Cold-inducible expression of the cell division cycle gene CDC48 and its promotion of cell proliferation during cold acclimation in zebrafish cells[1]

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Abstract A member of the ATPases associated with diverse cellular activities (AAA) family, the cell division cycle gene CDC48/VCP (valosin-containing protein)/p97, was cloned from zebrafish and found to be a major cold-inducible protein in fish cells. CDC48 mRNA levels increased significantly after reducing the temperature from 30 to 15°C for 25 days. CDC48 protein levels also increased 2.5-fold after 30 days at cold temperatures. When fish cells overexpressing CDC48 were exposed to a temperature of 15°C, cell proliferation was markedly enhanced in comparison with control cells. By contrast, expression of a mutant molecule with a tyrosine-805 to alanine substitution at the C-terminal phosphorylation site inhibited cell proliferation and induced apoptosis at low temperatures. Therefore, CDC48 may promote cell cycling and cell proliferation via C-terminal tyrosine phosphorylation during cold acclimation in fish cells.

Key words: CDC48; ATPases associated with diverse cellular activities; Cold acclimation; Zebrafish; Cell proliferation; Tyrosine phosphorylation

1. Introduction

Teleost fish possess cellular mechanisms that allow adaptation to a wide range of thermal environments [1,2]. Studies of biochemical adaptation to low temperatures have examined alterations in isoenzymes, such as acetylcholinesterase [3], lactate dehydrogenase [4,5], and myosin [6,7]. Such chemical responses are thought to be due to metabolic compensation in enzyme functions for the delay in various cellular responses at low temperatures. The enhanced desaturation of membrane phospholipids has also been found to offset cold rigidified membranes [8]. In cultured fish cells, ferritin H genes are transcriptionally induced during cold acclimation [9]. Therefore, cold acclimation is mediated by alteration in the mRNA and proteins present in cold-treated cells, and these alterations may directly affect cell proliferation and cell cycle progression. Therefore, elucidating the intracellular functions of cold-regulated genes is important for understanding the cold acclimation mechanism.

Our previous work using two-dimensional gel electrophoresis demonstrated that the major cold-inducible protein in cold-treated fish cells is a homolog of the mammalian valosin-containing protein (VCP) and the yeast cell division cycle gene (CDC) 48 protein [10]. Although the level of this protein increases with several days of cold treatment, it decreases markedly within one day when cells are returned to warm conditions. Nevertheless, the temperature-dependent regulation and cellular functions of this protein under cold conditions have not been characterized.

CDC48 is a member of the hexameric ATPases associated with diverse cellular activities (AAA) family [11–13]. Yeast mutants lacking the CDC48 gene have arrested cell division and abnormal spindles at temperatures of 10–15°C [11], and a mutation that results in loss of CDC48 ATPase activity causes an apoptotic phenotype in mouse cells [14]. CDC48 is thought to participate in the fusion of endoplasmic reticulum (ER) membranes during cell cycle progression [15–17] and protein degradation in the ER [18–25]. Tyrosine phosphorylation was suggested to regulate the function of CDC48/VCP/polypeptide of M_r 97 kDa (p97) in cell cycle progression [26–29]. Based on these studies, CDC48 appears to have essential roles in cell proliferation and cell cycle progression.

This study examined the cold-inducible expression of CDC48 mRNA and protein in cold-treated fish cells and the role of CDC48 in fish cells during cold acclimation. CDC48 cDNA was cloned, and cold-inducible gene expression was characterized in cultured zebrafish embryonic cells. The promotion of cell growth by CDC48 overexpression indicates that CDC48 is necessary for cell proliferation in the cold.

2. Materials and methods

2.1. Cell culture

A zebrafish embryo-derived cultured cell line (ZE cells) was maintained with Leibovitz's L-15 medium (Life Technologies) containing

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[1] The nucleotide sequence reported in this paper has been submitted to the DDBJ/EMBL/GenBank database with accession number AB093594.

Abbreviations: AAA, ATPases associated with diverse cellular activities; CDC48, cell division cycle gene CDC48; VCP, valosin-containing protein; p97, polypeptide of M_r 97 kDa; ER, endoplasmic reticulum; ZE cells, zebrafish embryo-derived cultured cell line; FBS, fetal bovine serum; CMV, human cytomegalovirus; BrdU, 5-bromo-2′-deoxyuridine; DAPI, 4′,6′-diamidine-2-phenylindole; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP end labeling

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5% fetal bovine serum (FBS; JRH Biosciences), and an antibiotic mixture of 100 units ml^{-1} penicillin G, 100 μg ml^{-1} streptomycin, and 0.25 μg ml^{-1} amphotericin B (Life Technologies) at 30°C.

