UbcH10 Is the Cancer-related E2 Ubiquitin-conjugating Enzyme

Yoshiaki Okamoto, Toshinori Ozaki, Kou Miyazaki, Mineyoshi Aoyama, Masaru Miyazaki, and Akira Nakagawara

Division of Biochemistry, Chiba Cancer Center Research Institute, Chiba 260-8717, Japan [Y. O., T. O., K. M., M. A., A. N.], and Department of General Surgery, Graduate School of Medicine, Chiba University, Chiba 260-0856, Japan [Y. O., M. M.]

ABSTRACT

Ubiquitin-dependent proteolysis by the 26S proteasome plays a pivotal role in cell cycle progression as well as in tumorigenesis. In this pathway, ubiquitin-conjugating enzyme (E2), together with ubiquitin ligase (E3), transfers ubiquitin to the specific substrate protein(s); however, little is known about the potential contribution of E2 to tumorigenesis. In this study, we examined the expression levels of 17 E2 genes in 25 different human normal tissues and 24 human cancerous cell lines by using a quantitative real-time reverse transcription-PCR. Among the E2 gene family, the expression level of UbcH10 was extremely low in many of the normal tissues but prominent in the majority of cancerous cell lines. Intriguingly, UbcH10 was expressed at high levels in primary tumors derived from the lung, stomach, uterus, and bladder as compared with their corresponding normal tissues, suggesting that UbcH10 is involved in tumorigenesis or progression of the tumor. To further investigate a possible contribution of UbcH10 to malignant transformation and tumor cell proliferation, NIH3T3 cells were transfected with the expression plasmid encoding UbcH10, and stable transfectants were subsequently established. UbcH10-overexpressing cells exhibited an increased incorporation of bromodeoxyuridine, an enhanced growth rate, an increase in saturation density, and a promotion of colony formation in soft agar medium as compared with parental NIH3T3 cells and the control transfectants. Collectively, our present results provide the first evidence that UbcH10 is highly expressed in various human primary tumors and that UbcH10 has an ability to promote cell growth and malignant transformation.

INTRODUCTION

Ubiquitination-dependent proteolysis is closely related to diverse cellular processes including cell cycle progression, signal transduction, and differentiation (1, 2). In this system, substrate proteins are processed for degradation by three distinct enzyme activities including the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3; Refs. 1 and 2). The E1 protein activates ubiquitin in an ATP-dependent manner and then transfers it to the E2 protein through a thiol-ester linkage. Eventually, ubiquitin is transferred from the E2 protein to a lysine residue of the specific substrate protein, which requires the E3 ligase activity. Polyubiquitinated proteins are recognized by the 26S proteasome and rapidly degraded (1, 2). Accumulating evidence suggests that E3 protein plays an important role in the regulation of the cell cycle progression as well as in tumorigenesis. MDM2, which acts as an E3 ubiquitin ligase for tumor suppressor p53, directly mediates the ubiquitination and subsequent degradation of p53 (3–5). Deubiquitination of p53 by HAUSP results in the stabilization of p53 to induce p53-dependent growth suppression and apoptosis (6). Overexpression of Eflp, which is a Ring finger-dependent E3 ligase and mediates proteolysis of the negative cell cycle regulator 14-3-3, promotes breast tumor growth (7).

In addition, the APC, which acts as an E3 ligase at mitosis, is required for the destruction of mitotic cyclins and thereby allows progression through mitosis and mitotic exit (8–11). These findings strongly suggest that down-regulation of the growth and/or tumor suppressor by ubiquitin-dependent breakdown contributes to cell cycle progression and/or tumor cell proliferation, respectively.

