lanes 1 and 6, 0 U/ml; lanes 2 and 7, 0.01 U/ml; lanes 3 and 8, 0.1 U/ml; lanes 4 and 9, 1 U/ml; lanes 5 and 10, 10 U/ml) for 10 min. Purified genomic DNA was completely digested by *SacI* and *MboII*, electrophoresed on 2.5% agarose gel, and transferred to nylon membrane. Filter was hybridized to Fl-end-labeled oligonucleotide probe (43/68) for mouse *wee1* DNA, and visualized by alkaline phosphatase-labeled anti-Fl Ab and chemiluminescence. (E) Cell cycle progression of the 28-4 Th1 clone. DNA synthesis of the 28-4 Th1 clone after anti-CD3 stimulation was measured by BrdU incorporation.

c-Fos transactivates the *wee1* gene at G₁/S

In rapidly dividing cells, including antigen-specific T cells, c-Fos/AP-1 expression normally increases transiently at early G₁ phase of the cell cycle (Shipp and Reinherz, 1987; Ullman *et al.*, 1990). Therefore, we studied the profile of induction of the *wee1* kinase gene by c-Fos/AP-1 throughout the cell cycle. Western blotting showed that c-Fos increased at 4–8 h and the *wee1* kinase increased at 16–32 h after anti-CD3 stimulation (Figure 4A), which corresponds to the G₁/S phase of cell cycle (Figure 4E).

The *cdc2* existed at low levels until 24 h, and increased at 32–48 h after stimulation (Figure 4A). The Tyr15 phosphorylation of *cdc2* was highest 16–32 h after stimulation. Phosphorylation of histone H1 was increased 32 h after stimulation (data not shown). The amount of *cdc25B* or *cdc25C* was increased 32 h after stimulation. Cyclin B was increased 32 h after stimulation (Figure 4A). A time course study of mRNA expression in 28-4 Th1 cells using an ABI7700 showed that *c-fos* mRNA and *wee1* mRNA increased in 28-4 Th1 cells between 1–3 and ~12 h after

Fig. 5. Profile of *c-fos*-expressing antigen-specific Th1 cells. (A) Flow cytometry analysis of the *c-fos*-expressing 75/15pH8 clone to measure cellular DNA content by propidium iodide staining. The 28-4 Th1 cells were transfected with *c-fos*-expressing 75/15pH8 vector and cultured for 6 months (upper panel). Clones residing in 2C or 4C were isolated by limiting dilution technique (middle panels). RNase protection assay to detect *c-fos* mRNA at the quiescent phase (9 days after antigen stimulation) (lower panel). RNA was hybridized to probes protecting 250 nucleotides of the mouse *c-fos* mRNA or 316 nucleotides of the mouse *GAPDH* mRNA, followed by electrophoresis on 5% polyacrylamide–8 M urea gel. Western blot of cell extracts obtained 9 days after antigen stimulation (lower panel). (B) Cell cycle progression of 4C clone stimulated with anti-CD3 Ab for 48 h in the absence or presence of aphidicolin or colcemid. DNA content measured by flow cytometry. (C) Percentage of G₂/M phase cells in the population of 2C (closed circles) or 4C (open circles) clones after anti-CD3 stimulation. (D) Western blot analysis of the cell extracts obtained 24 or 48 h after anti-CD3 treatment. Tyrosine phosphorylation of *cdc2* was detected by immunoprecipitation with anti-*cdc2* Ab and protein A–agarose beads, followed by western blotting using anti-pTyr Ab.

anti-CD3 stimulation, returning to a basal level within 18 h after stimulation (Figure 4B). In a nuclear run-on assay, *c-fos* was transcribed 1 h (G₁ phase) after anti-CD3 stimulation, and the *wee1* kinase gene was also transcribed during the G₁ phase at 4 h (Figure 4C and E). These results show that transactivation of the *c-fos* and *wee1* genes is sequential, and occurs within the G₁/S phase of the cell cycle.

