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Preferential Expression of the cDNA Encoding the Proteasome Subunit during the Growth/Differentiation Transition of *Dictyostelium* Cells

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A proteasome subunit-1 gene (DAPS-1) was isolated as one preferentially expressed during the transition from growth to differentiation in *Dictyostelium discoideum* cells, using the differential display method. The *DAPS-1* cDNA sequence with a length of 882 bp encodes a protein (Mr. 23.4 kDa) consisting of 213 amino acids. The deduced amino acid sequence of DAPS-1 showed 61% and 58% identity to the proteasome subunit Y of *Xenopus laevis* and *Homo sapiens,* respectively and 48% and 47% identity to the proteasome subunit LMP2 of *Homo sapiens* and *Orizas latipes,* respectively. Northern analysis revealed that a 1.0 kb of *DAPS-1* mRNA is predominantly expressed during the early stage of differentiation induced by starvation. This seems to indicate that the DAPS-1 protein may be involved in proteolysis coupled with active exchange of the cellular protein composition during the phase-shift of *Dictyostelium* cells from the proliferative to differentiated state.

Growth and differentiation of *Dictyostelium discoideum* Ax-2 cells are temporarily separated from each other and easily controlled by nutritional conditions. An available method for inducing synchronous growth of cells has been established (1). A phase-shift point (PS-point) from which a cell enters differentiation phase in response to starvation has been specified in the mid-late G_2 phase of vegetative cell cycle (2). Thus, this organism offers us a particularly useful system for elucidating the cellular and molecular mechanisms of growth/differentiation transition.

Three genes (*Quit1, Quit2* and *Quit3*) have been already isolated by differential plaque hybridization as ones expressed specifically or differently in differentiating cells starved just before the PS-point (3, 4, 5). *Quit1* and *Quit2* were found to encode the cAMP receptor 1 (CAR1) (3) and a novel Ca^{2+}-binding protein (CAF-1) (4), respectively. Another gene, *Quit3* is seems to regulate the synthesis of annexin VII that is involved in Ca*2*+-homeostasis in cells, by the natural antisense transcript via antisense RNA-RNA interaction (5). For exhaustive survey of other genes associated with the transition from growth to differentiation, we used Ax-2 cells synchronized by the temperature-shift method and adopted the differential display (DD) method (6). Among several genes isolated thus, we report here a gene (referred to as *DAPS-1*) encoding a proteasome subunit of *Dictyostelium,* as one preferentially expressed during transition from growth to differentiation.

In general, proteasomes are present in cells as both as 20S and 26S isoforms with molecular masses of 700 kDa and 2,000 kDa, respectively (7, 8, 9). The 26S proteasome is the central protease of the ubiquitin-dependent pathway of protein degradation (10, 11). The 20S proteasome subunits can be aligned with the α or β subunit. The β subunits contain the proteolytically active site, while the a subunits have targeting and regulatory functions (12, 13). The distinguishing characteristic of the α subunit is a highly conserved region at the N-terminus distinct from the β subunit.

Although the DAPS-1 presented here is a part of proteasomes, its preferential expression during the phase-shift of *Dictyostelium* cells from proliferation to differentiation seems to indicate that proteolysis through proteasomes may be closely involved in active exchange of protein constituents between the growth and differentiation phases.

MATERIALS AND METHODS

Cells and culture conditions. Dictyostelium discoideum Ax-2 was used in this study. Vegetative Ax-2 cells were grown axenically in

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HL-5 medium (14). Ten milliliters of the cell suspension was cultured in a 200-ml Erlenmeyer flask coated with Sigmacote (Sigma) at 22.0 °C at 150 rpm on a rotary shaker. Under these conditions, the generation time of Ax-2 cells was 7.5-8.0 hr.