2.2. Isolation of cDNA clones

Zebrafish embryos at the 24-h stage after fertilization were lysed in guanidium thiocyanate, and total RNA was harvested by pelleting through cesium chloride [30]. Poly(A) RNA was purified with oligo(dT)-resin (Oligotex, Takara). Double-stranded cDNA was prepared with oligo(dT) primer and ligated to a Marathon cDNA adaptor using a Marathon cDNA Amplification Kit (Clontech). To isolate a partial cDNA sequence of zebrafish CDC48, polymerase chain reaction (PCR) was performed with degenerate oligonucleotide primers with the following sequences: the 24-mer 5′ oligonucleotide set 5′-TGAYACRRTSGARGGYATMACTGG-3′, corresponding to the amino acid sequence homologous to yeast and mouse CDC48, (D/A)DT(M/V)EGITG [residues 120–128 of mammalian VCP (26)]; the 25-mer 3′ oligonucleotide set 5′-GCWCCRGTYTCATTNGCMACAGCTC-3′, corresponding to the consensus amino acid sequence RAVANETGA [residues 256–264 of mammalian VCP (26)], where Y = C+T, R = A+G, S = G+C, M = C+A, W = A+T, N = A+G+C+T. PCR was performed with an *Ex*Taq PCR Reaction Kit (Takara) using 10 ng of zebrafish 1-day embryo cDNA template and 1 ng of each sense and antisense primer in a total volume of 50 μl per sample. The samples were subjected to 35 amplification cycles (20 s at 94°C and 30 s at 65°C). The amplified 300-bp DNA fragment was purified after excision from an agarose gel, cloned into the *Eco*RV site of pT7Blue vector (Novagen), and sequenced.

To analyze the full-length sequence of the zebrafish gene, 5′- and 3′-rapid amplification of cDNA ends was performed with a Marathon cDNA Amplification Kit (Clontech). The sense (5′-GGCTTACAGGCCAATCCGTAAAGGT-3′, nucleotides 435–459) and antisense (5′-GAGGCTTCACACCAATAGCCTTGAA-3′, nucleotides 699–724) primers were used to amplify the 5′ or 3′ end of the zebrafish cDNA, respectively, with PCR with adapter primer AP1 from the kit (Clontech). PCR was performed according to the manufacturer's instructions using 10 ng of zebrafish 1-day embryo cDNA template and 1 ng of each primer in a total volume of 50 μl per sample. The samples were subjected to 40 amplification cycles (20 s at 94°C and 120 s at 70°C). The amplified DNA fragment was purified after excision from an agarose gel, cloned into the *Eco*RV site of pT7Blue-T vector (Novagen), and sequenced. To construct an expression vector, zebrafish CDC48 cDNA was amplified by PCR with a sense primer (5′-CCCGAATTCGGGAGAAGCAATATGGCTTCG-3′) containing an *Eco*RI site and an antisense primer (5′-GCGTCTAGATCCGTAAAGATCATCATCGTT-3′) containing an *Xba*I site. The cDNA for the CDC48 Tyr^{805} to alanine (Y805A) substitution mutant was amplified by PCR using a sense primer (5′-CCCGAATTCGGGAGAAGCAATATGGCTTCG-3′) containing an *Eco*RI site and an antisense primer (5′-GCGTCTAGATCCGGCAAGATCATCATCGTT-3′) containing an *Xba*I site. PCR was performed according to the manufacturer's instructions using 10 ng of cDNA template, and 1 ng