We next examined the state of the chromatin surrounding the chromosomal AP-1 site in the *wee1* promoter during the G₁/S phase. Nuclei obtained 4 h (G₁ phase) after anti-CD3 stimulation of 28-4 Th1 cells were analyzed by a DNase I hypersensitive site (HS) assay that specifically detects open chromatin structure in expressed genes (Gross and Garrad, 1988; Kornberg and Lorch, 1991). While an expected band of <835 bp was undetectable in unstimulated 28-4 Th1 cells after treatment with 1 U/ml DNase I, an ~600 bp band gradually emerged in the cells stimulated with anti-CD3 Ab (Figure 4D, compare lanes 4 and 9). Since the probe (43/68) bound to the genomic DNA region spanning the ATG initiation codon up to +68 bp of the *wee1* genome, the inducible DNase I HS was assigned to the position ~530 bp upstream of the ATG, placing it precisely within the region of the AP-1 binding motif. Additionally, constitutive DNase I HS was detected at the position ~330 bp upstream of the ATG (data not shown).

Transient increase in *c-fos* is a prerequisite for normal cell cycle progression

To study whether or not direct activation of the *wee1* gene by an immediate early gene product, *c-Fos*, at G₁/S phase was required for normal cell cycle progression, we transfected 28-4 T cells with *c-fos*-expressing 75/15pH8 vector, and stable transformants were obtained (Kawasaki *et al.*, 1999). After the transformants had been cultured for 1 month, we noticed an increasing number of the cells with tetraploid DNA content (4C), as determined by propidium iodide staining. Of the cells cultured for longer periods (>4 months), ~50% shifted to 4C (Figure 5A, upper panel). This shift to the 4C state was reproduced in >10 experiments, and was not observed in cells transfected with the control pH8 vector.

We subcloned each 2C and 4C clone by limiting dilution (Figure 5A, middle panel). Using an RNase protection assay, we quantitated *c-fos* mRNA expression 9 days following antigen stimulation (quiescent phase) and found that the 4C clones expressed higher amounts of *c-fos* mRNA than the 2C clones (Figure 5A). Western blot analysis confirmed that the amount of both the *c-Fos* and *wee1* proteins was significantly increased in the 4C clones (Figure 5A, lower panel). Thus, the Th1 cells go into aberrant mitosis when expression of an immediate early *c-fos* gene was not switched off.

Forty-eight hours after anti-CD3 treatment, the 4C clones shifted to an 8C DNA content (Figure 5B, upper panel). This shift was blocked by aphidicolin, a DNA synthesis inhibitor, suggesting that the shift from 4C to 8C required DNA synthesis (Figure 5B, middle panel). Anti-CD3 stimulation of cells in the presence of colcemid induced a shift from 4C to 8C, and cells remained in this state, indicating that these 8C cells are in the M phase of the cell cycle (Figure 5B, lower panel). We therefore assume that the 4C clones in resting state reside in G₁, and are formed when 2C cells undergo S phase DNA replication without intervening mitosis. Entrance into G₂/M was also significantly delayed in the 4C clones after anti-CD3 stimulation: whereas the 2C clones entered G₂/M normally at 24 h, the 4C clones entered G₂/M at 48 h after stimulation and remained there until 96 h (Figure 5C). We therefore compared the 2C and 4C clones for expression of cell cycle regulators known to act primarily in the G₂/M phase. Western blot analysis showed that expression of c-Fos and *wee1* was higher in the 4C clones, while the peak levels of cyclin B, *cdc2*, *cdc25B* and *chk1* were similar between the 2C and 4C clones in the G₂/M phase (Figure 5D). Since *wee1* is a tyrosine-protein kinase that specifically phosphorylates *cdc2* at Tyr15, we immunoprecipitated *cdc2* and analyzed phosphotyrosine content by western blot analysis with anti-*cdc2*-pTyr Ab. Tyrosine phosphorylation of *cdc2* was detected only in the 4C clones at the G₂/M phase (Figure 5D).