Synchronization of the cell-cycle phase. Cell synchronization was performed using the temperature shift method (1) with a slight modification. Exponentially growing cells (1.0-1.5 $\times$ 10^6 cells/ml) at 22.0 °C with a generation time of about 7.6 hr were shifted to 9.4 °C, shaken for 14.5-hr and then reshifted to 22.0 °C. Under these conditions, cell doubling occurred over about a 2-hr period after a lag phase of about 1 hr. T7 cells (just before the PS-point), 7 hr after the shift-up from 9.4 °C to 22.0 °C, were harvested, starved by washing twice in 20 mM phosphate buffer (PB) (KH_2PO_4/Na_2HPO_4, pH 6.5), and then shaken at 1 $\times$ 10^7 cells/ml for 2 hr at 150 rpm to obtain T7+2 cells as just differentiating ones. T4+2 cells were also prepared by starving T4 cells for 2 hr in PB, as starved but not differentiated cells. As another control, T9 cells obtained by incubating T7 cells for 2 hr in the growth (HL-5) medium were used.

Developmental condition. Synchronized or nonsynchronized Ax-2 cells were harvested at the desired phases and washed twice in PB by centrifugation at 2,500 rpm for 1 min. The cell pellet was suspended in PB and aliquots (500 μl) of the cell suspension were plated at a density of about 1-1.5 $\times$ 10^6 cells/cm^2 on 1.5% non-nutrient agar, followed by incubation for 24-36 hr at 22.0 °C.

Isolation of total RNAs and Northern hybridization. Total RNAs were prepared according to the method of Nellen et al. (15) from T7+2 cells, T9 cells, and T4+2 cells. They were also prepared from non-synchronized cells at various developmental stages. The total RNA samples were heat-treated at 65.0 °C for 15 min to denature the RNA and then quickly chilled on ice. The samples (10 or 15 μg) were separated on 1.0% formaldehyde agarose gel and transferred on to the nylon membrane (Amersham) by upward capillary transfer. The membranes were immobilized by bake for 2 hr at 80.0 °C and were stored at room temperature or 4.0 °C. Prehybridization was carried out at 63.0 °C for 2 to 4 hr in a solution containing 5 $\times$ Denhardt solution (0.02% Ficoll 400, 0.02% bovine serum albumin (BSA), and 0.02% polyvinylpyrrolidone (pvp)), 5 $\times$ SSPE (43.8 g of NaCl, 6.9 g of $NaH_2PO_4H_2O$ and 1.85 g of EDTA; pH 7.4) or 5 $\times$ SSC (43.8 g of NaCl and 22.05 g of sodium citrate), 0.5% sodium dodecyl sulfate (SDS), and 20 mg-100 mg of denatured salmon sperm DNA per ml. Hybridization was carried out at 63.0 °C for 20 hr in the same solution containing the ^{32}P-labeled cDNA probe by use of the Megaprime DNA labeling system (Amersham). The membranes were washed twice with a washing solution (1 $\times$ SSC and 0.1% SDS) at room temperature for 15 min and then washed twice with a solution (0.1 $\times$ SSC and 0.1% SDS) at 63.0 °C for 5-10 min. The membranes were applied to X-ray films for 2 to 7 days at $-$80.0 °C.

Differential display of total RNAs. Differential display (DD) was performed by the method of Liang et al. (16) with the RNAimage kit (GenHunter). DNA-free total RNAs of T4+2 cells, T7+2 cells, and T9 cells were reverse transcribed with three one-base anchored oligo(dT) primers (5′-AAGCTTTTTTTTTTTG-3′; H-T11G, 5′-AAGCTTTTT-TTTTTA-3′; H-T11A and 5′-AAGCTTTTTTTTTTC-3′; H-T11C) that annealed at the start of the poly(A) tails of mRNAs, followed by polymerase chain reaction (PCR) amplification with the anchored primers and six arbitrary primers (5′-AAGCTTTCCTGGA-3′; H-AP25, 5′-AAGCTTGCCATGG-3′; H-AP26, 5′-AAGCTTCTGCTGG-3′; H-AP27, 5′-AAGCTTACGATGC-3′; H-AP28, 5′-AAGCTTAGC-AGCA-3′; H-AP29, 5′-AAGCTTCGATCGT-3′; H-AP30). After size-fractionation of amplified cDNA fragments of 3′-termini of mRNAs on a 6% denaturing polyacrylamide gel electrophoresis and subsequent retrieval, the cDNA fragments of interest were reamplified, cloned into the PCR-TRAP cloning vector (Gene Hunter) and sequenced.

