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The Study of Trnascriptional Regulated Gene, *hrp*$^{2+}$, in Yeast

In Soon Choi†

Department of life Sciences, Silla University, Pusan 617-736

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Abstract This study was designed to clone the SNF2/SW12 helicase-related genes from the fission yeast *Schizosaccharomyces pombe* and thereafter to elucidate the common functions of the proteins in this family. The *hrp*$^{2+}$ gene was cloned by polymerase chain reaction amplification using degenerative primers from conserved SNF2 motifs within the *ERCC6* gene, which encodes a protein involved in DNA excision repair. Like other SNF2/SW12 family proteins, the deduced amino acid sequence of Hrp2 contains DNA-dependent ATPase/7 helicase domains as well as the chromodomain and the DNA binding domain. This configuration is similar to that of mCHD1 (mouse chromo-ATPase/helicase-DNA-dinding protein 1), suggesting that Hrp2 is a *S. pombe* homolog of mCHD1, which is thought to function in altering the chromatin structure to control the gene expression.

To characterize the function of Hrp2, 4 Uracil-Hrp2 fusion protein, it was purified near homogeneity by affinity chromatography on Ni^{2+}-NTA agarose, DEAE-Sepharose ion exchange and Sephacryl S-200 gel filtration chromatographies. The purified fusion protein exhibited DNA-dependent ATPase activity, which was stimulated by both double-stranded and single-stranded DNA. To determine the steady-state level of hrp^{2+} transcripts during growth, cells were cultured in medium and collected at every 2hr to prepare total RNAs. The northern blot analysis showed that the level of hrp^{2+} transcripts reached its maximum before the cells entered the exponential growth phase and then decreased gradually. This result implies that Hrp2 may be required at early stages of cell growth.

Key words: mCHD1, SNF2/SW12 family, Hrp2 protrein, chromodomain

Introduction

The yeast SWI2/SNF2 gene was identified in genetic spectrum of differently regulated yeast genes [1,2]. Transcriptional dependence on SWI2/SNF2 appears to be related to the nature of the core promoter sequence, the number of binding sites for upstream activators, and the type of activator. The SWI2/SNF2 polypeptide contains the characteristic seven conserved protein motifs that are present in a large and rapidly growing group of nucleoside triphosphate (NTP)-binding proteins that include DNA and RNA helicases [3-5].

The number of proteins assigned to the SNF2 family has increased rapidly over the last few years and continues to expand. Interestingly, members of the SNF2-like family exhibit an impressive range of biological functions. The activities include gene-specific transcriptional activation (SNF2 subfamily), transcriptional repression (MOT1), destabilization of reconstituted nucleosomes (SNF2 and SNF2L subfamilies), transcription coupled repair (ERCC6 subfamily), nucleotied excision repair of nontranscribed region of the genome (RAD16), recombination repair (RAD54 subfamily), chromosome segregation (lodestar) and chromatin compaction (CHD1 subfamily). Some of the proteins in the SNF2 family contain sequence motifs, which help identify the function conserved within the subfamily. All members of the SNF2 subfamily contain a chromodomain motif. This motif is found in a variety of proteins involved in transcriptional regulation [6] and it has been suggested that it may be involved in protein-protein interactions. CHD1 subfamily contains a chromodomain motif, which is found in a few other proteins and is proposed to play a role in chromatin compaction [7-9]. Finally, a RING finger motif is found in all the proteins in the RAD16 subfamily. This motif is related at the sequence and structural levels to the zinc finger motif [10,11]. It is found in many proteins that interact with DNA (including the DNA repair protein RAD18, the p53-associated protein MDM2 and the protooncogene mel-18) and suggested that it is involved in DNA binding [10].

