Regulation of Wee1 kinase in response to protein synthesis inhibition

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Abstract To investigate the mechanism coupling growth (protein synthesis) with cell division, we examined the relationship between the tyrosine kinase Wee1 that inhibits Cdc2-Cdc13 mitosis-inducing kinase by phosphorylating it, and protein synthesis inhibition in fission yeast. The *wee1-50* mutant showed supersensitivity to protein synthesis inhibitor, cycloheximide. Wee1 was essential for the G_2 delay upon a partial inhibition of protein synthesis. Indeed, the protein synthesis inhibition caused an increase in the Wee1 protein by the Sty1/Spc1 MAPK-dependent transcriptional and the Sty1/Spc1 MAPK-independent post-transcriptional regulations. Further, the results indicated that the post-transcriptional regulation is important for the G_2 delay.

Key words: Wee1; Protein synthesis inhibition; Cycloheximide; Cell cycle; Sty1 MAPK

1. Introduction

Growing cells maintain a constant cell size as they proliferate by coupling growth with cell division [1]. The nature of the coupling mechanism remains to be clarified.

It is well established that CDKs (cyclin-dependent kinases) play a central role both in the G_1/S and G_2/M transition during the cell cycle in all eukaryotes [2]. The Cdc2 kinase in fission yeast *Schizosaccharomyces pombe* is essential for both cell cycle transitions. The activity of Cdc2 is regulated by several mechanisms including phosphorylation, binding to a cyclin subunit and CDK inhibitors. B-type cyclins Cdc13 [3–5] and Cig2 [6–8] act as G_2/M cyclin and G_1/S cyclin, respectively. Rum1 is an inhibitor of the Cdc2-Cdc13 and Cdc2-Cig2 cyclin B kinases [9,10]. The onset of mitosis in fission yeast is controlled by the inhibitory phosphorylation of Cdc2 on tyrosine-15 [2,11,12]. The tyrosine phosphorylation of Cdc2, which inhibits its in vitro kinase activity [13], is catalysed by the tyrosine kinases Wee1 and Mik1 [13–15] and is removed by the tyrosine phosphatase Cdc25 [13,16,17]. This mechanism is used by the DNA structure checkpoint to prevent mitosis in the presence of unreplicated or damaged DNA [18,19].

Although CDK activity is known to be essential for cell cycle control, the mechanism coupling cell division with growth has been little understood. In fission yeast, there are two cell size controls, at G_1/S and G_2/M, respectively [20,21]. The *wee1*$^+$ gene was originally identified as a genetic element of mitotic size control [15]. Previously, the *cdc25* mutant was isolated as an allosuppressor increasing the efficiency of weakly suppressing *sup* alleles, and the allosuppressor phenotype was eliminated by the *wee1* mutation [22]. This result suggested that the G_2 delay is important for the cell growth in the perturbation of protein synthesis, and Wee1 is important for this mechanism. It has been suggested that, in *Δrum1wee1-50* double mutant, there is no size control either in G_1/S or in G_2/M [23]. However, the relationship between the negative cell cycle regulators and protein synthesis remains to be clarified.

It has been shown that mutations of translation initiation factors cause cell cycle arrest in G_1 in budding yeast [24,25], and in G_1 and G_2 in fission yeast [26,27]. The G_1-cyclin Cln3 in budding yeast or the B-type cyclins (Cig2 and Cdc13) and the phosphatase Cdc25 in fission yeast are candidates to be involved in a mechanism coupling growth with cell division through a rate-limiting initiation of translation of their mRNA [26–29]. In one of the fission yeast mutants defective in the translation initiation factor, the general protein synthesis was reduced by 80% [27]. In *Drosophila* and humans, it has been demonstrated that Cdc25 and cyclins are important for the coordination of growth and cell division [30,31]. These results indicate that the positive regulators of the cell cycle are rate-limiting factors upon serious inhibition of protein synthesis.

Previously, the effect of protein synthesis inhibition on cell cycle progression was examined [32], and it was shown that a transient cycloheximide (CYH) treatment (100 μg/ml) partially synchronized mitosis, division, and DNA synthesis. In this report, we investigated the relationship between Wee1 and protein synthesis inhibition. We found that the *wee1* mutant shows supersensitivity to CYH. Wee1 was essential for the G_2 delay induced by a partial inhibition of protein synthesis. Finally, we demonstrated that Wee1 is up-regulated upon the protein synthesis inhibition.