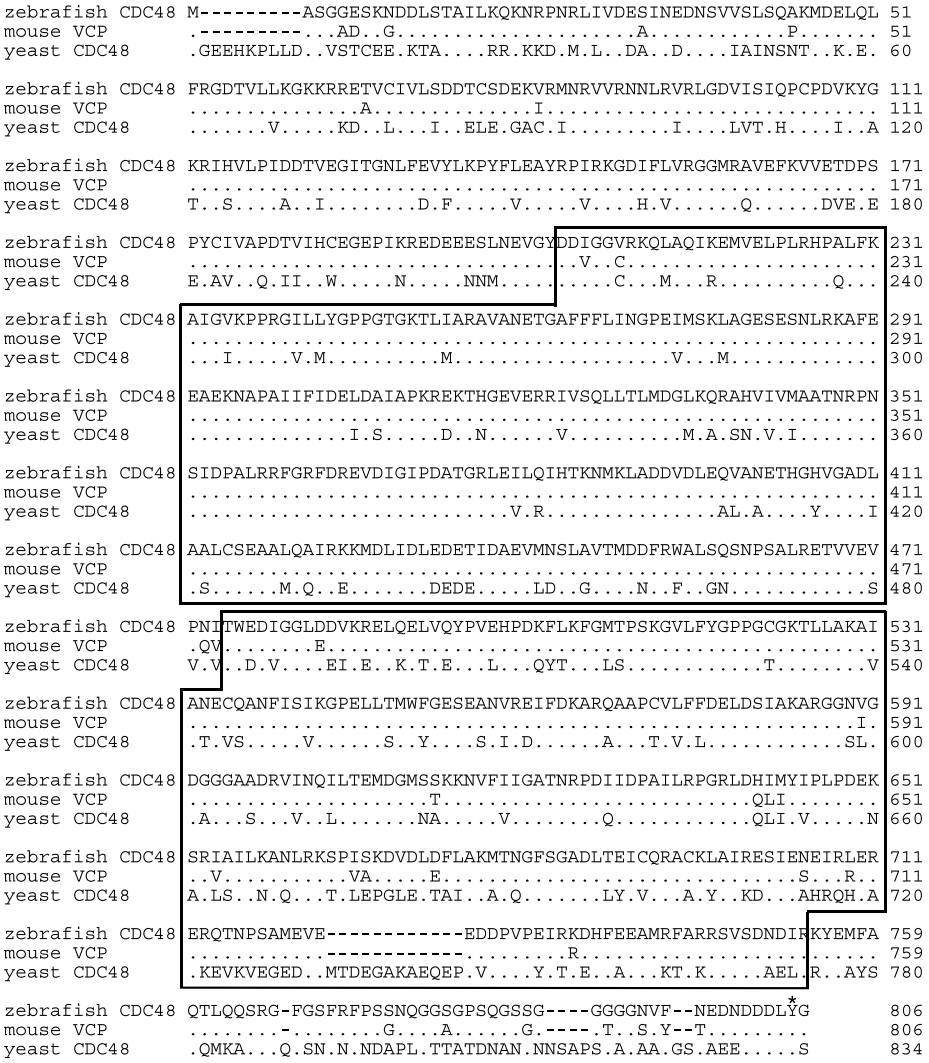

Fig. 1. Alignment of the deduced amino acid sequences of zebrafish CDC48, mouse VCP, and yeast CDC48. CDC48 cDNA encoded a protein of 806 amino acids with an M_r of 97 kDa that duplicated the ATPase binding domains (boxed regions) containing a nucleotide binding consensus sequence. The asterisk marks a possible tyrosine phosphorylation site. Identical residues in two or more sequences are indicated with dots.

of each primer in a total volume of 40 μl per sample. The samples were subjected to 30 amplification cycles (30 s at 94°C, 30 s at 58°C, and 180 s at 72°C). The amplified DNA fragment was purified after excision from an agarose gel, and subcloned into the *Eco*RI and *Xba*I sites of the p3×FLAG-CMV-14 Expression Vector (Sigma), downstream from the human cytomegalovirus (CMV) promoter, to fuse a FLAG tag sequence to the C-terminus of the open reading frame (ORF).

2.3. Sequence analysis

Nucleotide sequences were determined with a DNA sequencer (ABI Model 373, Perkin Elmer) and a Thermo Sequence II Dye Terminator Cycle Sequencing Kit (Amersham).

2.4. Immunoblotting

To characterize the cold induction of zebrafish CDC48, the culture temperature of ZE cells was reduced from 30°C to 12, 15, 17, 20, or 25°C, and maintained for 5, 10, 15, 20, 25, 30, 35, or 40 days. The level of CDC48 protein in cold-treated ZE cells was examined by Western blot analysis. A rabbit polyclonal antibody against CDC48 was raised against a synthetic peptide, FLEAYRPIRKGDIFL-VRGGM, corresponding to 20 amino acid residues in the zebrafish CDC48 sequence (residues 138–158). Cells were harvested and lysed with sodium dodecyl sulfate (SDS) buffer [50 mM Tris–HCl, pH 6.8, containing 10% glycerol, 2% SDS, 2% 2-mercaptoethanol, 2% bromophenol blue, and protease inhibitors mix (Roche)]. Cell lysate aliquots (10 μg) were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) [31]. The separated proteins were transferred to a nitrocellulose membrane, and signals were detected with primary antibody and anti-rabbit IgG conjugated with horseradish peroxidase (Amersham) by developing with the Enhanced Chemiluminescence Reagent (ECL, Amersham). Protein bands were stained with Coomassie Brilliant Blue R250 in control experiments. FLAG-tagged CDC48 and CDC48-Y805A mutant proteins expressed in ZE cells were detected by Western blot analysis with an anti-FLAG monoclonal antibody (M2, Sigma).

2.5. Northern blotting

Total RNA was extracted from ZE cells using Trizol (Life Technologies). Each RNA aliquot (8 μg) was electrophoresed on a 0.8% agarose/formaldehyde gel and transferred to a Hybond-N^+ nylon membrane (Amersham). The blot was hybridized with a ^{32}P-labeled probe corresponding to a 2.4-kb zebrafish CDC48 cDNA fragment that was prepared by the random primer method (*Bca*BEST DNA Labeling Kit, Takara) at 60°C in hybridization solution (Rapid-Hyb Buffer, Amersham), according to the manufacturer's instructions. A 28S ribosomal RNA probe was used as an internal control.

2.6. Gene transfection

The FLAG-tagged CDC48 cDNA constructs for wild-type CDC48 and a mutant with a Y805A, and mock vector were transfected into ZE cells by electroporation [32]. The cells were harvested and resuspended in Leibovitz's L-15 medium without FBS. The cell suspension containing the vectors (20 μg) was added to a 0.4-cm gap cuvette, and transfected with an electroporator (Electroporator II, Invitrogen) at a capacitance of 1000 μF.