Recently, the number of proteins enlisted in this family is increasing rapidly through genome sequencing projects or homology-based cloning [12, 13]. This study was aimed to isolate unidentified SNF2/SWI2 family proteins and to investigate the conservation of sequence and function within eukaryotes. The fission yeast *Schizosaccharomyces pombe* was used for this study, which serves as an important model

†Corresponding author
Phone: 82-51-309-5348, Fax: 82-51-309-5176
E-mail: ischoi@silla.ac.kr

system for the study of basic processes in higher eukaryotes.

These work is very important to determine the *in vivo* role (s) of the protein and will enable us to grasp the insight about the various processes in *S. pombe*. Hrp2 has been known to contain DNA-binding activity. However, like other SNF2 family, it exhibits no helicase activity in spite of the presence of the conserved helicase domains. In addition, deletion of hrp^{2+} gene resulted in accelerated cell growth and overexpression of Hrp2 protein caused a reduction in growth rate.

MATERIALS AND METHODS

Strains, cell culture, and genetic methods

E. coli strains DH5*a* (*F- endA1, hsd17, (r-, mk-), supE44, thi-1, recA1, gyrA96, relA1, lacIqZ-M15*) and XL1 blue (stratagene, USA) was used as a host for propagation of plasmids. Yeast *S. pombe* strain JY741 were (*h- ade6- M210 leu1-32 ura4-D18*) was used for this study. *E. coli* strains were grown on LB media (1% tryptone, 1% sodium chloride, 0.5% yeast extract). Yeast strain was grown in YE (2% glucose, 0.5% yeast extract) medium supplemented with appropriate amino acids. Standard molecular biology techniques were employed as described [14]. Transformation of *S. pombe* was performed by dimethyl sulfoxide (DMSO)-enhanced lithium method [15,16].

Isolation of RNA and Northern blot analysis

Total RNA was prepared according to Jang *et al.* (17). RNA was denatured, fractionated on 1.2% agarose gel containing 0.66 M formaldehyde, and transferred onto S&S Nytran membrane. The probe and filter hybridization and washing conditions were employed as described (14).

Preparation of DNA probe by random priming

The DNA fragment was labeled with [a-^{32}P] dCTP (3,000 Ci/mmole) by random primed DNA labeling method [18]. The labeling reaction was carried out in 20 *ul* of the standard random priming buffer containing 50 *ng* of DNA, 30 *uCi* of [a-^{32}P] dCTP, dATP, dGTP, dTTP, and 2 unit of Kenow enzyme for 1 hr at 37^{0}C.

Western blotting

Cells were grown to 5×10^6 cell/ml and harvested. Total proteins were extracted in breakage buffer (100 mM Tris-HCl (pH 8.0), 20% glycerol, 1 mM DTT). About 50 *ug* of total protein was loaded in each lane on an 8% SDS-polyacrylamide gel and subsequently wet-transferred to a immobilon-P membrane (Millipore, USA). The blot was probed with a 1 : 1000 dilution of affinity purified anti-Hrp2 antibodies [19].

Preparation of whole cell extract (WCE)

The cells were grown in one liter of TMM (MM with 1 mM of thiamine) to OD_{595}=2.0 were harvested and washed three times with fresh MM. After dilution to OD_{595} = 0.1 into 15 L of fresh MM, cells were further cultured at 30^{0}C for 12 hr, collected and washed with extraction buffer (20 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, pH 8.0). Then, cells were quickly frozen in liquid nitrogen and stored at -70^{0}C until use. The stored cells were washed once in extraction buffer and lysed in 150 ml of extraction buffer by glass beads with 6×30 sec pulses on a BioSpec products beadbeater, and clarified by centrifugation for 2 hr at 43 K in a Beckman Ti70 rotor. The supernatant was dialyzed against the same buffer and referred to as whole cell extract.

Gel electrophoresis mobility shift assay (EMSA)

Complexes formed with the Hrp2 protein and dsDNA were measured in reaction mixtures (20 *ul*) containing 20 mM HEPES-KOH, pH 7.4, 2 mM DTT, 0.2 *mg*/ml BSA, 5% glycerol, 50 fmol of substrate and the indicated amount of Hrp2 protein in the absence or presence of 4 mM $MgCl_2$ and 1 mM ATP. After 30 min of incubation at 30^{0}C, complexes formed were analyzed by 4% polyacrylamide (30 : 1) gel in $0.5\times$TBE buffer [20].