2. Materials and methods

2.1. Yeast strains and general methods

The following *S. pombe* strains were used: 972 (*h*$^-$), DH157-1A (*h*$^+$ *wee1-50*), #136 (*ura4 Δrum1::ura4*$^+$), GL192 (*h*$^+$ *cdc2-3w Δcdc25::ura4*$^+$), IY1045 (*h*$^-$*leu1 ura4 ade6 Δnim1::LEU2*), cdc10 (*h*$^-$ *cdc10-129*), cdc25 (*h*$^+$ *cdc25-22*), PN1076 (*h*$^-$ *leu1 ura4 ade6-M216 Δcig2::ura4*$^+$), PN117 (*h*$^-$ *cdc13-117*), JM1160 (*h*$^+$ *leu1 ura4 ade6 Δsty1::ura4*$^+$), DH278-10A (*h*$^-$ *wee1*$^+$*:HA:ura4*$^+$ *cdc25*$^+$*:*

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13Myc:kan^r), and DH249-1B (*h*$^-$ *leu1 ura4 Δsty1::ura4*$^+$ *wee1*$^+$*:HA:ura4*).

2.2. General genetic and biological methods

We used general genetic and biological methods for the study of fission yeast [33]. DNA contents of cells were examined by FACS analysis (Becton-Dickinson FACSCalibur). Thirteen copies of the human c-myc (Myc) epitope were integrated into the genome in front of the terminator of the *cdc25*$^+$ gene by a PCR-based gene targeting method [34]. The rate of [^{35}S]methionine incorporation into protein was determined as described [29].

2.3. Cytological techniques

Cytological techniques were performed as described [35–39]. Calcofluor white (Sigma) was used to monitor cell wall growth, and DAPI (4′,6-diamidino-2-phenylindole)(Sigma), for observing the chromatin region.

2.4. Western and Northern blot analysis

Preparation of cell extracts and Western blots were performed as detailed earlier [36,37]. For detection of HA-tagged protein, Cdc13, Cdc2, tyrosine-15-phosphorylated Cdc2, and Cdc25-Myc, anti-HA (HA11; BAbCO), anti-Cdc13 (provided by Dr. P. Nurse), anti-PSTAIR (provided by Dr. M. Yamashita), anti-phospho-Y15-Cdc2 (#9111S; BioLabs), and anti-Myc (9E10; BAbCO) antibodies, respectively, were used as primary antibodies. Horseradish peroxidase-conjugated sheep anti-mouse IgG (NA931, Amersham) or donkey anti-rabbit IgG (NA934, Amersham) as secondary antibodies and a chemiluminescence system (ECL, Amersham) were used to detect bound primary antibodies. Total RNA was isolated by the hot-phenol method, and then subjected to Northern blot analysis.

3. Results and discussion

3.1. Wee1 is important for the G_2 delay upon protein synthesis inhibition

To investigate the relationship between protein synthesis and cell cycle regulators, we examined the effect of the inhibition of the peptide elongation step in protein synthesis by the inhibitor CYH on the cell growth of the mutants defective in cell cycle regulators (Fig. 1A). The *wee1-50* mutant showed supersensitivity to CYH at the permissive temperature 30°C, whereas other mutants defective in the CDK inhibitor Rum1, the transcription factor Cdc10, the mitotic inducer Cdc25 phosphatase, and B-type cyclins (Cig2 and Cdc13) were insensitive to CYH. Further, the *Δnim1* mutant, in which Wee1 is constitutively active, was insensitive to CYH. These results indicate that Wee1 is important for the cell growth upon protein synthesis inhibition. To confirm that Wee1 plays a major role in the cell growth upon protein synthesis inhibition, we used the *cdc2-3wΔcdc25* double mutant. The *cdc2-3w* mutation is a dominant active allele that relieves the requirement for Cdc25 but leaves Cdc2 responsive to Wee1 [40]. This mutant was also insensitive to CYH.

