J. Biochem. 120, 494-497 (1996)

Cloning and Expression of cDNA Encoding a Human Ubiquitin-Conjugating Enzyme Similar to the *Drosophila bendless* Gene Product[1]

Tomoko Yamaguchi,* Nam-Soon Kim,* Shingo Sekine,* Hiroaki Seino,† Fumio Osaka,† Fumiaki Yamao,† and Seishi Kato*,[2]

*Sagami Chemical Research Center, 4-4-1 Nishi-Ohnuma, Sagamihara, Kanagawa 229; and †National Institute of Genetics, Mishima, Shizuoka 411

Received for publication, March 4, 1996

cDNA encoding a novel human ubiquitin-conjugating enzyme has been cloned from an epidermoid carcinoma KB cDNA library. This clone encodes a protein of 152 amino acids with a calculated M_r of 17,137. The amino acid sequence showed 80% identity with the *Drosophila*'s *bendless* gene product (ubiquitin-conjugating enzyme E2). The corresponding transcripts are highly expressed in heart, skeletal muscle, and testis. The product expressed in *Escherichia coli* exhibited the ability to form a thiol ester linkage with ubiquitin in a ubiquitin-activating enzyme E1-dependent manner. These results suggest that the obtained cDNA encodes a novel human E2 which may be involved in protein degradation mainly in the muscles and testis.

Key words: bacterial expression, cDNA, human homolog, ubiquitin-conjugating enzyme.

The ubiquitin system is one of the intracellular protein degradation systems, and is composed of ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2, and ubiquitin-ligase E3 (*1, 2*). Ubiquitin (Ub) is activated by the enzyme E1 to form an E1-Ub thiol ester. The activated Ub is then transferred from E1 to a specific thiol of E2, forming an E2-Ub thiol ester. Finally, the Ub of E2-Ub is donated to a target protein either alone or in conjunction with the E3 protein. The ubiquitinated protein is degraded by 26S proteasome in an energy-dependent manner. Several human cDNA clones encoding E2s have been isolated: yeast RAD6 homolog (*3, 4*), keratinocyte-derived E2 (*5*), CDC34 homolog (*6*), yeast UBC8 homolog (*7*), UbcH5 (*8-10*), UbcH6 (*11*), UbcH7 (*11*), and UBE2I (*12*).

During the construction of a human full-length cDNA bank (*13*), we found a cDNA encoding an E2-like protein similar to the *Drosophila bendless* gene (*ben*) product (*14, 15*). This paper presents the sequence analysis of this cDNA clone and the Ub thiol ester formation activity of the encoded protein expressed in *Escherichia coli*.

MATERIALS AND METHODS

Reagents—Restriction enzymes, modification enzymes, and *E. coli* JM109 competent cells were purchased from Takara Shuzo. *Bsa*I, pMAL™-c2, amylose resin and factor Xa were obtained from New England Biolabs. *E. coli* DH12S competent cells for electroporation were purchased from GIBCO-BRL. Helper phage M13KO7 was from Pharmacia. Human Multiple Tissue Northern Blots were purchased from Clontech. IPTG was from Nacali Tesque. cAMP-dependent protein kinase and yeast inorganic pyrophosphatase were from Sigma. [γ-^{32}P]ATP and [α-^{32}P]-dCTP were from Amersham.

cDNA Library—Human epidermoid carcinoma cell line KB cells were cultured in MEM medium supplemented with 10% fetal bovine serum. Total mRNA was isolated from cells using the guanidinium/cesium chloride method (*16*). Poly(A)$^+$RNA was prepared using oligo(dT)-cellulose column chromatography (*17*). A cDNA library was prepared using a dT-tailed pKA1 vector primer according to the capping method (*13*). The ligation mixture was used to transform *E. coli* DH12S using an electroporation apparatus (GIBCO-BRL).

Sequencing—The deleted clones for sequencing the entire region of the cDNA were prepared using a deletion kit (Takara Shuzo). Single-stranded DNAs were isolated from the *E. coli* culture medium after infection with helper phage M13KO7 and used as a template for sequencing. The sequencing reaction was performed by the dideoxy method using a fluorescent dye-labeled primer kit (Applied Biosystems). The reaction mixture was analyzed on a 373A automated DNA sequencer (Applied Biosystems).