RESULTS AND DISCUSSION

Isolation of hrp^{2+} gene from S. pombe

To isolate new members of SNF2 helicase related genes from *S. pombe*, this study took advantage of the highly conserved sequence elements within the *ERCC6* gene and generated the degenerative oligo primers [21-25]. From these results, the SNF2 gene family was isolated and then designated as hrp^{2+} gene. Analysis of the nucleotide sequence revealed that the length of hrp^{2+} ORF is 5,780 bp, coding for an 1548 amino acid polypeptide with an estimated molecular mass of 172 KDa. Amino acid sequence analysis showed that Hrp2 has a chromo (C) domain, ATPase/7 helicase (H) domain, and one DNA binding (D) domain (Fig.1). This configuration implies that Hrp2 belongs to the CHD1 subfamily, which comprises of SYGP-4, an *S. cerevisiae* CHD protein and mCHD-1. The C domain has been found in proteins that are implicated in chromatin compac-

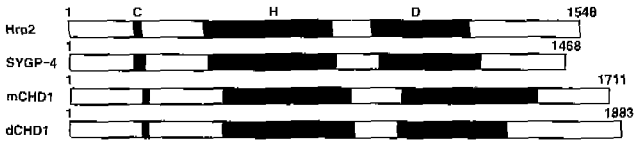

Fig. 1. Comparison of Hrp2 with CHD1 subfamily members. Schematic representation of Hrp2, SYGP-4, mCHD1 and dCHD1 to indicate the location of the homologous segment in the proteins.C, H, and D are indicated Chromodomain, ATPase/7 helicase domain, and the region of DNA binding domain, respectively. The numbers at the ends of diagrams denote the length of each polypeptide. Schematic drawing of the hrp^{2+} gene shows that the protein product encodes 1,548 amino acids.

tion. The sequence conservation of Hrp2 C domain with those of CHD1 subfamily members implies their functional relatedness in the modification of chromatin structure [26-28]. CHD1 was identified as a mammalian DNA-binding protein that contains three signature sequence motifs, which are chromodomain, SW12/SNF2 ATPase domain, and DNA binding motifs [27]. CHD1 shows a sequence-selective preference for binding to (A+T)-rich tracts *in vitro*. Homologs of CHD1 have been reported in Drosophila, bird, and human [28]. However biochemical and genetics analyses of CHD1 function as an ATP-dependent, nucleosome or chromatin-perturbing complex have not yet been proven. Here, Hrp2, a homolog of CHD1, is purified from fission yeast and show that it has a DNA-dependent ATPase activity but no helicase activity (data not shown).

Induction kinetis of Hrp2 production

A cell line overproducing the 4 Uracil-Hrp2 fusion protein was constructed [28]. Five ura+ transformants were selected and analyzed for the expression of Hrp2 using western blot analysis (Fig.2). To determine the optimum time point of Hrp2 production after depletion of thiamine from the medium, the protein expression was monitored by western blot analysis. The 172-KDa Hrp2 was appeared after 6 hr, reached its maximum after 10 hr and kept constant expression levels through 14 hr.

The levels of expression were high enough to be seen in the gel stained with coomasie blue as well as in the blot (Fig.2). Thus, I determined the induction time point to 10 hr for the further purification of Hrp2 protein.