Next, to investigate the relationship between cell growth and protein synthesis inhibition, we examined the effect of various concentrations of CYH on cell growth, total protein synthesis, and cell morphology of wild-type cells grown in EMM minimal medium at 30°C. In the presence of CYH, the percentage of unseptated cells growing in a monopolar manner increased, whereas that of septated cells decreased (Fig. 1B). A 5 μg/ml concentration of CYH, which inhibited the rate of total protein synthesis of cells growing in EMM medium by 50%, was the most effective for the accumulation of the unseptated monopolar cells (Fig. 1B). Higher concentrations (up to 50 μg/ml) of CYH, which inhibited the rate of total protein synthesis by 80%, resulted in random arrest of the cell cycle (Fig. 1B). In the case of YPD rich medium, we examined the effect of various concentrations of CYH (1–100 μg/ml) on cell growth and found that a 10 μg/ml concentration of CYH was the most effective for the accumulation of the unseptated monopolar cells. In the presence of this concentration of CYH in YPD medium, unseptated monopolar cells increased, whereas septated cells decreased (Fig. 1C), as in the case of EMM medium containing 5 μg/ml CYH. The cell length of the septated cells (cell size) increased by 15% in the presence of CYH, compared with that in the absence of CYH (Fig. 1D). To investigate if a partial inhibition of protein synthesis would induce a delay in G_2 phase, we examined yeast cells growing in the presence of CYH for their DNA content by flow-cytometry analysis (FACS), for their chromatin region by staining with DAPI, and for their tubulin structure by using TAT1 antibody. The unseptated cells had one hemispherical nucleus with two copies of the DNA content and cytoplasmic microtubules (Fig. 1E,F), which are characteristic of the G_2 delay. Another inhibitor of the peptide elongation step, anisomycin, had an effect similar to that of CYH (data not shown). These results indicate that a partial inhibition of protein synthesis in exponentially growing cells caused a delay in G_2 phase.

Further, to investigate if Wee1 is important for the G_2 delay upon protein synthesis inhibition, we examined the septation index, the cell size, and the tubulin structure of the *wee1-50* mutant cells grown in the presence of CYH at the permissive temperature 30°C. In the presence of CYH, the decrease in septation index and the increase in cell size observed in wild-type cells did not occur in the *wee1-50* mutant cells (Fig. 1C,D), and mitotic cells and abnormally septated small cells were observed in the case of the mutant (Fig. 1F). These results suggested that Wee1 is important for the G_2 delay upon a partial inhibition of protein synthesis. To confirm that Wee1 plays a major role in the CYH-induced G_2 delay, we examined the effect of CYH on the cell growth of the *cdc2-3wΔcdc25* double mutant. In this mutant, the increase in the unseptated monopolar cells and the decrease in the septated cells were observed as in the case of wild-type cells (Fig. 1C).

In the presence of CYH, a small population of G_1 cells was observed in the *wee1-50* mutant cells (Fig. 1E). This result suggested that the protein synthesis inhibition in the mutant cells induced G_1 delay. The G_1 regulation might be important for the cell growth of the mutant upon protein synthesis inhibition.

3.2. Wee1 is positively regulated upon protein synthesis inhibition

To investigate if Wee1 is positively regulated upon a partial inhibition of protein synthesis, we examined the Wee1 protein level in the presence of 10 μg/ml CYH. Since the positive regulators of Cdc2, Cdc13 and Cdc25, have been shown to be rate-limiting factors upon serious protein synthesis inhibition caused by the mutation of translational initiation factor [26–29], we examined the levels of Cdc13 and Cdc25 proteins. The Wee1 protein level increased three-fold in the presence of CYH (Fig. 2), whereas the levels of Cdc13 and Cdc25 proteins did not change under this condition (Fig. 2). Consistent with the increase in Wee1 protein level, the phosphorylation level of Cdc2 on the tyrosine-15 also increased in the presence of CYH (Fig. 2).