Discussion

We cloned and sequenced the promoter region of the mouse *wee1* kinase gene up to -1069 relative to the ATG. The promoter did not contain a consensus TATA box similar to that found in the promoters of cyclin A (Henglein *et al.*, 1994), cyclin D1 (Philipp *et al.*, 1994) or other cell cycle regulators (Azizkhan *et al.*, 1993). However, we did identify one AP-1 binding motif in the *wee1* promoter region (-418/-412) with the sequence GGAGTCA, which is one base different from the consensus AP-1 binding motif TGAGTCA. We showed that c-Fos, induced by antigen stimulation, bound c-Jun to make an AP-1 heterodimer to bind this putative AP-1 motif *in vivo* and *in vitro*. Similar one-base deviations from the consensus AP-1 motif have been shown to be functional, e.g. the motif CGAGTCA in the human $\alpha 2$ (I) collagen promoter (Chung *et al.*, 1996). We also found that consensus motifs other than AP-1 did not contribute significantly to the transactivation of *wee1* kinase following anti-CD3 stimulation. Site-directed mutagenesis and antisense experiments showed that both the presence of an intact AP-1 binding motif and the induction of c-Fos were required for transactivation of the mouse *wee1* kinase gene. Transactivation of the *c-fos* and *wee1* kinase genes occurred sequentially during the G₁/S phase, and the chromatin structure around the AP-1 motif existed in an open conformation at the G₁/S phase, as revealed by DNaseI HS analysis. Thus, c-Fos/AP-1 directly transactivates the *wee1* kinase gene during the G₁/S phase following antigen stimulation.

Under prolonged expression of *c-fos* gene, however, expression of the *wee1* kinase gene was sustained at higher levels in Th1 cells. Such T cells were found to have a

4C DNA content and to be arrested in the cell cycle, but to progress to an 8C state after receptor ligation. This shift to 8C was blocked by the DNA synthesis inhibitor aphidicolin, indicating that DNA synthesis is required for entrance into 8C. In contrast, receptor ligation in the presence of colcemid caused 4C clones to shift to the state with 8C DNA content, and arrest in this state. Thus, the 8C and 4C cells are assumed to represent the cells in the M and G₁ phases of the cell cycle, respectively. Normally, c-Fos expression increases transiently in early G₁. However, when the expression of *c-fos* is prolonged, either experimentally or as a result of disease conditions such as tumors (Miller *et al.*, 1984; Wang *et al.*, 1991) or the tumor-like cell growth seen in arthritis (Shiozawa *et al.*, 1992, 1997), the *wee1* kinase activity was increased and its substrate *cdc2* was inactivated by phosphorylation at Tyr15, and the cells went into aberrant mitosis. This was indeed the case for the Th1 cells overexpressing *c-fos* in the present study.

These findings are consistent with those of Broek *et al.* (1991), who showed that *S.pombe* underwent DNA duplication twice in one cell cycle when *cdc2* was physically disrupted. Correa-Bordes and Nurse (1995) showed that expression of the cyclin-dependent kinase inhibitor *p25^{rum1}* increases in early G₁ and inhibits *p34^{cdc2}/p56^{cdc13}*, thereby preventing premature mitosis when the cells are in G₁/S. Human herpesvirus 7 infection induces significant cell cycle abnormalities in T cells, including the accumulation of polyploid cells in G₂/M, which is due to inactivation of *cdc2* (Secchiero *et al.*, 1998). Megakaryocytes normally mature to polyploid cells as a result of DNA replication proceeding in the absence of mitosis, a process called endomitosis. This polyploidization of megakaryocytes was shown to be caused by inactivation of cyclin B1-dependent *cdc2* kinase (Zhang *et al.*, 1996), further supporting the idea that cells go into aberrant mitosis and remain in the G₂/M phase when *cdc2* is continuously inactivated. Collectively, these findings suggest that a transient increase in c-Fos and *wee1* kinase expression at G₁/S is required for normal cell cycle progression, and that aberrant mitosis can result if the expression of these genes is not subsequently down-regulated.

cdc25 has been implicated in the checkpoint control that prevents initiation of mitosis in cells before DNA replication is complete (Enoch and Nurse, 1990). However, O'Connell *et al.* (1997) have shown that *wee1* kinase, which is phosphorylated by *chk1*, may also be important in the G₂ checkpoint. *cdc25* appears to function to promote the rapid and efficient cell cycle progression through G₂/M, while the *wee1* kinase seems more important in the overall control of mitosis. *cdc25* has been shown to be up-regulated via a positive feedback mechanism in which *cdc25* is phosphorylated by kinases including *cdc2*/cyclin B, and in turn stimulates *cdc2*/cyclin B activity (Hoffmann *et al.*, 1993). In the present study, we observed that expression of either *cdc25* or *chk1* was not increased in the 4C clones, suggesting that the inhibition of mitotic cell division observed in the 4C clones was unlikely to be due to increased expression of *cdc25* or *chk1*.