2.7. Cell proliferation assay

Exponentially growing (< 70% confluence) cells were harvested and plated in triplicate on 12-well plates (Costar) at a density of 8.0×10^4 cells/well. The cells were incubated overnight at 30°C, and then the culture temperature was reduced from 30 to 15°C and maintained at 15°C for 4 days. After the cells were suspended by trypsinization, they were counted with a hemacytometer.

2.8. BrdU labeling and immunofluorescence analysis of DNA synthesis

DNA synthesis was monitored by measuring the incorporation of the artificial thymidine nucleotide analog 5-bromo-2′-deoxyuridine (BrdU) (Sigma) into newly synthesized DNA. Cells (1×10^5 cells/wells) were plated on glass coverslips in 12-well culture plates. After incubation for the first 24 h in complete medium without BrdU, the medium was replaced with medium containing 10 μM BrdU and the cells were incubated for an additional 24 h for BrdU incorporation. After fixation in methanol for 15 min at −20°C, and denaturation of DNA with 2 N HCl for 30 min at room temperature, the cells were stained with an anti-BrdU mouse monoclonal antibody (Sigma) and fluorescein-conjugated anti-mouse IgG (Molecular Probes), and counterstained with 4′,6′-diamidine-2-phenylindole (DAPI).

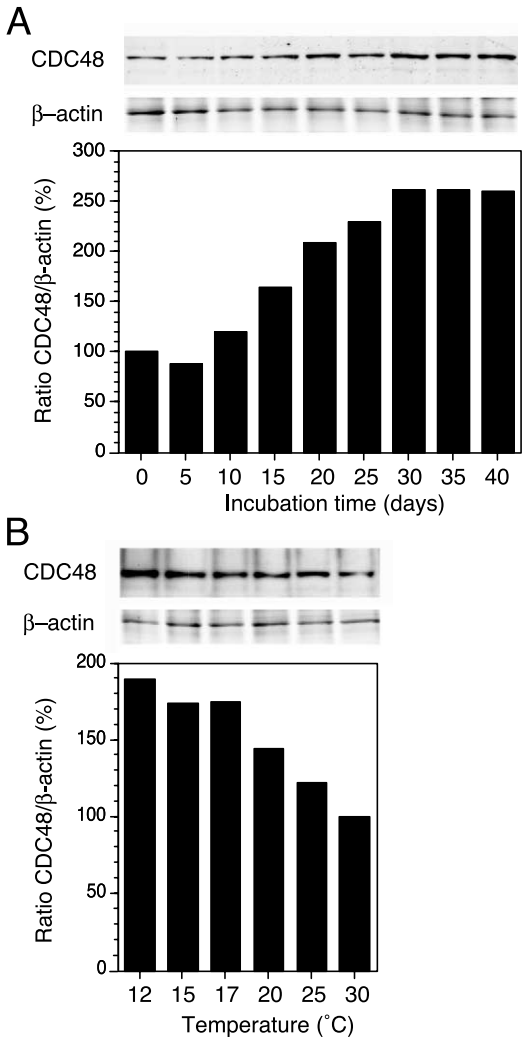

Fig. 2. Induction of CDC48 in cold-treated ZE cells. The levels of CDC48 protein in cold-treated ZE cells were examined by Western blot analysis. A: The ZE cell culture temperature was reduced from 30 to 15°C, and cells were incubated for 5, 10, 15, 20, 25, 30, 35, or 40 days at 15°C. B: The culture temperature was reduced from 30°C to 12, 15, 17, 20, or 25°C, and maintained at each temperature for 25 days. Cell lysates were prepared and subjected to SDS–PAGE. After transfer of separated proteins to nitrocellulose membranes, the blots were stained with anti-CDC48 antibody. β-Actin stained with Coomassie Brilliant Blue R250 was used as an internal control. The relative expression levels of CDC48 protein/β-actin were calculated by densitometry.

2.9. TUNEL assay for microscopic analysis of apoptosis

Apoptosis was observed using a terminal deoxynucleotidyl transferase-mediated dUTP end labeling (TUNEL) assay. Cells were fixed in 4% paraformaldehyde–PBS (phosphate-buffered saline) for 25 min and stained using the DeadEnd colorimetric TUNEL system (Promega).

3. Results

The full-length sequence of zebrafish CDC48 cDNA was isolated from a cDNA library of zebrafish 1-day embryos. This clone had a 2418-bp ORF that encoded a protein of 806 amino acid residues with a M_r of 97 kDa (Fig. 1). A

FASTA search of the SwissProt/PIR protein sequence database [33] revealed that the amino acid sequence showed 96% and 69% identity with mouse VCP [26] and yeast CDC48 [11], respectively. The AAA domains were duplicated from Val^{201} to Val^{469} and Thr^{474} to Tyr^{755}, and contained the Walker A and B motifs, characteristic of the nucleotide binding consensus sequence. These residues are highly conserved in zebrafish CDC48, mouse VCP, and yeast CDC48. The Tyr^{805} residue of fish CDC48 was conserved as the major site of tyrosine phosphorylation, as in other known CDC48 proteins [26]. Therefore, the isolated cDNA clone was found to encode zebrafish CDC48, a member of the AAA family.