Northern Blot Hybridization—An *Eco*RI-*Hin*dIII cDNA fragment was labeled with [α-^{32}P]dCTP using a random primer labeling kit (Takara Shuzo) and used as a probe for Northern blot hybridization (*16*).

Expression of the E2 cDNA—The coding region of the E2 cDNA was amplified by the PCR method using two synthetic oligonucleotide primers: 5′-GCGGTCTCGATGGCC-GGGCTGCCCCGCA-3′ and 5′-GCGTCGACTTAAATAT-TATTCATGGCAT-3′. The amplified product was digested with *Bsa*I, blunted by Klenow fragment treatment, and

[1] The nucleotide sequence reported in this paper has been submitted to the GenBank™/EMBL/DDBJ data bank with the accession number D83004.

[2] To whom correspondence should be addressed. Phone: +81-427-42-4791, Fax: +81-427-42-5091

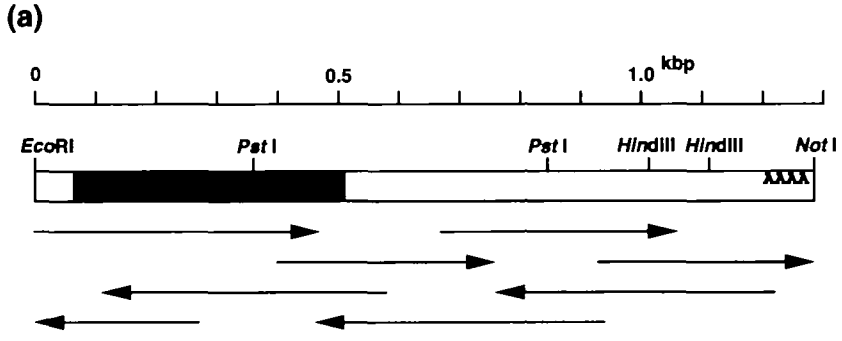
Abbreviations: IPTG, isopropyl-1-thio-β-D-galactopyranoside; Ub, ubiquitin.

digested with *Sal*I. The cDNA fragment was inserted between *Xmn*I and *Sal*I of pMAL™-c2. The resulting plasmid pMALHP686 was introduced into *E. coli* JM109. All experiments on the expression and purification of the fusion protein and the separation of E2 from the fusion protein by factor Xa were performed using the manufacturer's protocol.

Ub Thiol Ester Formation Assay—The thiol ester formation with Ub was measured using $(His)_6$-tagged mouse recombinant E1 and recombinant Ub fused to a phosphorylation tag for cAMP-dependent protein kinase (Osaka *et al.*, manuscript in preparation). The bacterially expressed Ub was purified and labeled with the catalytic subunit of cAMP-dependent protein kinase in the presence of [γ-^{32}P]ATP. The recombinant E2 (100 ng) and $(His)_6$-tagged mouse E1 (200 ng) were incubated with ^{32}P-labeled Ub in a reaction mixture containing 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM ATP, 0.1 mM DTT, and 20 U of yeast inorganic pyrophosphatase for 5 min at room temperature. The reaction was stopped by the addition of the SDS gel sample buffer, from which DTT had been omitted. A replicate sample was boiled for 1 min in the presence of 0.1 M DTT. All samples were analyzed by SDS-PAGE and ^{32}P-labeled Ub-containing bands were visualized by autoradiography.

RESULTS AND DISCUSSION

The partial amino acid sequence encoded by one cDNA clone picked from the KB cDNA library showed similarity to the sequences of Ub-conjugating enzymes registered on the protein database. Full-sequence analysis revealed that this clone, HP00686, contained a total of 1,283 bp including a 5′-noncoding region of 63 bp, an open reading frame of 459 bp, a 3′-noncoding region of 681 bp, and a poly (A) tail of 80 bp (Fig. 1). The first ATG codon is preceded by an