Previous studies have shown that both the amount and the activity of *wee1* kinase are elevated during the S phase, and both decrease rapidly as the cells enter the M phase

(Watanabe *et al.*, 1995; Michael and Newport, 1998). Our antigen-specific Th1 cells also behaved as such after receptor ligation. Michael and Newport (1998) showed that proteolytic degradation of *wee1* kinase is required for the timely initiation of mitosis. Since degradation of *wee1* kinase is inhibited when DNA replication is blocked, these authors speculated that a factor which is unknown may be responsible for maintaining elevated *wee1* expression during DNA duplication and prior to entry into the M phase. We propose that this factor is c-Fos/AP-1, a direct transactivator of the *wee1* gene, and that c-Fos/AP-1 acts to prevent premature entry of G₁/S cells into G₂/M.

In summary, c-Fos/AP-1 directly transactivates the *wee1* kinase gene during the G₁/S phase following antigen stimulation in Th1 cells, and the resulting increase in *wee1* kinase activity is likely to be responsible for preventing premature mitosis while antigen-specific Th1 cells are in the G₁/S phase of the cell cycle. We have an impression that c-Fos/AP-1 and *wee1* kinase act as a brake to halt cellular G₂/M transition in the face of G₁ progression, and c-Fos/AP-1, on the other hand, acts as an accelerator to drive cellular machines to pass through G₁/S efficiently. Cells will arrest at G₁ when an immediate early *c-fos* gene is not transcribed (Nishikura and Murray, 1987; Riabowol *et al.*, 1988).

Materials and methods

Cells

The KLH-specific mouse CD4 Th1 clone 28-4 (Asano and Tada, 1989) and antigen-specific *c-fos*-expressing T-cell transformant 75/15pH8 were maintained as described (Kawasaki *et al.*, 1999).

Isolation of the mouse *wee1* promoter

The promoter of the mouse *wee1* gene was isolated by nested PCR (GenomeWalker; Clontech, Palo Alto, CA). The walking adaptor was ligated to the genomic DNA fragments digested by *EcoRI*, *ScaI*, *DraI*, *PvuII* or *SspI*. Primary PCR was conducted in a 25 µl reaction containing 2.5 mM MgCl₂, 400 µM each dNTP, 1× LA Buffer (Takara, Kyoto, Japan), 6% DMSO, 3% glycerol, 1.25 U of LA Taq DNA polymerase (Takara), 200 nM adaptor sense primer (5'-GTAATACGACTCACTA-TAGGGC-3'), 200 nM *wee1* gene-specific antisense primer (-168/-139; 5'-CTGCGCGGCGGCAACTCCGAGGCCCGTCAA-3') and 50 ng of adaptor-ligated genomic DNA (Clontech) at 94°C for 20 s and 68°C for 5 min for 40 cycles. Secondary PCR used nested adaptor primer (5'-ACTATAGGGCAGCGTGGT-3') and nested *wee1*-specific antisense primer Mb2. Subcloned pT7Blue vector was confirmed to contain *wee1* gene by sequencing. The transcription start site was determined by the oligonucleotide-capping method (Maruyama and Sugano, 1994). Primary PCR was conducted in a 25 µl reaction containing 400 µM each dNTP, 1× GCII Buffer (Takara), 1.25 U of LA Taq DNA polymerase, 200 nM adaptor sense primer 1RC (5'-CAAGGTACGCCACAGCGTATG-3'), 200 nM *wee1* gene-specific antisense primer (265/284; 5'-AGCAGC-AAGTCGTCTCCAG-3') and 1 µl of cap site cDNA from mouse kidney (Nippon Gene, Toyama, Japan) at 95°C for 20 s, 50°C for 20 s and 72°C for 5 min for 35 cycles. Secondary PCR used nested adaptor primer 2RC (5'-GTACGCCACAGCGTATGATGC-3') and nested *wee1*-specific antisense primer (170/189; 5'-AGAAGGCAGGGGGCGAATCCG-3'). The PCR products were extracted from the gel and directly sequenced.