cDNA sequencing. cDNA sequencing was carried out using the ABI PRISM Dye Terminator cycle sequencing kit (Perkin Elmer). PCR reaction was performed using 10-50 ng single strand DNAs or 100-500 ng double strand DNAs as the template DNA. PCR products were purified by ethanol precipitation and then dried. This dried PCR products were resuspended in 25 μl of the 310 Genetic Analyzer Buffer and were analyzed by a ABI Prism 310 Genetic Analyzer (Perkin Elmer). For multiple sequence alignments the programs of GENETYX-MAX were used.

Plaque hybridization. The cDNAs encoding the full length of mRNAs were screened by the plaque hybridization method from T7+2 cDNA library (3) that was constructed in λ-ZAPII (Stratagene). The λ-phages (approximately 50,000) were plated on the surface of the λ top agarose (1.0% trypton, 0.5% NaCl, 0.7% agarose, 10 mM $MgSO_4$ and 0.2% maltose) in a 95mm $\times$ 135mm dish, and were imprinted by gently layering a nylon membrane (Hybond-N^+; Amersham). After denaturation with alkali solution (0.5 M NaOH and 1.5 M NaCl), the DNAs were irreversibly bound to the membrane by baking at 80.0 °C and then hybridized to the ^{32}P-labeled fragment probes. The membranes were washed and exposed to autoradiographic X-ray films. Hybridizing plaques, identified by aligning the film with the library plate, were put on a microfuge tube containing SM buffer (5.8 g of NaCl, 2.0 g of $MgSO_4.7H_2O$, 50 ml of 1 M Tris-HCl (pH 7.5) and 5.0 ml of 2.0% gelatin) and 4% chloroform. The plaque stocks thus obtained were used for *in vivo* excision into the XL-1 blue MRF′ strain.

RESULTS AND DISCUSSION

Since 32 kinds of cDNA fragments were detected as ones specifically expressed during the transition of *Dictyostelium* cells from the PS-point to differentiation, they were withdrawn and reamplified using both primers. The reamplified cDNAs were used as each probe for Northern blot analysis. As a result, a cDNA frag-

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TTTTTTTTTTTTTTTTTTATATTTTTTTCTTTGTTAAATTATTCACATTTATATATATAT    60

AAATTTATATATATATTTATTAAATATATTAAATAATGGAGGCCCCTGAATGGTTAGATA   120
                                  M  E  A  P  E  W  L  D  N      9

ATGCCGTTGATTTAGGTACTTCTATTATGGCAGTAGAATATGATGGTGGTGTTATTATGG   180
  A  V  D  L  G  T  S  I  M  A  V  E  Y  D  G  G  V  I  M  G   29

GTGCAGATTCAAGAACTACCACTGGTGCATATATCGCAAATAGAGTTACAAACAAAATTA   240
  A  D  S  R  T  T  T  G  A  Y  I  A  N  R  V  T  N  K  I  T   49

CCCCAATTCATGAAAGAATTTATTGTTGTAGATCAGGTTCAGCTGCTGATACTCAAGCAA   300
  P  I  H  E  R  I  Y  C  C  R  S  G  S  A  A  D  T  Q  A  I   69

TTTCTGATTATGTTAGATATTATCTTGAAATGCATACTTCAGAATTATGTGATGAACCAG   360
  S  D  Y  V  R  Y  Y  L  E  M  H  T  S  E  L  C  D  E  P  D   89

ATGTAAAAACCGCAGCATGTCTTTTCCAATTATTATGTTATAGTAATAAAAACAATTTAA   420
  V  K  T  A  A  C  L  F  Q  L  L  C  Y  S  N  K  N  N  L  M  109