All known E2 proteins are structurally related and share a conserved domain of M, 16,000 that carries the cysteine residue required for the formation of ubiquitin-E2 thiol ester (12). E2 protein catalyzes the E3-dependent multiple ubiquitination that leads to degradation of substrate proteins, and various E2 and E3 proteins function in cognate pairs and provide specificity in substrate protein ubiquitination (12, 13). Recent work provides evidence that various E2 proteins play a cell cycle-regulatory role. It has been shown that UbcH10 or Ubc4 is required for APC-dependent ubiquitination of mitotic cyclins (9, 14, 15), and dominant-negative UbcH10 blocks the ubiquitination as well as the destruction of mitotic cyclins and causes cells to accumulate in mitosis (16). In addition, Ubc2/Rad6 and Ubc3/CDC34 are specifically involved in the ubiquitination-dependent degradation of the cyclin-dependent kinase inhibitor p27 (17). Intriguingly, the expression level of UbcH10 is up-regulated in NIH3T3 cells transformed by a EWS-FLI1 fusion gene associated with Ewing’s sarcoma (18). Recently, it has been shown that the expression levels of the ubiquitin-conjugating enzyme gene Ubc9 are increased in human lung adenocarcinomas compared with those of their corresponding normal tissues (19). Thus, certain E2 proteins could be closely linked to the cell cycle progression and/or tumorigenesis. However, little is known about the potential contribution of E2 protein to the tumorigenic response mediated by ubiquitination-dependent proteolysis.

In the present study, we examined by quantitative real-time RT-PCR the expression patterns of 17 E2 genes in 25 different human normal tissues, 24 human cancerous cell lines, and various primary tumors and their corresponding normal tissues. We found that UbcH10 was highly expressed in numerous cancerous cell lines and various primary tumors as compared with matched normal tissues. Additionally, overexpression of UbcH10 in NIH3T3 cells promoted deregulated cell growth and also induced anchorage-independent growth.

MATERIALS AND METHODS

Cell Culture and Transfection. Human osteosarcoma cell lines OST and SAOS-2; human colorectal adenocarcinoma cell lines COLO320, SW480, and LoVo; human hepatocellular carcinoma cell line HepG2; human breast cancer cell lines MB453 and MB231; human malignant melanoma cell line G-361; human amelanotic melanoma cell line C32TG; human thyroid medullary carcinoma cell line TCT2C2; human pancreas adenocarcinoma cell line ASPC-1; and human lung adenocarcinoma cell line A549 were obtained from American Type Culture Collection. Human esophageal squamous cell carcinoma cell line EC-GI-10 was obtained from RIKEN Cell Bank. Human gastric carcinoma cell line Kato-III was obtained from Japanese Cancer Research Resources Bank. Human rhabdomyosarcoma cell lines RMS-NK and ASPS-KY were kind gifts to Dr. A. Nishimura (National Cancer Center Research Institute). Human neuroblastoma cell

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lines SMS-KAN, SMS-KCN, IMR32, SK-N-AS, SK-N-DZ, and T2G were kind gifts from Dr. G. M. Brodeur (The University of Pennsylvania). Human neuroblastoma cell line NB-1 was kindly provided by Dr. S. Miyake (Kyoto Prefectural University of Medicine). Cells were maintained in DMEM or RPMI 1640 supplemented with 10% heat-inactivated CS (Invitrogen, Carlsbad, CA), 2 mM t-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37°C in an atmosphere of 5% CO2 in air. For transfection, NIH3T3 cells were stably transfected with the backbone expression plasmid (pcDNA3) or the expression plasmid encoding human UbcH10 by using Lipofectin transfection reagent (Invitrogen) according to the manufacturer’s instructions. Forty-eight h after transfection, cells were grown in culture medium containing 800 μg/ml G418 for 2 weeks. The resulting drug-resistant colonies were isolated and assayed for UbcH10 expression by Western analysis.

**Tumor Samples.** Surgically resected tumor tissues and their corresponding normal tissues used in this study were obtained as frozen specimens from Chiba Cancer Center Hospital between 1995 and 1996 (Chiba, Japan). These tumors included six lung adenocarcinomas, three gastric adenocarcinomas, two uterine carcinomas, and six bladder carcinomas.

**Plasmids.** To obtain full-length human UbcH10 cDNA, total RNA prepared from human fetal liver was reverse transcribed using oligo(dt) and Superscript II reverse transcriptase (Invitrogen). The subsequent PCR was performed with the following oligonucleotide primers: UbcH10 sense, 5′-CGCCGATTCAGCGGTTCTCCAAAAC-3′; and UbcH10 antisense, 5′-TATAGGCGGCGACAAAAGGAGACAGACAT-3′. These sense and antisense oligonucleotide primers were synthesized based on the nucleotide sequence databases (GenBank and European Molecular Biology Laboratory) and contained an engineered EcoRI and NotI restriction site (underlined), respectively. The specific amplified fragment was gel purified, digested completely with EcoRI and NotI, and subcloned into the identical restriction sites of the pcDNA3-FLAG expression plasmid to give pcDNA3-FLAG-UbcH10. These expression plasmids was sequenced to confirm in-frame fusion of the UbcH10 and FLAG tag.