Plasmids

The *wee1* promoter (-1069/-158) was subcloned in the pT7Blue vector (Novagen, Madison, WI), digested by *Sall* and *BamHI*, and subcloned into the pGL3-Enhancer vector (Promega, Madison, WI) to generate a firefly luciferase reporter (pwee1WT-luc). The -520 deletion containing the AP-1 motif was constructed using sense primer (-502/-501; 5'-TAAACATAGACTCTGGGTGG-3') and antisense primer M2b (-187/-158; 5'-AGGCCCCGTCAAGCTCAGACGGGGCGGACGG-3'). The -382 deletion lacking the AP-1 motif was constructed using sense primer (-382/-356; 5'-TTCTCCCCGTGGGCT-3') and antisense

primer M2b. Reference +1 was the initiating ATG codon. For site-directed mutagenesis (Kunkel, 1985), a 50 µl reaction containing 40 µM each dNTP, 1× reaction buffer, 2.5 U of *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA), 125 ng of sense primer (5'-GTG-GGGCGGAGTTGGCGAGTTCCCCG-3'), 125 ng of antisense primer (5'-GCGGGAAGTCCGCAACTCCGCCCCAC-3') and 50 ng of pwee1WT-luc was carried out at 95°C for 30 s, followed by amplification at 95°C for 30 s, 55°C for 1 min and 68°C for 2 min for 18 cycles. Ten units of *DpnI* were added to each amplification reaction to digest non-mutated DNA. Luciferase reporter genes containing 5× AP-1 motif (GGAGTCA) of the mouse *wee1* promoter, 4× consensus Sp1 motif (GGGGCGGGGC) or 6× consensus c-Myc motif (CACGTG) were constructed in pGL3-Enhancer vectors. 7× pAP1-luc plasmid (Stratagene) or 5× pNF-κB-luc plasmid (Stratagene) was used as consensus AP-1 or NF-κB motifs. Mouse pcDNA-c-fos and pcDNA-c-jun expression vectors were constructed from pYN3181 and pJal1 plasmids (Dr Nakabeppu, Kyushu University).

Transient transfection

We transfected the 28-4 Th1 clone (1 × 10⁷) isolated 9 days after antigen stimulation with 40 µg of pGL3-Enhancer vector containing various *wee1* promoters using a Bio-Rad electroporator (200 V, 975 µF) in 0.4 ml of RPMI supplemented with 20% fetal calf serum (FCS). After overnight culture, cells were stimulated with anti-CD3 monoclonal Ab (mAb). As an internal control for transfection efficiency, 2 µg of pRL-TK (Promega) containing herpes simplex virus thymidine kinase promoter in the upstream of *Renilla* luciferase were co-transfected.

Antisense oligonucleotides

Sense and antisense phosphorothioate analogs (-6/15) of *c-fos* mRNA including the ATG (Ivanov and Nikolic-Zugic, 1997) consisted of the sequences 5'-TCGACCATGATGTTCTCGGGT-3' and 5'-ACCCGA-GAACATCATGGTCA-3', respectively. They (1 µg) were transfected into the 28-4 Th1 clone by electroporation. Cells were cultured overnight and then stimulated with anti-CD3 mAb (5 µg/ml; 145-2C11; Cedarlane, Ontario, Canada) coated on 24-well plates. Oligonucleotides were also added just prior to anti-CD3 stimulation.

Luciferase assay

Cell lysates (20 µl) were assayed using a dual-luciferase reporter assay (Promega) in Luminoskan (LabSystems, Tokyo, Japan), the activity of firefly luciferase being divided by the activity of *Renilla* luciferase (Mitsuda *et al.*, 1997).