TGGCTGGTATTATTGTAGCTGGTTGGGATAAACATCAAGGTAGTGTATACAATATTTCGC   480
  A  G  I  I  V  A  G  W  D  K  H  Q  G  S  V  Y  N  I  S  L  129

TTGGAGGTTCAATGGTTAAACAACCATTTGCTATTGGTGGTTCAGGTTCAACCTATATTT   540
  G  G  S  M  V  K  Q  P  F  A  I  G  G  S  G  S  T  Y  I  Y  149

ATGGTTATTGTGATTCCAAATTTAAACCAAAAATGACTAAAGATCGATGTATTGAATTTG   600
  G  Y  C  D  S  K  F  K  P  K  M  T  K  D  R  C  I  E  F  V  169

TTCAAAATTCATTGGCTCTTGCTATGTTCAGAGATGGTTCATCTGGTGGTGTAATTAGAT   660
  Q  N  S  L  A  L  A  M  F  R  D  G  S  S  G  G  V  I  R  L  189

TATGTATTATCGACAAAAACGGTGTAGAACGTAAAATGATTCCTGGAAATAATTTACCAA   720
  C  I  I  D  K  N  G  V  E  R  K  M  I  P  G  N  N  L  P  R  209

GATTCTGGGAGGGATAATTTTCGAATATTTAAATAATGATTGGAATTAACCATGCATATA   780
  F  W  E  G  *                                               213

ATATTATTGTAAAGAAATAACTAAAAAAAAAAACAAAAAAAAAAAAAATTGAAAAAAAAAA   840

TAAATAAAAATAAAAAATTTCTAAATATCCCAAAAAAAAAAA                     882
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FIG. 1. cDNA sequence and deduced amino acid sequence of the *Dictyostelium* proteasome subunit (*DAPS-1*). The asterisk indicates the stop codon. This nucleotide sequence is deposited in the DDBJ, EMBL and GenBank databases with the accession number AB007024.

ment was found to be expressed predominantly in T7+2 cells (just-differentiating cells) as compared with T9 and T4+2 cells still being in the cell cycle. This cDNA fragment (about 200bp) was cloned into the PCR-TRAP cloning vector (GeneHunter). Extraction and purification of the plasmid DNAs were performed using alkaline lysis (17, 18) and polyethylene glycol (PEG) precipitation method with a modification. This cDNAs sequence was analyzed by a ABI prism 310 Genetic Analyzer (Perkin Elmer) using single or double stranded cDNAs. Since the search of the determined sequence revealed high homology to the proteasome subunits of various organisms, this gene was refereed to as differentiation associated proteasome subunit-1 (*DAPS-1*). From T7+2 cDNA library (3) of 600,000 clones, 26 clones were found to hybridize positively to the short probe. To obtain the cDNAs encoding the full length of *DAPS-1*, the longest positive clone with a 882 bp insert was isolated and sequenced. As shown in Fig. 1, the cDNA clone potentially encodes a protein consisting of 213 amino acids with an estimated molecular mass of 23.4 kDa. The predicted amino acid sequence showed high homology to the proteasome subunit Y (*β* subunit) of *Xenopus laevis* (63%) (19), *Homo sapiens* (60%) (20) and *Lampetra japonica* (59%) (19) peptides and also to the proteasome subunit LMP2 (*β* subunit) of *Homo sapiens* (21) and *Xenopus laevis* (48%) (19), *Mus musculus* (22, 23) and *Orizas latipes* (47%) (24) peptides (Fig.2).