**Immunofluorescence.** Transfected cells were grown on coverslips. After incubation for 36 h, cells were washed with ice-cold PBS, fixed with 3.7% formaldehyde in PBS for 30 min, permeabilized with 0.2% Triton X-100 for 5 min, and then blocked in PBS supplemented with 3% BSA. The cells were sequentially treated with a monoclonal anti-FLAG antibody (M2; Sigma Chemical Co., St. Louis, MO) and a rhodamine-conjugated goat antimouse IgG antibody (Cell Signaling Technology, Beverly, MA). Enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Piscataway, NJ) was used for detection.

**BrdUrd Incorporation during DNA Synthesis.** Cells were seeded at a density of 1 × 10^3 cells/well in 60-mm dish. After incubation for 36 h, cells were treated with 10 μM BrdUrd (Roche Molecular Biochemicals, Indianapolis, IN) for 10 min. Cells were then washed three times with ice-cold PBS and fixed with 70% ethanol for 20 min at −20°C. After washing with PBS, cells were incubated with a monoclonal anti-BrdUrd antibody diluted 1:100 in incubation buffer [66 mM Tris-Cl (pH 7.5), 0.66 mM MgCl2, and 1 mM β-mercaptoethanol] for 1 h at 37°C, washed three times with PBS, incubated with a FITC-conjugated goat anti-FLAG secondary antibody diluted 1:20 in PBS for 1 h at 37°C, and again washed three times with PBS. Cell nuclei were stained with propidium iodide. The stained cells were visualized under a confocal laser scanning microscope (Olympus, Tokyo, Japan).

**Cell Proliferation.** To evaluate cell proliferation, cells were plated on 24-well cell culture dishes at a density of 5 × 10^3 cells/well in 1 ml of culture medium containing 10% or 2% CS. Cells were allowed to adhere to the bottom of the cell culture dish for 24 h. At the indicated time points, cells were trypsinized, and cell counting was carried out in triplicates using a Coulter Counter (Coulter Electronics Ltd., Hialeah, FL).

**Quantitative Real-Time RT-PCR.** Total RNA was isolated using TRizol reagent (Invitrogen) according to the manufacturer’s protocol. Twenty-five μg of total RNA per lane were electrophoresed in a formaldehyde-containing 1% agarose gel and transferred onto a nylon membrane filter (Hybond-N+; Amersham Pharmacia Biotech) by capillary diffusion in 20× SSC. The filter was fixed by UV irradiation and then hybridized overnight at 65°C in a solution containing 7.5% dextran sulfate, 1 mM NaCl, 0.1% N-lauroyl sarcosine, 2.5 μg/ml heat-denatured salmon sperm DNA, and a radiolabeled probe. The filter was washed twice in 0.5× SSC/0.1% N-lauroyl sarcosine at 50°C and subjected to autoradiography.
Expression of 17 Ubiquitin-conjugating Enzymes (E2) Genes in Human Normal Tissues and Cancer Cell Lines. To search for genes encoding ubiquitin-conjugating enzymes (E2s), we performed a BLAST analysis of nucleotide and protein sequence database (DDBJ) using both full-length and amino acid residues 60–91 of Ubch5A as query. As listed in Table 1, 17 clones, including Ubch5A, matched this search. To examine the tissue distributions of 17 E2 genes, the primer/probe set was designed for each gene, and total RNA prepared from 25 different human normal tissues was subjected to the quantitative real-time RT-PCR analysis. The expression level of each E2 mRNA was normalized to that of GAPDH. Fig. 1A shows the expression patterns for these genes in each of the normal tissues. First, we observed that all of the E2 mRNAs that we examined were undetectable in skeletal muscle and expressed at extremely low levels in colon and heart. Among them, Ubch1A, Ubch1B, and Ubch5C were expressed at high levels in placenta and pancreas, and Ubch1B, Ubch5B, Ubch12, Ubch13, and Ubch17 were highly expressed in testis. In addition, the expression levels of Ubch6, Ubch16, and Ubch19 were high in pancreas. Of note, expression levels of Ubch10 and Ubch17 in the majority of normal tissues were significantly lower than those of the other E2 genes.