To characterize the cold induction of CDC48, antiserum was raised against a synthetic peptide corresponding to 20 residues of the amino acid sequence (residues 138–158) of zebrafish CDC48. This antiserum specifically immunoblotted a 97-kDa protein band in cell lysates, which corresponded to CDC48 by Western blotting (Fig. 2A). CDC48 induction in ZE cells started to increase after the first 10 days following temperature reduction from 30 to 15°C, and remained at high levels during the 30-day acclimation period. Thirty days after the reduction to 15°C, the CDC48 protein level was 2.5 times higher than the level in control cells at 30°C. Furthermore, CDC48 protein was induced in ZE cells at a range of cold-

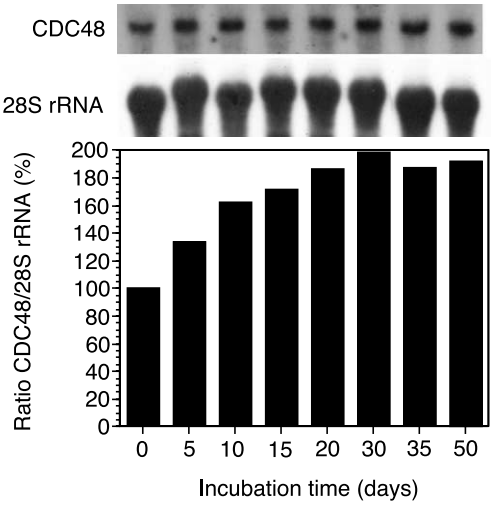

Fig. 3. Expression of CDC48 in cold-treated ZE cells. The culture temperature was reduced from 30 to 15°C, and cells were incubated for 5, 10, 15, 20, 30, 35, or 50 days. Total RNA was isolated from the cold-treated cells and subjected to Northern blot analysis. Membranes were hybridized with a ^{32}P-labeled full-length CDC48 cDNA fragment as a probe. A 28S ribosomal RNA probe was used as an internal control.

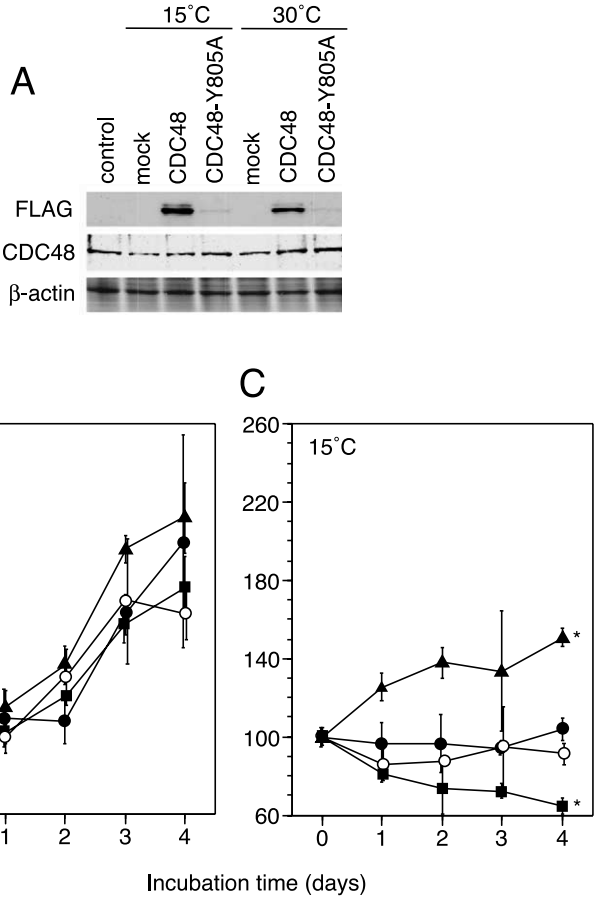

Fig. 4. Effect of CDC48 overexpression on cell proliferation at low temperatures. After transfection with cDNA constructs for CDC48 and CDC48-Y805A, the expression of the transgene and cell proliferation were examined. Transfected cells were preincubated at 30°C for one day, and then the culture was incubated at 15°C for four days, or left at 30°C as a control. The transfected cells were harvested 2 days after the temperature shift, and induction was analyzed by Western blotting using anti-FLAG antibody and anti-CDC48 antibody (A). Viable cell numbers are given as percentage of initial cell numbers (B,C). Each value represents the mean ± S.D. of triplicate measurements performed on a 12-well plate. Asterisks denote significant differences in the cell numbers in the cells overexpressing CDC48 and mutant CDC48 as compared with the mock cells by repeated measure ANOVA ($P < 0.05$). Open circles, non-transfected cells; solid circles, mock vector; solid triangles, cells overexpressing CDC48; solid squares, cells overexpressing CDC48 mutant.