In vivo DMS footprinting

The 28-4 Th1 clone (1 × 10⁷) stimulated with anti-CD3 mAb was suspended in RPMI medium containing 10% FCS and 0.1% DMS (Pfeifer *et al.*, 1990). Nuclei were isolated, and DNA was purified by reaction with 300 µg/ml proteinase K, 50 mM Tris-HCl pH 8.0, 25 mM EDTA and 0.2% SDS at 37°C for 3 h (Wijnholds *et al.*, 1988), and dissolved in 1 M piperidine. Strand scission reaction was performed at 90°C for 30 min. DNA samples were prepared for linker-mediated PCR (LMPCR) (Garrity and Wold, 1992) with the following modifications. A 30 µl reaction containing 40 mM NaCl, 10 mM Tris-HCl pH 8.9, 5 mM MgSO₄, 0.01% gelatin, 0.3 pmol *wee1* gene-specific primer1 (-354/-337; 5'-GGTTTGAAAAACAGGGA-3'), 240 µM each dNTP, 1 U of Vent polymerase (New England BioLabs, Beverly, MA) and DNA (2 µg) was carried out at 95°C for 5 min, 50°C for 30 min and 76°C for 10 min. This was mixed with 45 µl of ligation solution containing 188 mM Tris-HCl pH 7.6, 14 mM MgCl₂, 33 mM dithiothreitol (DTT) and 0.008% bovine serum albumin, 1.7 mM ATP, 100 pmol unidirectional linker (LMPCR1, 5'-GCGGTGACCCGGGAGATCTGAATTC-3'; LMPCR2, 5'-GAA-TTCAGATC-3') and 4.5 U of T4 DNA ligase (Promega), and incubated at 17°C overnight. Purified DNA was amplified in a 100 µl reaction containing 40 mM NaCl, 20 mM Tris-HCl pH 8.9, 5 mM MgSO₄, 0.01% gelatin, 0.01% Triton X-100, 670 µM each dNTP, 10 pmol *wee1*-specific primer2 (-360/-344; 5'-GCGCCCTCCCTGTTTTT-3'), 10 pmol linker primer LMPCR1 and 3 U of Vent polymerase at 95°C for 1 min, 55°C for 2 min and 76°C for 3 min for 18 cycles. Five microliters of labeling mixture containing 40 mM NaCl, 20 mM Tris-HCl pH 8.9, 5 mM MgSO₄, 0.01% gelatin, 0.01% Triton X-100, 2 mM each dNTP, 2.3 pmol TET-labeled *wee1*-specific primer3 (-366/-344; 5'-TACGTGCGG-CCCTCCCTGTTTTT-3') and 1 U of Vent polymerase were added. The labeling reaction was carried out at 95°C for 4 min, 58°C for 2 min and 76°C for 10 min for two cycles. Labeled DNA was electrophoresed at 3000 V for 2 h in an ABI377 (Perkin Elmer, Norwalk, CT). The DNA fragment peaks were identified by GENESCAN (version 2.0.2) software

(Perkin Elmer), and were 25 nucleotides longer than the original footprinting products because of the 25 nucleotide linker.

EMSA

Nuclear extract (5 µg), incubated for 30 min with 0.5 pmol of digoxigenin (DIG)-labeled oligonucleotide probes, was run on a 4% polyacrylamide gel, transferred to a positively charged nylon membrane, and visualized by alkaline phosphatase-labeled F(ab)₂ anti-DIG Ab and chemiluminescence reaction (Kawasaki *et al.*, 1999). The double-stranded AP-1 motif (−434/−400) of the mouse *wee1* promoter was 5′-GCCTAGGCTGTG-GGGCGGAGTCAGCGAGTTCCC-3′ and 5′-GCGGGAACCTCGTGA-CTCCGCCCCACAGCCTAG-3′. Mutated AP-1 motif was 5′-GCC-TAGGCTGTGGGGCGGAGTTGGCGAGTTCCC-3′ and 5′-GCGGGA-ACCTCGCCAACCTCGGCCCCACAGCCTAG-3′. Non-labeled oligonucleotides (50 pmol) were added as competitor. Supershift was performed by adding 0.5 µg of anti-c-Fos Ab (sc-52; Santa Cruz Biotechnology, Santa Cruz, CA), anti-c-Jun Ab (PC-06; Calbiochem-Novabiochem Corp., La Jolla, CA), JunB (sc-046; Santa Cruz), JunD (sc-047; Santa Cruz) or normal rabbit IgG (sc-2027; Santa Cruz).

Immunoprecipitation

Two hundred micrograms of protein in 200 µl of lysis buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF)] were incubated with anti-cdc2 Ab (Upstate Biotechnology, Lake Placid, NY) at 4°C for 3 h, followed by reaction with protein A-agarose beads (Boehringer Mannheim, Mannheim, Germany) at 4°C overnight. After heating at 95°C for 5 min, supernatant was separated by 12% SDS-PAGE and transferred to an Immobilon-P membrane. The membrane was then incubated either with biotin-conjugated anti-phosphotyrosine mAb (4G10; Upstate Biotechnology) or anti-cdc2 Ab (Upstate Biotechnology), followed by reaction with horseradish peroxidase (HRP)-conjugated streptavidin (Upstate Biotechnology) or HRP-conjugated anti-rabbit Ig Ab (Amersham Pharmacia Biotech, Uppsala, Sweden) and chemiluminescence.