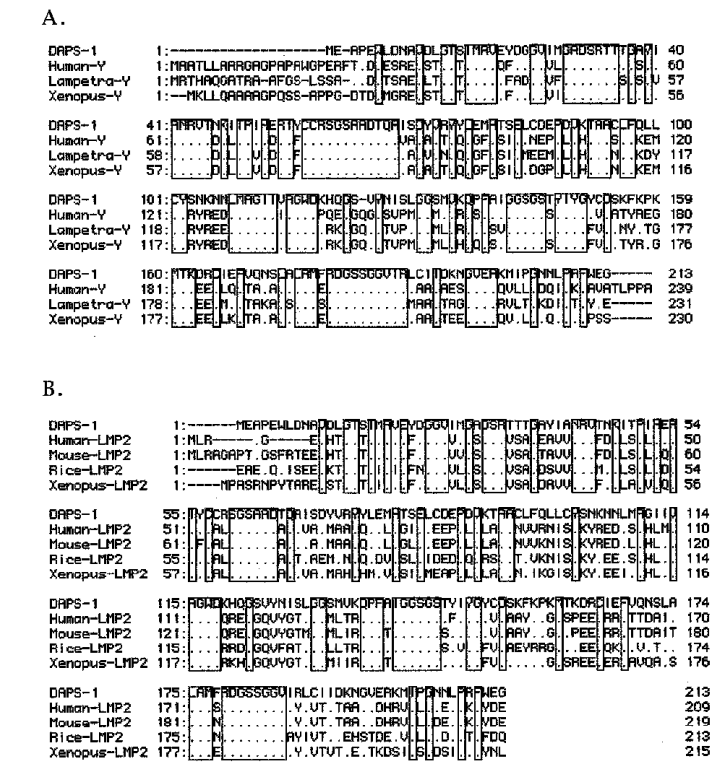

FIG. 2. Comparison of the amino acid sequences of DAPS-1 and proteasome subunits of various organisms. A. the proteasome subunit Y of *Homo sapiens, Lampetra japonica* and *Xenopus laevis.* B. the proteasome subunit LMP2 of *Homo sapiens, Mus musculus, Orizas latipes,* and *Xenopus laevis.* Dots (·) indicate amino acids identical with DAPS-1. Hyphens (-) represent gaps in the alignment.

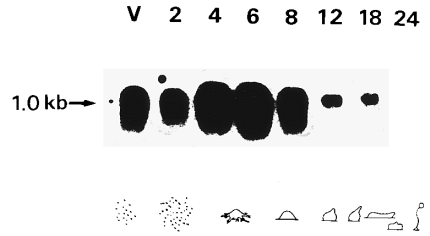

FIG. 3. Developmental change of the *DAPS-1* expression. Non-synchronized Ax-2 cells growing in HL-5 medium were harvested, washed in 20 mM phosphate buffer (KH_2PO_4/Na_2HPO_4, pH 6.5) and allowed to develop on 1.5% non-nutrient agar at 22.0 °C. Lane V, vegetative growth phase. At the indicated times (hr) of incubation, total RNAs were isolated from cells and blotted for Northern hybridization to the *DAPS-1* cDNA. The expression reaches to the maximal level 4-6 hr after starvation, followed by rapid decrease. The gross morphology of each developmental stage is shown at the bottom of the figure.

In general, the proteasome is an essential component of the ATP-dependent proteolytic pathway in eukaryotic cells and is responsible for the degradation of most cellular proteins. The proteasome has been highly conserved during eukaryotic evolution and simpler forms are even found in archaebacteria and eubacteria. Ubiquitin-mediated proteolysis of proteasome is known for the specific degradation of cyclins during the cell cycle (25), but its role during development is less understood. From *Dictyostelium* growth-phase cells, two *α*-type subunits of proteasome have been isolated by Schauer *et al.* (26). They have suggested that the *Dictyostelium* proteasome is consisting of four seven-membered rings or disks collectively forming a barrel-shaped structure and locate both in the cytosol and, in higher concentrations, in the nucleus. The developmental change of *DAPS-1* expression was illustrated in Fig. 3: *DAPS-1* mRNA being maximally expressed 4-6 hr (around the aggregation-stream stage) after starvation, followed by drastic decrease from the mound to tipped aggregate stage. Thus, the proteasome including DAPS-1 protein might be involved in proteolysis by which the cellular protein composition is actively exchanged during the early step of transition from growth to differentiation in *Dictyostelium* cells, though the developmental kinetics of other proteasome subunits is presently unknown and remains to be elucidated.

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