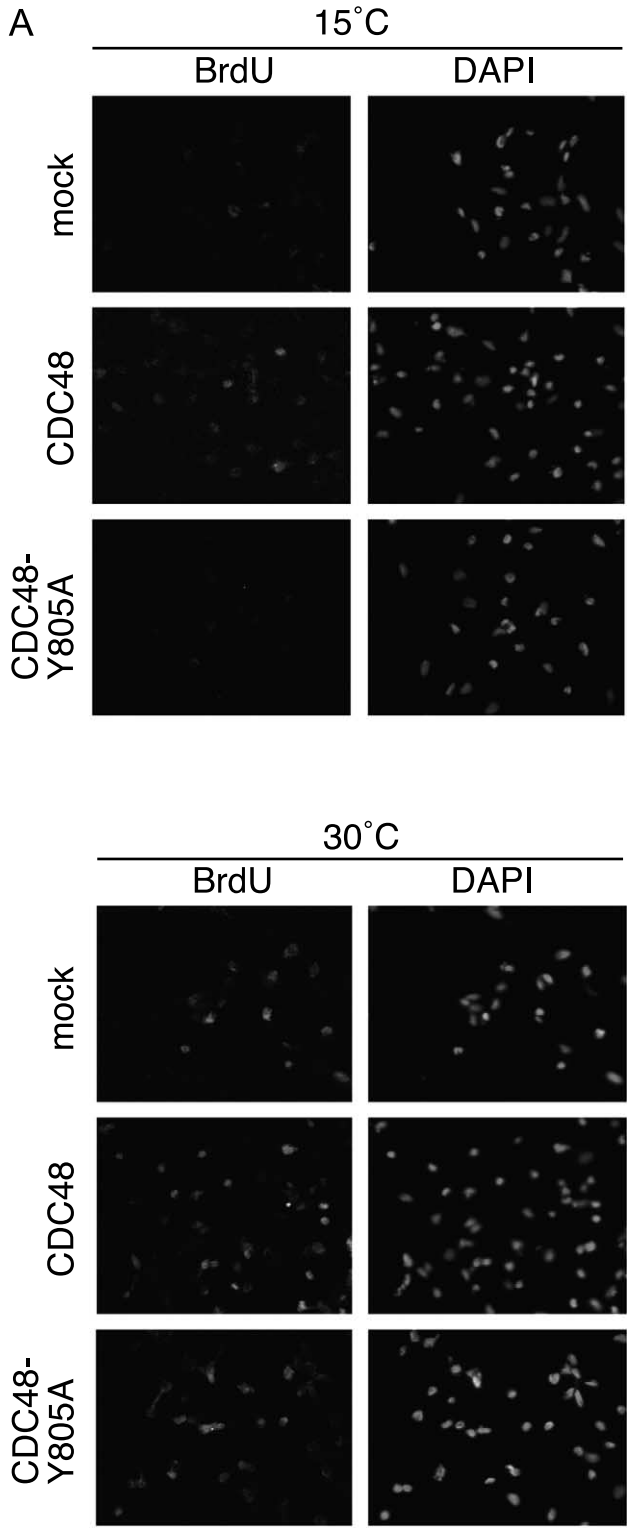

Fig. 5. Effect of overexpression of CDC48 on DNA synthesis. A: ZE cells were transiently transfected with expression vectors encoding CDC48, CDC48-Y805A, or mock vector. The transfected cells were preincubated at 30°C for 1 day, and then incubated at 15°C for 2 days and labeled with BrdU on the last day. B: After immunofluorescent staining with anti-BrdU monoclonal antibody for nuclei that had undergone DNA synthesis, all nuclei were counterstained with DAPI to allow quantification of the total number of nuclei in the same microscopic field. Each value represents the mean ± S.D. for the data for five different samples. Asterisks denote significant differences in the BrdU-positive cells as compared with the control and mock cells ($P < 0.05$).

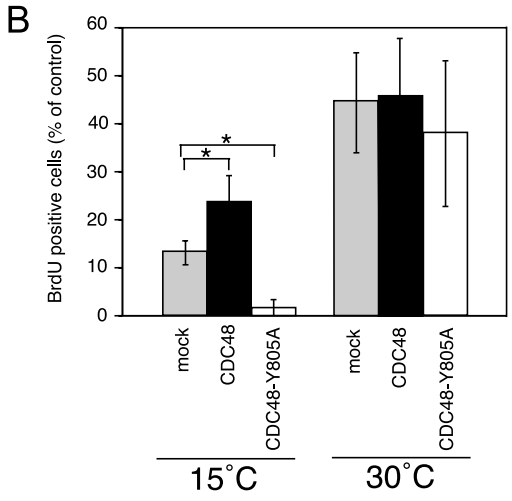

Fig. 5 (*Continued*).

acclimated temperatures from 12 to 25°C, and CDC48 levels increased in a temperature-dependent manner (Fig. 2B). Northern hybridization also showed the time course of CDC48 mRNA expression (Fig. 3). A single 4-kb band was found by hybridization with a ^{32}P-labeled full-length CDC48 cDNA probe. The CDC48 mRNA started to increase during the first 5 days after temperature reduction from 30 to 15°C, and remained high during the 30-day acclimation period.