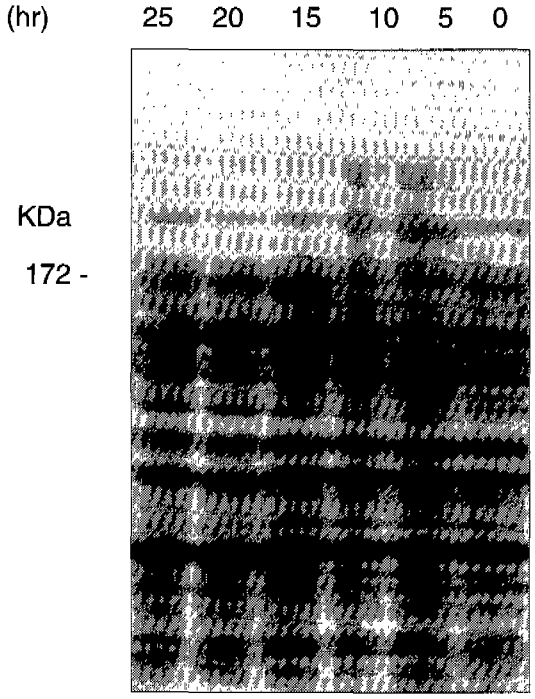

Fig. 2. Induction kinetics of Hrp2 protein. Cells were grow MM with 1 mM thiamine, washed three times fresh MM and diluted in MM $O.D_{595} = 0.1$. Cells were further cultured at 30℃ and collected at each time point indicated. After preparation of whole cell extract, the expression of Hrp2 monitored by Western blot analysis. Figure shows the SDS-PAGE (8%) pattern stained with Coomassie brilliant blue.

Purification of Hrp2 protein

Hrp2 protein was purified by combination of affinity chromatography on Ni^{2+}-NTA agarose, DEAE-sepharose ion exchange and sephacryl S-200 gel filtration chromatographies [28]. Peak fractions were pooled, dialyzed against equilibration buffer (20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, pH 8.0) and loaded onto an DEAE sepharose column (2 × 15 cm). Peak fractions containing full sized Hrp2 were pooled (Fig. 3 fraction #6~#10,20 ml), dialyzed against equilibration buffer and reconcentrated. The protein was used in the further experiments.

Transcription and DNA binding property

To determine the steady-state level of hrp^{2+} transcripts during growth, cells were cultured in rich medium and collected at every 2 hr to prepare total RNAs. The nothern blot analysis showed that the level of hrp^{2+} transcripts reached its maximum before the cells entered the exponential growth phase and then decreased gradually (Fig. 4). This expression pattern was similar to that of hrp^{1+} and hrp^{3+} transcripts, implying that Hrp2 may be required at early stages of cell growth.

The mouse CHD1, a homolog of Hrp2, is a DNA binding protein. Thus, I examined whether Hrp2 also have the activity to interact with double stranded oligonucleotides. As seen in Fig. 5, the Hrp2 protein dose indeed bind to the

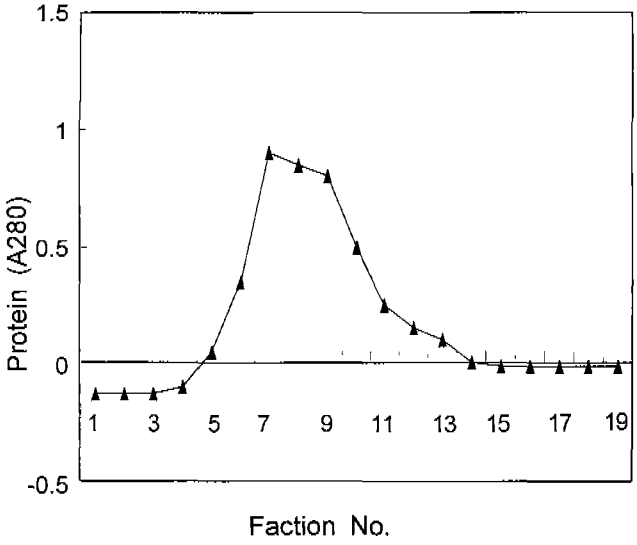

Fig. 3. Ni^{2+}-NTA agarose gel elution profile. Eluates were collected and the protein concentration were measured at A_{280}. Each fraction was loaded (30 $\mu\ell$) onto SDS - polyacrylamide gel (8%) and the gel was stained with Coomassie brilliant blue.