(b)

```
ACTCGTGCGTGAGGCGAGAGGAGCCGGAGACGAGACCAGAGGCCGAACTCGGGTTCTGAC    60
AAGATGGCCGGGCTGCCCCGCAGGATCATCAAGGAAACCCAGCGTTTGCTGGCAGAACCA   120
   M  A  G  L  P  R  R  I  I  K  E  T  Q  R  L  L  A  E  P      19
GTTCCTGGCATCAAAGCCGAACCAGATGAGAGCAACGCCCGTTATTTTCATGTGGTCATT   180
V  P  G  I  K  A  E  P  D  E  S  N  A  R  Y  F  H  V  V  I      39
GCTGGCCCTCAGGATTCCCCCTTTGAGGGAGGGACTTTTAAACTTGAACTATTCCTTCCA   240
A  G  P  Q  D  S  P  F  E  G  G  T  F  K  L  E  L  F  L  P      59
GAAGAATACCCAATGGCAGCCCCTAAAGTACGTTTCATGACCAAAATTTATCATCCTAAT   300
E  E  Y  P  M  A  A  P  K  V  R  F  M  T  K  I  Y  H  P  N      79
GTAGACAAGTTGGGAAGAATATGTTTAGATATTTTGAAAGATAAGTGGTCCCCAGCACTG   360
V  D  K  L  G  R  I  C  L  D  I  L  K  D  K  W  S  P  A  L      99
CAGATCCGCACAGTTCTGCTATCGATCCAGGCCTTGTTAAGTGCTCCCAATCCAGATGAT   420
Q  I  R  T  V  L  L  S  I  Q  A  L  L  S  A  P  N  P  D  D     119
CCATTAGCAAATGATGTAGCGGAGCAGTGGAAGACCAACGAAGCCCAAGCCATAGAAACA   480
P  L  A  N  D  V  A  E  Q  W  K  T  N  E  A  Q  A  I  E  T     139
GCTAGAGCATGGACTAGGCTATATGCCATGAATAATATTTAAATTGATACGATCATCAAG   540
A  R  A  W  T  R  L  Y  A  M  N  N  I  *                      152
TGTGCATCACTTCTCCTGTTCTGCCAAGACTTCCTCCTCTTTGTTTGCATTTAATGGACA   600
CAGTCTTAGAAACATTACAGAATAAAAAAGCCCAGACATCTTCAGTCCTTTGGTGATTAA   660
ATGCACATTAGCAAATCTATGTCTTGTCCTGATTCACTGTCATAAAGCATGAGCAGAGGC   720
TAGAAGTATCATCTGGATTGTTGTGAAACGTTTAAAAGCAGTGGCCCCTCCCTGCTTTTA   780
TTCATTTCCCCCATCCTGGTTTAAGTATAAAGCACTGTGAATGAAGGTAGTTGTCAGGTT   840
AGCTGCAGGGGTGTGGGTGTTTTTATTTTATTTTATTTTATTTTATTTTTGAGGGGGGAG   900
GTAGTTTAATTTTATGGGCTCCTTTCCCCCTTTTTTGGTGATCTAATTGCATTGGTTAAA   960
AGCAGCTAACCAGGTCTTTAGAATATGCTCTAGCCAAGTCTAACTTTATTTAGACGCTGT  1020
AGATGGACAAGCTTGATTGTTGGAACCAAAATGGGAACATTAAACAAACATCACAGCCCT  1080
CACTAATAACATTGCTGTCAAGTGTAGATTCCCCCCTTCAAAAAAAGCTTGTGACCATTT  1140
TGTATGGCTTGTCTGGAAACTTCTGTAAATCTTATGTTTTAGTAAAATATTTTTTGTTAT  1200
TCT                                                            1203
```

Fig. 1. **Structure of the cDNA.** (a) Restriction map of the cDNA and the sequencing strategy. The solid box represents the open reading frame. (b) The nucleotide sequence of the cDNA and the deduced amino acid sequence. The putative poly (A) addition signals are underlined.