To examine the role of CDC48 protein in cell proliferation at low temperature, zebrafish CDC48 cDNA was transiently transfected and overexpressed in ZE cells. A vector was constructed that expressed a CMV promoter-driven CDC48 cDNA with a FLAG sequence tag. The ZE cells were transfected with CDC48 constructs and mock vector by electroporation, and the transfected and control cells were preincubated at 30°C for one day; the culture temperature was then reduced from 30 to 15°C. Western blotting using anti-CDC48 antibody and anti-FLAG monoclonal antibody showed that FLAG-tagged CDC48 and the CDC48-Y805A mutant were produced in the transfected cells at both temperatures 2 days after the temperature shift (Fig. 4A). Total CDC48 levels in the transfected cells overexpressing CDC48 or the CDC48-Y805A mutant were approximately 1.5-fold higher than in control cells as detected with anti-CDC48 antibody. By contrast, since the anti-FLAG antibody had low sensitivity on Western blotting, the overexpressed FLAG-tagged CDC48 was barely detected in the CDC48 mutant-transfected cells, suggesting that a population of the transfected cells strongly overexpressing CDC48-Y805A was eliminated by apoptosis.

When cells overexpressing CDC48 were cooled to 15°C with increased CDC48 levels, cell numbers were significantly increased after 4 days as compared with control cells transfected with mock vector (160 vs. 100%) (Fig. 4C). By contrast, there were only slight differences between the transfected cells and cells incubated at 30°C (Fig. 4B). In cells expressing the CDC48-Y805A mutant, cell numbers were significantly decreased after 4 days at 15°C compared with cells transfected with mock vector (60 vs. 100%). No apparent differences in cell numbers were found between control cells and cells transfected with mock vector.

In order to confirm the significant role of CDC48 in cell proliferation under cold conditions, DNA synthesis was mea-

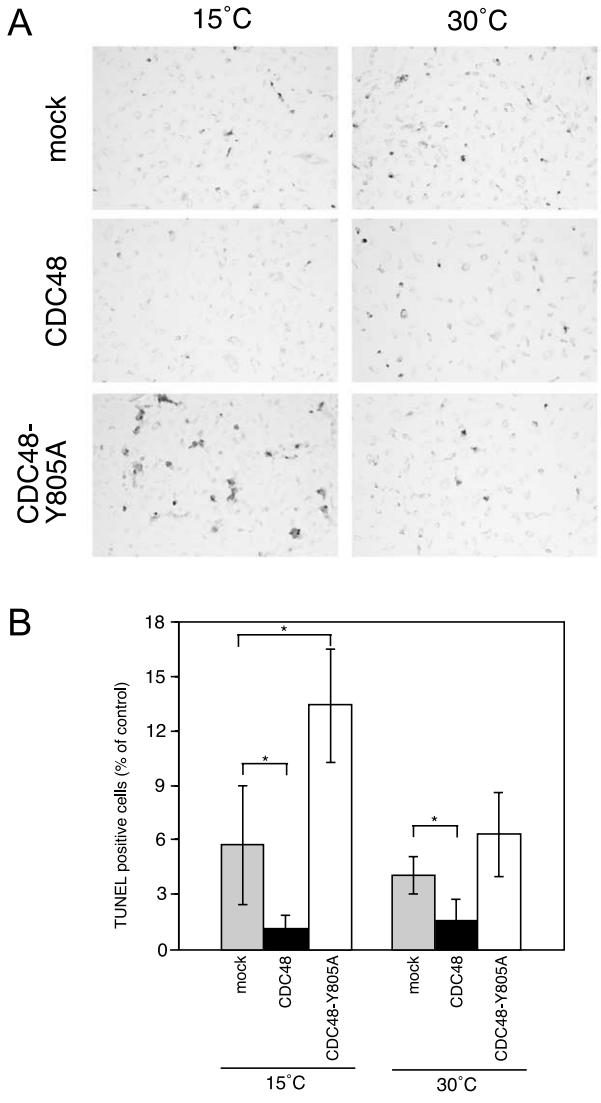

Fig. 6. Effect of overexpression of CDC48 and mutant CDC48 on the induction of apoptosis by TUNEL staining. Each value represents the mean ± S.D. for the data of five different samples. Asterisks denote significant differences in the cell numbers in the CDC48-overexpressing cells and CDC48 mutant-overexpressing cells as compared with the mock cells ($P < 0.05$).

sured using a BrdU incorporation assay. Cells transfected with CDC48 cDNA showed significantly higher BrdU labeling, while cells overexpressing the CDC48 mutant showed reduced BrdU labeling under cold conditions (Fig. 5). There were no apparent differences in the levels of BrdU incorporation between mock cells and CDC48-transfected cells at 30°C.

The percentage of TUNEL-positive apoptotic cells with CDC48 overexpression was significantly lower than that of mock cells at 15 and 30°C (Fig. 6). Larger numbers of apoptotic cells were found in the cells overexpressing the CDC48 mutant as compared with the mock and CDC48-overexpressing cells at 15°C (Fig. 6). This anti-apoptotic function of CDC48 overexpression and the induction of apoptosis with the loss of tyrosine phosphorylation under cold conditions at 15°C were more pronounced than under warm conditions at 30°C (Fig. 6). These findings indicate that overexpression of CDC48 enhanced cell cycle progression and inhibited apoptosis, especially under cold conditions.