Western blotting

The 28-4 Th1 cells (5×10^6) stimulated with anti-CD3 mAb were suspended in 500 µl of hypotonic buffer (10 mM Tris-HCl pH 7.5, 3 mM MgCl₂, 1% NP-40, 10 mM 2-mercaptoethanol and 1 mM PMSF) on ice for 10 min. The nuclear fraction was precipitated by centrifugation at 10 000 g for 1 min, and dissolved in 100 µl of RIPA buffer (10 mM Tris-HCl pH 7.5, 1% SDS, 1 mM PMSF, 1 mM Na₃VO₄). Fifty micrograms of protein were separated by SDS-PAGE and transferred to Immobilon-P membrane (Millipore, Bedford, MA) as described (Kawasaki *et al.*, 1999). The membrane was incubated with anti-c-Fos Ab (sc-52; Santa Cruz Biotechnology), anti-cyclin B1 Ab (sc-752; Santa Cruz), anti-cdc2 Ab (06-194; Upstate Biotechnology), anti-phospho-Tyr15-cdc2 Ab (C0228; Sigma-Aldrich, St Louis, MO), anti-wee1 Ab (KAP-CC005E; StressGen Biotechnologies Corp., Victoria, BC, Canada), anti-cdc25B Ab (sc-326; Santa Cruz), anti-cdc25C Ab (sc-327; Santa Cruz) or anti-chk1 Ab (sc-7233; Santa Cruz) for 2 h, followed by reaction with HRP-conjugated Ab and enhanced chemiluminescence system (ECL; Amersham Pharmacia Biotech).

RNase protection assay

Total RNA was prepared from T cells 9 days after antigen stimulation using the RNeasy total RNA kit (Qiagen, Hilden, Germany). Biotinylated antisense probe protecting 250 nucleotides of the mouse *c-fos* mRNA was transcribed from the pTRI-c-fos/exon4-Mouse plasmid (Ambion, Austin, TX) using SP6 RNA polymerase. Biotinylated antisense probe protecting 316 nucleotides of the mouse *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) mRNA was transcribed from the pTRI-GAPDH-Mouse plasmid (Ambion). Total RNA (10 µg) was hybridized with these probes, followed by digestion with RNase A (5 µg/ml) and RNase T1 (100 U/ml) at 37°C for 30 min. The protected fragments were electrophoresed, transferred to positively charged nylon membrane (Boehringer), and visualized by alkaline phosphatase-conjugated streptavidin and chemiluminescence (Tropix, Bedford, MA).

Quantitative RT-PCR

mRNA was quantified by RT-PCR using TaqMan Gold RT-PCR reagents in an ABI PRISM 7700 (Perkin Elmer) (Gibson *et al.*, 1996; Heid *et al.*, 1996). The primers and TaqMan probes were: 5′-ACTGAGATTGCCAATCTGCT-3′ (forward), 5′-GGCAGACCTCC-AGTCAAAT-3′ (reverse) and 5′-CTGCAAGATCCCCGATGACCTT-3′ (TaqMan probe) for *c-fos*; 5′-GAAAGAACTCAAGAAAGCCC-3′

(forward), 5′-ATATAGTAAGGCTGACAGAGCG-3′ (reverse) and 5′-AGTGCCCGTTCTCAGCTGCAA-3′ (TaqMan) for *wee1*; and 5′-GGTTGATCTGCCAGTAGCAT-3′ (forward), 5′-ATCCAAGTAG-GAGAGGAGCGAG-3′ (reverse) and 5′-TAAGTACGCACGGCCGG-TACAGTGAA-3′ (TaqMan) for 18S *rRNA*. A 100 µl reaction containing 5.5 mM MgCl₂, 200 µM each dNTP, 1× TaqMan RT Buffer, 2.5 µM random hexamer, 0.4 U/µl RNase inhibitor, 1.25 U/µl MultiScribe reverse transcriptase and 100 ng of total RNA was carried out at 25°C for 2 min, 48°C for 30 min and 95°C for 5 min. Fifty microliters of the RT solution were mixed with 5.5 mM MgCl₂, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 400 µM dUTP, 0.01 U/µl AmpErase uracil *N*-glycosylase, 1× TaqMan Buffer A, 0.025 U/µl AmpliTaq Gold DNA polymerase and 200 nM each forward and reverse primers. The mixture was amplified at 95°C for 20 s and at 60°C for 1 min for 50 cycles.