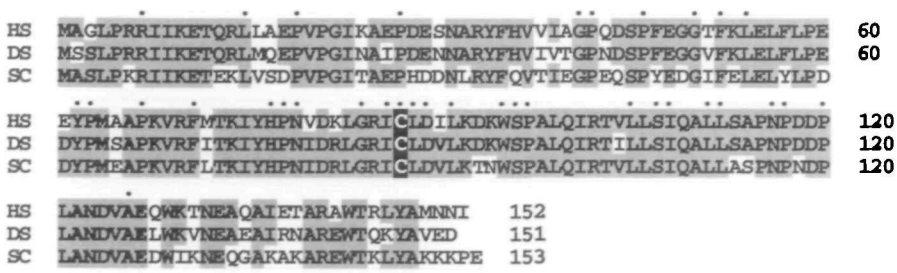


Fig. 2. **Alignment of Ub-conjugating enzyme E2 amino acid sequences deduced from human cDNA (HS), *Drosophila* gene (DM), and *Saccharomyces cerevisiae* gene (SC).** Identical amino acids among the sequences are shaded. The cysteine residue involved in thiol ester bond with Ub is blackened. Dots over the sequence indicate amino acid residues which are conserved in other E2 proteins.

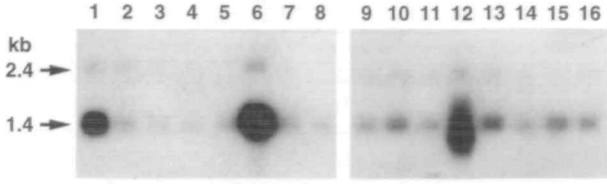


Fig. 3. **Northern blot hybridization.** 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas; 9, spleen; 10, thymus; 11, prostate; 12, testis; 13, ovary; 14, small intestine; 15, colon; 16, peripheral blood leukocytes.

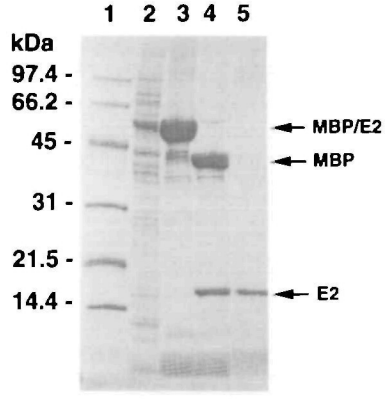


Fig. 4. **SDS-PAGE analysis of expression product of maltose-binding protein and human E2 fusion gene in *E. coli*.** 1, molecular weight markers; 2, lysates of *E. coli* cells carrying expression vector pMALHP686 for the fusion protein after IPTG induction; 3, the fusion protein purified on an amylose column; 4, the factor Xa-digested product of the fusion protein; 5, the separated E2.

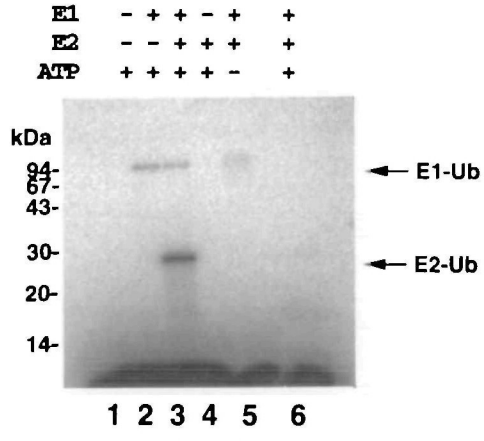


Fig. 5. **Ub thiol ester formation assay of recombinant E2.** ^{32}P-labeled Ub (lane 1) was mixed with mouse recombinant E1 (lane 2), recombinant E2 (lane 4) in the presence of ATP, or E1+E2 in the presence (lanes 3 and 6) or absence (lane 5) of ATP. The reaction mixtures were analyzed by SDS-PAGE without adding DTT (lanes 1-5) or after 5 min boiling with 0.1 M DTT (lane 6).

in-frame termination codon and is in agreement with Kozak's rule (*17*). The putative poly(A) addition signal, AGTAAA, is found at position 1,181. The open reading frame encodes a protein of 152 amino acids with a calculated M_r of 17,137.

As a result of homology search using the GenBank™/EMBL/DDBJ database, the determined nucleotide sequence partially matched the following human EST sequences: 5 clones from fetal liver and spleen (accession numbers R83392, T83898, T96902, T98324, T99403), 4 from melanocytes (H84799, H98983, N44818, W03614), 3 from infant brain (H10694, R54347, T23480), 2 from bone (T30096, T30100), 2 from fetal lung (W00674, W05757), 1 from heart (T32724), 1 from fetal heart (N84246), 1 from placenta (N39781), and 1 from multiple sclerosis (N49129). One clone (T99509) ends near position 640, which is preceded by the putative poly(A) addition signal AATAAA. This clone may have resulted from alternative poly(A) addition.