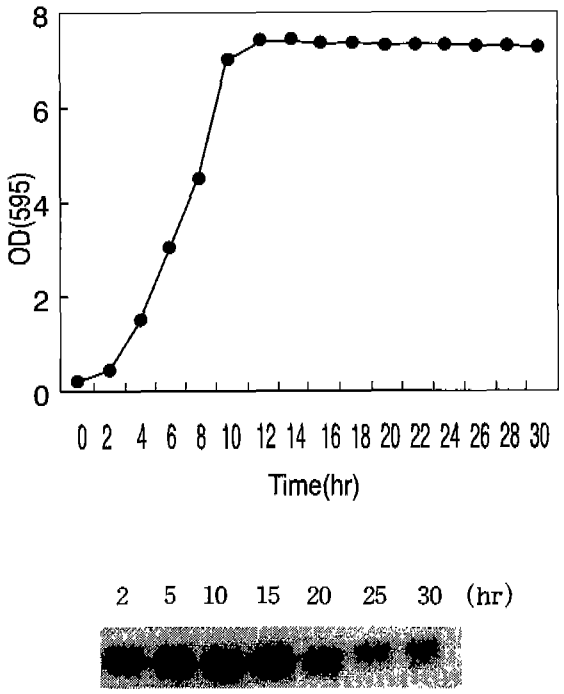

Fig. 4. The change of *hrp*$^{2+}$ transcript level during growth stage. Cells in log phase were diluted to concentration of 1×10^5 cells/ml and were grown with vigorous shaking (A). The $O.D_{595}$ was measured at each time point. (B) RNA was extracted, and then Nothern blot analysis was performed.

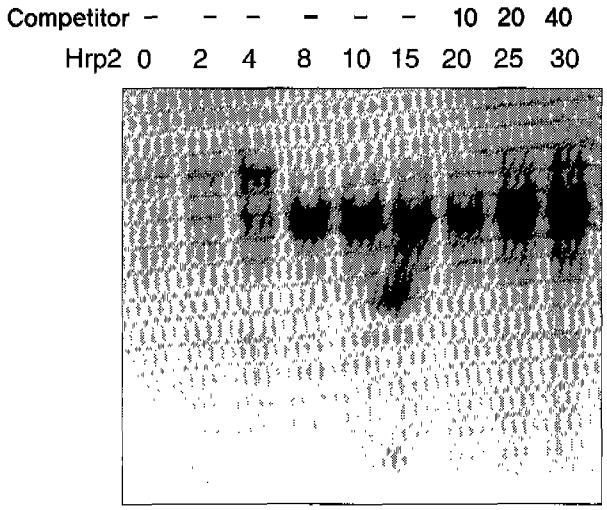

Fig. 5. DNA binding of Hrp2. An EMSA was carried out with ^{32}P-end labeled flus end duplex DNA probe (50 fmol per lane) and increasing amount of the Hrp2 protein. The numbers at the top of figure indicate the fold molar excess of proteins to the DNA probe. The competitor is the cold flush end duplex DNA. The bound DNA protein complexes are indicated with arrow.

DNA fragment, as evidenced by the formation of a single retarded band. The amount of binding increased with increasing amounts of protein and the binding was eliminated by the addition of increasing amounts of unlabeled DNA fragment (Fig. 5).

In this report, I present the subcellular localization and purification of Hrp2 protein, mCHD1 known as a constituent of bulk chromatin is released into the cytoplasm when cells enter mitosis and is reincorporated into chromatin during telophase-cytokinesis [29]. Moreover, dCHD1 was localized to sites of extended chromatin and regions associated with high transcriptional activity on polytere chromosomes from salivary glands of third instar larvae [29]. There observations an open chromatin structure and Hrp2 also functions with similar manner in the cells.

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