4. Discussion

A cold-inducible protein was first identified in cultured rainbow trout cells, and was found to be a homolog of mammalian VCP and yeast CDC48 [10]. This study examined the physiological role of this protein in fish cells at low temperatures. The CDC48 gene was cloned, and cold-inducible gene expression and promotion of cell proliferation were characterized in ZE cells. The deduced amino acid sequence of zebrafish CDC48 was highly homologous with mammalian VCP and yeast CDC48 (Fig. 1). The sequence contained two ATPase binding domains, including the Walker A and B motifs (very similar to mouse VCP), and a possible phosphorylation site at Tyr^{805}. Therefore, due to the high degree of homology in the amino acid sequence and domain structure, the cloned protein is a zebrafish homolog of mammalian VCP and yeast CDC48.

The transcription and acclimation of zebrafish CDC48 mRNA and protein were upregulated by cold temperatures (Figs. 2 and 3). Previous studies of the mouse CDC48 homolog p97 showed that the *cis*-acting elements necessary for basal transcription activity reside within 410 bp of the flanking region. This upstream segment contains consensus binding sites for several transcriptional activators, and has been implicated in basal gene transcription and growth factor/cytokine-responsive, developmental, and stress-induced expression [34]. The 5′-flanking region of zebrafish CDC48 contains similar *cis*-acting elements for basal gene transcription, signal transduction, and cell cycle regulation (unpublished data). Therefore, expression of the CDC48 gene may be regulated by transcriptional factors in response to cell proliferation, and cell cycle progression under cold conditions.

CDC48 overexpression significantly enhanced cell proliferation and protected cells from apoptosis under cold conditions. In the overexpression experiments, when CDC48-transfected cells were exposed to low temperatures, cell proliferation was maintained in these cells, but not in control cultures. This effect on cell proliferation is in line with previous evidence showing that CDC48 functions to regulate the cell cycle in various organisms, such as yeast, *Arabidopsis*, mouse, and human [11,14,35,36]. Nevertheless, there are several reports describing that CDC48 has a variety of biological functions, including a role in regulating apoptosis [14,37,38]. Recently, Thoms [39] found that CDC48 possesses general chaperone activity that can distinguish between native and non-native proteins in the absence of cofactors. The chaperone activity of CDC48 may be required to disassemble the SNARE (soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptors) complex in membrane fusion, as well as to recognize proteins that are to be processed or degraded after release from the ER [12]. In yeast, CDC48 is required for the export of ER proteins into the cytosol, a process in which misfolded proteins from the ER are retranslocated, polyubiquitinated, and targeted to cytosolic proteasomes, and it plays an important physiological role in protein quality control [20,21,39,40]. Therefore, the enhanced chaperone activity with the induction of CDC48 in fish cells at cold temperatures may recognize and degrade misfolded proteins

that are abnormally produced under cold conditions and prevent the cells undergoing apoptosis.

According to the results of studies of cold-sensitive yeast mutants, cell division arrest occurs at the large budded cell stage, in which there is an undivided nucleus and bundles of microtubules spread throughout the cytoplasm from an unseparated spindle pole body [11]. Therefore, yeast CDC48 plays an important role in M phase of the cell cycle at low temperature, and these mutant phenotypes are consistent with a defect in homotypic fusion of the inner nuclear and ER membranes. In fish cells, the fact that CDC48 overexpression enhances cell proliferation at low temperatures suggests that accumulated CDC48 protein in cold-acclimated cells compensates for the defect in membrane fusion function at M phase of the cell cycle.

The loss of the tyrosine phosphorylation site inhibited cell proliferation and induced apoptosis, while the overexpression of CDC48 promoted cell proliferation at 15°C (Fig. 4). A signal transduction pathway mediated by protein tyrosine phosphorylation depending on the cell cycle phase has been reported in murine T cells after stimulation of the T cell antigen receptor [26]. Tyrosine phosphorylation of CDC48 may coordinate assembly of the ER [26–29,35]. This protein is mainly attached to the ER, but relocalized from the ER to the nucleus or to the centrosome in a cell cycle-dependent manner [28,29,35]. This regulation is triggered by a membrane-associated tyrosine kinase and protein-tyrosine phosphatase activity [28,29,35]. For zebrafish, this tyrosine phosphorylation of CDC48 protein may be essential for cellular function in the survival and growth of fish cells at low temperatures. CDC48 protein was inducible in fish cells in response to cold temperatures. Higher levels of phosphorylated CDC48 protein may be required to promote cellular functions in the cold than are required at the normal growth temperature of 30°C.

In conclusion, CDC48 was induced during cold acclimation in zebrafish cells, and overexpression of this protein in cultured fish cells promoted cell proliferation and repressed apoptosis at 15°C by regulating Tyr^{805} phosphorylation at the C-terminal. Therefore, CDC48 might control cell proliferation via changes in both the quantity and quality of this protein.

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