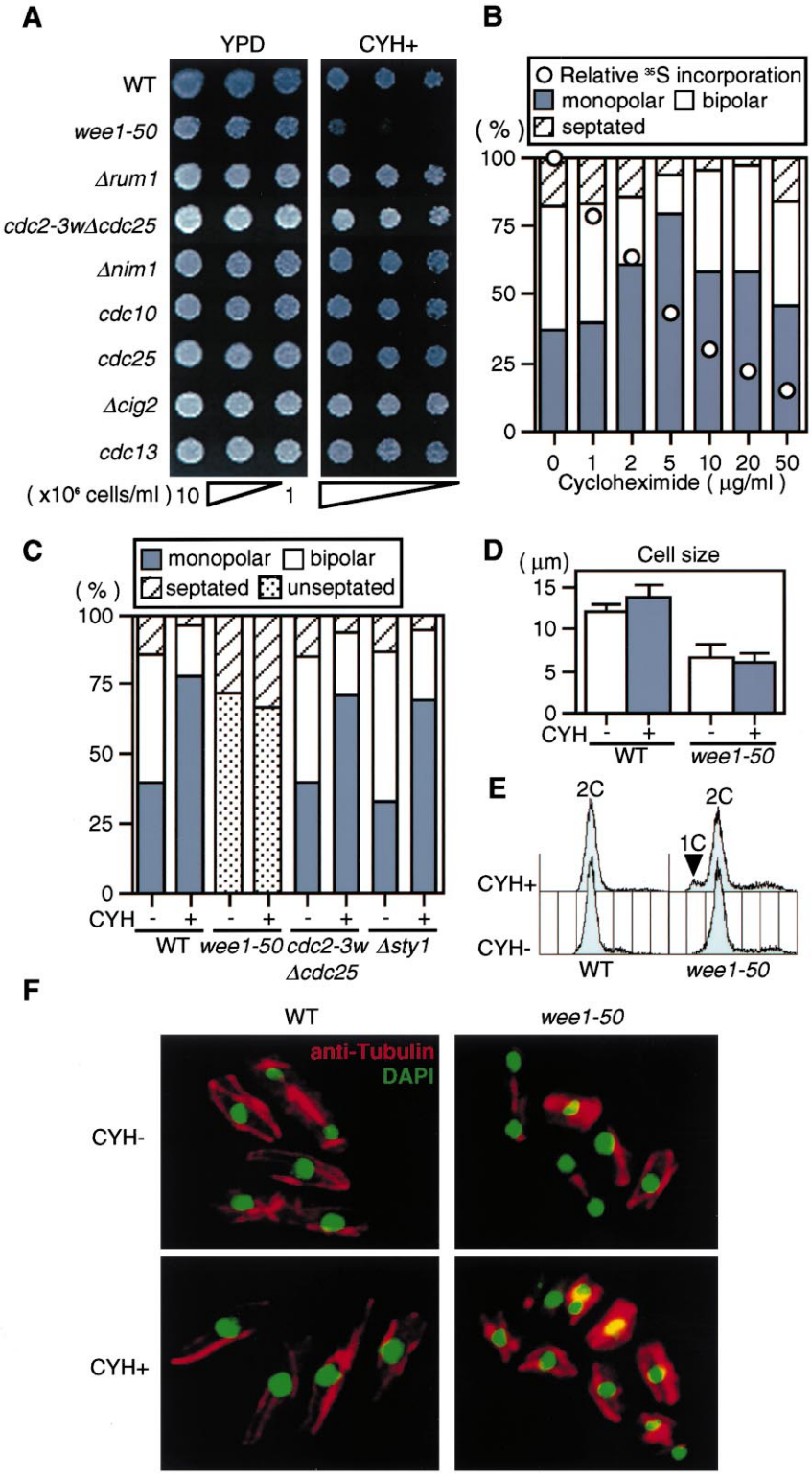

Fig. 1. Wee1 is important for the cell growth upon protein synthesis inhibition. A: Wild-type (972), *wee1-50* (DH157-1A), *Δrum1* (#136), *cdc2-3wΔcdc25* (GL192), *Δnim1* (IY1045), *cdc10* (cdc10-129), *cdc25* (cdc25-22), *Δcig2* (PN1076), and *cdc13* (PN117) mutant cells cultured in YPD medium were serially diluted (10–1×10^6 cells/ml), spotted onto YPD solid medium containing 5 μg/ml CYH, and cultured at 30°C for 3 days. B: Wild-type cells (972) grown to early-log phase (3–4×10^6 cells/ml) in EMM medium were treated with various concentrations of CYH, collected for [^{35}S]methionine incorporation measurements, and observed for cell morphology. The value of the incorporation in EMM without CYH was expressed as 100%. C: Either CYH (final concentration, 10 μg/ml) (CYH+) or carrier EtOH alone (CYH−) was added to cultures of wild-type (972), *wee1-50* (DH157-1A), *cdc2-3wΔcdc25* (GL192), and *Δsty1* (JM1160) cells grown to early-log phase (3–4×10^6 cells/ml) in YPD medium at 30°C. After a 4 h incubation at 30°C, the cells of the CYH+ and CYH− cultures were taken for the observation of the cell morphology. As the *wee1-50* mutant cells are smaller than the wild-type cells, it is hard to distinguish monopolar from bipolar in the unseptated cells. D–F: The wild-type (972) and the *wee1-50* (DH157-1A) cells of the same cultures taken in (C) were used for the measurement of the cell length of the septated cells (cell size) (D), FACS analysis (E), staining with TAT1 (anti-Tubulin, red) and DAPI (green) (F).