The deduced amino acid sequence showed high similarity to the Ub-conjugating enzyme E2 family, particularly the *Drosophila ben* product, with 80% identity and 90% similarity, and *Saccharomyces cerevisiae* putative E2 (accession number Z50111), with 70% identity and 84% similarity (Fig. 2). The levels of identity with other members of the E2 family are between 27 and 49%: *Drosophila* UBC4 (49%/145 amino acids), and yeast UBC4 (47%/145 amino acids). The single cysteine residue in this clone is presumably involved in thiol ester bond formation, and is conserved in all members of the E2 family. From these amino acid sequence similarities, this protein seems to be a human homolog of the *Drosophila ben* product, so it has been named UbcH-ben.

Northern blot hybridization was carried out to examine the expression pattern of UbcH-ben. As shown in Fig. 3, a main band of 1.4 kb and a faint band of 2.4 kb appeared in all tissues examined. The obtained cDNA may be derived from the 1.4 kb transcript. High levels of expression in the heart and skeletal muscles suggest that UbcH-ben is involved in the degradation of muscle-related proteins. The testis also showed a high level of expression of UbcH-ben, but the transcript is shorter than those in the other tissues, which may result from alternative poly(A) addition or alternative splicing. In brain, the relatively high ratio of 2.4 kb transcript is characteristic compared with other tissues.

In order to obtain recombinant UbcH-ben, the coding region of the UbcH-ben cDNA was subcloned into an

expression vector for preparing a fusion protein with maltose-binding protein. *E. coli* cells harboring the expression vector produced a large amount of fusion protein in the soluble fraction after IPTG induction (Fig. 4). The fusion protein was purified by amylose column chromatography. The molecular mass of the fusion protein, 60 kDa, coincided with the calculated value. The E2 part including the first methionine was separated from the fusion protein by digestion with factor Xa, giving a product of 17 kDa.

The Ub thiol ester formation of the *Drosophila ben* product has not been reported. That of UbcH-ben was measured by our own method using ^{32}P-labeled recombinant Ub and mouse recombinant E1 (manuscript in preparation). As shown in Fig. 5, the mouse E1 formed a thiol ester with Ub in the presence of ATP. When the recombinant UbcH-ben was added to this reaction mixture, the band of E1-Ub became weak, and instead, a new band corresponding to E2-Ub appeared. Both bands of E1-Ub and E2-Ub disappeared under reducing conditions, so that these conjugates have a thiol ester linkage. The maltose-binding protein–UbcH-ben fusion protein also exhibited Ub thiol ester formation (data not shown). Since the fusion protein possesses a unique cysteine residue originating in the E2 part, the thiol ester bond formation must occur at this cysteine residue. These results confirm that UbcH-ben encoded by the cloned cDNA catalyzes Ub thiol ester formation in an E1-dependent manner.

The *ben* mutation was isolated as a mutation altering synaptic connectivity between giant fibers and tergotrochanteral jump muscle motoneurons (*14, 15*). Since the *ben* mutation causes morphological abnormalities within the visual system, *ben* is thought to function in a number of developmental processes. Based on the results of Northern blot hybridization, the human structural homolog of *ben* is expressed in various tissues. Furthermore, the existence of the yeast homolog implies that this E2 is related to a basic biological process in the cell. To elucidate the role of this protein, we need to identify the expressing cells in the tissues and the timing of the expression as well as to identify the target protein ubiquitinated by E2. The cloned cDNA and recombinant E2 protein will help us to investigate the physiological role of this protein.