Nuclear run-on assay

Nuclei of 28-4 Th1 cells (1×10^7) stimulated with anti-CD3 mAb were collected and stored in nuclei freezing buffer (50 mM Tris-HCl pH 8.3, 5 mM MgCl₂, 40% glycerol, 0.1 mM EDTA) at −70°C. Nuclei were incubated with [α -³²P]UTP (>3000 Ci/mmol; Amersham Pharmacia Biotech) (Greenberg and Ziff, 1984), and run-on transcripts were hybridized to 10 µg of denatured plasmids pcDNA3.1 (Invitrogen, Carlsbad, CA), mouse *c-fos* cDNA (pcDNA-c-fos), mouse *wee1* cDNA (pcDNA-wee1) or mouse 18S *rRNA* (pcDNA-ribosomal RNA). Plasmids were denatured in 0.1 M NaCl for 30 min at room temperature, neutralized with 6× SSC and dot-blotted onto nylon membrane. After incubation for 48 h, membrane was washed and reacted with 10 µg/ml RNaseA at 37°C for 30 min. Dried filters were subjected to autoradiography.

DNase I HS analysis

Nuclei of the 28-4 Th1 clone (1×10^7) stimulated with anti-CD3 mAb were collected and resuspended in 200 µl of DNase I digestion buffer (60 mM KCl, 15 mM NaCl, 15 mM Tris-HCl pH 7.5, 6 mM MgCl₂, 0.05 mM CaCl₂, 10% glycerol, 0.5 mM DTT), followed by incubation with DNase I (Nippon Gene) on ice for 10 min (Matsukawa *et al.*, 1997). After adding 10 mM EDTA, nuclei were lysed at 37°C overnight and 10 µg of genomic DNA were completely digested with *SacI* and *MboII*. The DNA was electrophoresed and transferred to nylon membrane. Filter was hybridized to fluorescein (Fl)-end-labeled mouse *wee1* probe (43/68; 5′-GTAGGAGCCGCTTACAGTCTGCGGCA-3′) and visualized by applying alkaline phosphatase-labeled anti-Fl Ab and chemiluminescence reaction.

Cell cycle assay

Cellular DNA content was measured by Cycle TEST PLUS (Becton Dickinson, San Jose, CA) using FACStarPLUS and FACSCalibur (Becton Dickinson). Cells (1×10^6) were incubated with trypsin at room temperature for 10 min, and with trypsin inhibitor and RNase A at room temperature for 10 min, followed by staining with propidium iodide and spermine tetrahydrochloride (Vindelov *et al.*, 1983). Fluorescence intensity was measured in a FACStarPLUS (Becton Dickinson). To measure DNA synthesis, bromodeoxyuridine (BrdU; 10 µM) was added and incubated for 30 min. Cells were fixed with 70% ethanol and reacted with 1.5 M HCl, followed by neutralization with 0.1 M Na₂B₄O₇. Specimens were incubated with anti-BrdU Ab (PharMingen, San Diego, CA), followed by the reaction with fluorescein isothiocyanate-conjugated anti-mouse Ig Ab (PharMingen). After RNase treatment, cells were stained with propidium iodide (5 µg/ml) and analyzed by FACSCalibur.

Acknowledgements

We thank Dr Y.Nakabeppu, Kyushu University, Fukuoka, Japan, for pYN3181 (*c-fos*) and pJacl1 (*c-jun*) plasmids, and Dr H.Yasuda, Tokyo University of Pharmacy and Life Science, Tokyo, Japan, for mouse *wee1* kinase cDNA. We also thank Dr M.Lamphier for critical reading of the manuscript. This work was supported in part by grant-in-aid for scientific research to S.S. (B) 07557228, (B) 08457153, (B) 09470127 and (B) 11557026 of the Ministry of Education, Science, Sports and Culture.

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Received February 19, 2001; revised and accepted July 2, 2001