Next, to investigate if the expression of the *wee1*$^+$ gene is regulated at the transcriptional level, we examined the *wee1*$^+$ mRNA level in the presence of CYH by Northern blot analysis. In the presence of CYH, the levels of *wee1*$^+$ mRNA increased substantially (Fig. 3A). In contrast, the levels of *cdc2*$^+$ and *cdc13*$^+$ mRNAs were unchanged (data not shown).

3.3. The Wee1 protein increases by the Sty1-dependent transcriptional and the Sty1-independent post-transcriptional regulations upon protein synthesis inhibition

As Sty1/Spc1 MAPK is activated by a range of stimuli including the protein synthesis inhibitor anisomycin [41,42], we examined if the induction of *wee1*$^+$ mRNA and the increase in Wee1 protein in the presence of CYH are mediated by the Sty1/Spc1-MAPK pathway. In the *Δsty1* mutant cells, the induction of *wee1*$^+$ mRNA was completely abolished (Fig. 3A), but increase in the Wee1 protein was observed (Fig. 3B). Further, the increase in the monopolar cells and the decrease in the septated cells were also observed in this mutant (Fig. 1C). These results suggested that Wee1 protein is positively regulated at the transcriptional and the post-transcriptional levels in response to protein synthesis inhibition, and that the Sty1 MAPK is required for the transcriptional regulation. It is also indicated that the Sty1-independent post-transcriptional regulation of Wee1 is important for the CYH-induced G_2 delay. The post-transcriptional regulation remains to be clarified. The basal level of Wee1 protein in the *Δsty1* mutant was lower than that in wild-type cells (Fig. 3B, compare WT CYH− and *Δsty1* CYH−), suggesting that the Sty1/Spc1 MAPK might be involved in the regulation of Wee1 protein level under normal conditions.