REFERENCES

1. Hershko, A. and Ciechanover, A. (1992) The ubiquitin system for protein degradation. *Annu. Rev. Biochem.* **61**, 761-807
2. Jentsch, S. (1992) The ubiquitin-conjugation system. *Annu. Rev. Genet.* **26**, 179-207
3. Schneider, R., Eckerskorn, C., Lottspeich, F., and Schweiger, M. (1990) The human ubiquitin carrier protein E2 (M_r=17000) is homologous to the yeast DNA repair gene RAD6. *EMBO J.* **9**, 1431-1435
4. Koken, M.H.M., Reynolds, P., Jaspers-Dekker, I., Prakash, L., Prakash, S., Bootsma, D., and Hoeijmakers, J.H.J. (1991) Structural and functional conservation of two human homologs of the yeast DNA repair gene RAD6. *Proc. Natl. Acad. Sci. USA* **88**, 8865-8869
5. Liu, Z., Diaz, L.A., Haas, A.L., and Giudice, G.J. (1992) cDNA cloning of a novel human ubiquitin carrier protein. An antigenic domain specifically recognized by endemic pemphigus foliaceus autoantibodies is encoded in a secondary reading frame of this human epidermal transcript. *J. Biol. Chem.* **267**, 15829-15835
6. Plon, S.E., Leppig, K.A., Do, H.-N., and Groudine, M. (1993) Cloning of the human homolog of the CDC34 cell cycle gene by complementation in yeast. *Proc. Natl. Acad. Sci. USA* **90**, 10484-10488
7. Kaiser, P., Seufert, W., Höfferer, L., Kofler, B., Sachsenmaier, C., Herzog, H., Jentsch, S., Schweiger, M., and Schneider, R. (1994) A human ubiquitin-conjugating enzyme homologous to yeast UBC8. *J. Biol. Chem.* **269**, 8797-8802
8. Scheffner, M., Huibregtse, J.M., and Howley, P.M. (1994) Identification of a human ubiquitin-conjugating enzyme that mediates the E6-AP-dependent ubiquitination of p53. *Proc. Natl. Acad. Sci. USA* **91**, 8797-8801
9. Rolfe, M., Beer-Romero, P., Glass, S., Eckstein, J., Berdo, I., Theodoras, A., Pagano, M., and Draetta, G. (1995) Reconstitution of p53-ubiquitinylation reactions from purified components: The role of human ubiquitin-conjugating enzyme UBC4 and E6-associated protein (E6AP). *Proc. Natl. Acad. Sci. USA* **92**, 3264-3268
10. Jensen, J.P., Bates, P.W., Yang, M., Vierstra, R.D., and Weissman, A.M. (1995) Identification of a family of closely related human ubiquitin conjugating enzymes. *J. Biol. Chem.* **270**, 30408-30414
11. Nuber, U., Schwarz, S., Kaiser, P., Schneider, R., and Scheffner M. (1996) Cloning of human ubiquitin-conjugating enzymes UbcH6 and UbcH7 (E2-F1) and characterization of their interaction with E6-AP and RSP5. *J. Biol. Chem.* **271**, 2795-2800
12. Watanabe, T.K., Fujiwara, T., Kawai, A., Shimizu, F., Takami, S., Hirano, H., Okuno, S., Ozaki, K., Takeda, S., Shimada, Y., Nagata, M., Takaichi, A., Takahashi, E., Nakamura, Y., and Shin, S. (1996) Cloning, expression, and mapping of UBE2I, a novel gene encoding a human homologue of yeast ubiquitin-conjugating enzymes which are critical for regulating the cell cycle. *Cytogenet. Cell Genet.* **72**, 86-89
13. Kato, S., Sekine, S., Oh, S.-W., Kim, N.-S., Umezawa, Y., Abe, N., Yokoyama-Kobayashi, M., and Aoki, T. (1994) Construction of a human full-length cDNA bank. *Gene* **150**, 243-250
14. Muralidhar, M.G. and Thomas, J.B. (1993) The Drosophila *bendless* gene encodes a neural protein related to ubiquitin-conjugating enzymes. *Neuron* **11**, 253-266
15. Oh, C.E., McMahon, R., Benzer, S., and Tanouye, M.A. (1994) *bendless*, a *Drosophila* gene affecting neuronal connectivity, encodes a ubiquitin-conjugating enzyme homolog. *J. Neurosci.* **14**, 3166-3179
16. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory press, New York
17. Kozak, M. (1987) An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.* **15**, 8125-8148