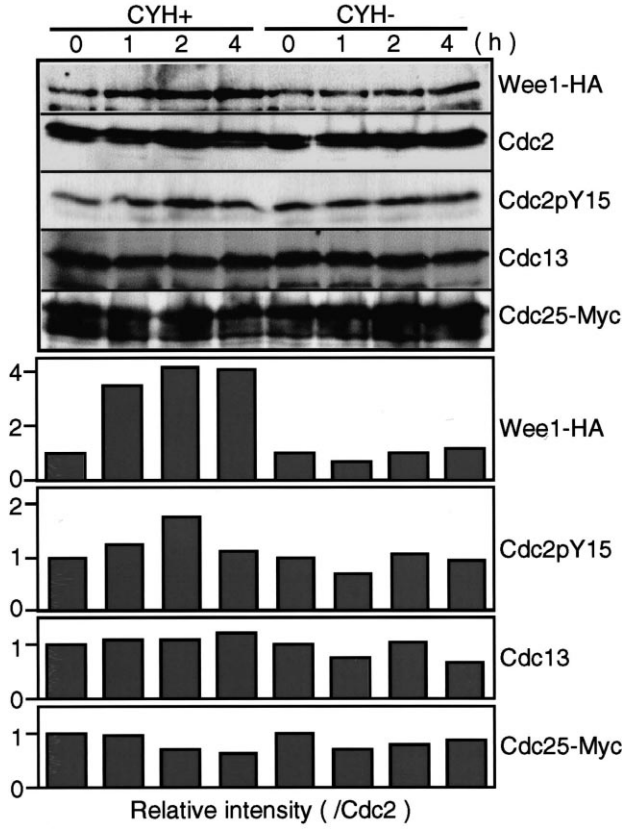

Fig. 2. Wee1 protein increases upon protein synthesis inhibition. Either CYH (final concentration, 10 μg/ml; CYH+) or carrier EtOH alone (CYH−) was added to cultures of wild-type cells having *wee1*$^+$*:HA* and *cdc25*$^+$*:13Myc* (DH278-10A) grown to early-log phase (3–4×10^6 cells/ml) in YPD medium at 30°C. After the addition of CYH, the cells of the CYH+ and CYH− cultures were taken at the indicated times. The total proteins (50 μg) were run on SDS–polyacrylamide gels. The Wee1-HA protein, the tyrosine phosphorylation of Cdc2, the Cdc13 protein, and the Cdc25-Myc protein in the samples were analysed by Western blotting with antibodies specific for the HA epitope (Wee1-HA), Cdc2 phosphorylated on tyrosine-15 (Cdc2pY15), Cdc13, and the Myc epitope (Cdc25-Myc), respectively. The blots were reprobed with an anti-PSTAIR antibody to visualise the total amount of Cdc2. The intensity of each band was quantified by the Intelligent Quantifier (Bio Image).

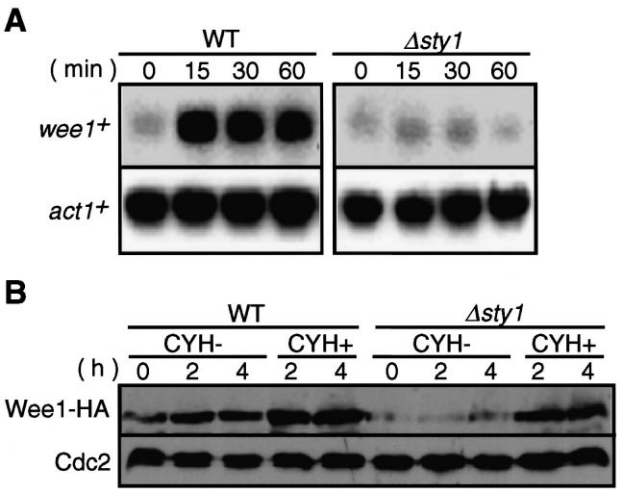

Fig. 3. The Wee1 protein level increases by Sty1-dependent transcriptional and Sty1-independent post-translational regulations. A: Wild-type (972) and *Δsty1* mutant cells (JM1160) grown to early-log phase (3–4×10^6 cells/ml) in YPD were treated with CYH (10 μg/ml). The levels of *wee1*$^+$ and *act1*$^+$ mRNAs at the indicated time were determined by Northern blotting. B: Either CYH (final concentration, 10 μg/ml; CYH+) or carrier EtOH alone (CYH−) was added to the cultures of wild-type cells having *wee1*$^+$*:HA* (DH278-10A) and the *Δsty1* mutant having *wee1*$^+$*:HA* (DH249-1B) grown to early-log phase (3–4×10^6 cells/ml) in YPD medium at 30°C. The cells of the cultures were taken at the indicated times. The total proteins (50 μg) were run on SDS–polyacrylamide gels. The Wee1-HA protein in the samples was analysed by Western blotting.

Here we demonstrated that fission yeast cells have a mechanism inducing G_2 delay in response to a partial inhibition of protein synthesis (50% reduction in general protein synthesis), by positively regulating the negative cell-cycle regulator, Wee1. It has been shown that the positive regulators of the cell cycle are rate-limiting factors upon serious inhibition of protein synthesis [26–29]. Upon protein synthesis inhibition, cells would have two mechanisms inducing the cell-cycle delay, i.e. by positively regulating the negative cell-cycle regulators and by negatively regulating the positive regulators.

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