We next measured the expression levels of the above-mentioned E2 gene family in 24 human cell lines derived from cancers of multiple origins using quantitative real-time RT-PCR. As seen in Fig. 1B, Ubch1A, Ubch10, and Ubch16 were expressed at high levels in osteosarcoma Osteosarcoma and neuroblastoma TCGW cells showed high levels of Ubch6, Ubch10, and Ubch17 mRNA. In contrast to the results obtained from the expression analysis in normal tissues, Ubch10 was expressed at a high level in the majority of cancerous cell lines examined as compared with the other E2 genes.

Ubch10 mRNA Is Expressed at High Levels in Primary Cancerous Tissues of the Lung, Stomach, Uterus, and Bladder. The elevated level of Ubch10 expression in certain cancerous cell lines suggests that Ubch10 might be expressed at high levels in human primary tumor tissues. Therefore, we performed quantitative real-time RT-PCR to examine the expression level of Ubch10 in various human tumor tissues derived from six lung adenocarcinomas, three gastric adenocarcinomas, two uterine carcinomas, and six bladder carcinomas as well as their corresponding normal tissues. Our results showed that Ubch10 mRNA expression was significantly increased in all of the
tumor tissues we examined as compared with the matched normal tissues, suggesting that up-regulation of UbcH10 expression contributes to the occurrence or progression of various human tumors (Fig. 2A). To validate the expression changes detected by quantitative real-time RT-PCR analysis, we performed Northern blot analysis with the same RNA samples used for the quantitative real-time RT-PCR analysis. As shown in Fig. 2B, UbcH10 mRNA (approximately 2.0 kb) was highly expressed in all of the investigated tumor tissues. In contrast, UbcH10 mRNA was expressed at extremely low levels in matched adjacent normal tissues, correlating well with the results obtained by the quantitative real-time RT-PCR analysis for UbcH10.

Generation of Stable Transfectants Overexpressing UbcH10. To examine a possible role of UbcH10 in the cell growth regulation, mouse fibroblast NIH3T3 cells were stably transfected with a cytomegalovirus promoter-driven expression plasmid pcDNA3 (containing resistance to G418) encoding human full-length UbcH10 tagged with the FLAG peptide on its NH2 terminus (pcDNA3-FLAG-UbcH10). The transfected cells were selected in the presence of G418. After 2 weeks of selection, we obtained several drug-resistant transfectants. Western blot analysis using a monoclonal anti-FLAG antibody revealed that UbcH10-2, UbcH10-12, UbcH10-17, UbcH10-20, UbcH10-40, and UbcH10-59 cells expressed relatively larger amounts of UbcH10 than UbcH10-7 and UbcH10-15 cells, whereas FLAG-UbcH10 was not detected in the parental NIH3T3 cells and NIH3T3 cells transfected with an empty plasmid (V-15 and V-29; Fig. 3A). The equal protein loading was confirmed by reprobing the blot with a polyclonal anti-actin antibody (Fig. 3B). The equal protein loading was confirmed by reprobing the blot with a polyclonal anti-actin antibody (bottom panel). A similar result was obtained in a separate experiment in which UbcH10-20 cells were used. Therefore, we selected UbcH10-17 and UbcH10-20 cells for further analysis.

Overexpression of UbcH10 Stimulates Growth and Colony-forming Activity in NIH3T3 Cells. We next examined the possible effect(s) of UbcH10 on the initiation of DNA synthesis. To this end,
V-15 and UbcH10-17 cells were grown in the presence of BrdUrd (at a final concentration of 10 μM), and the number of cells that were stained with an antibody against BrdUrd was scored. As shown in Fig. 4, UbcH10-17 cells incorporated BrdUrd much more than the control transfectants. On average, 52.3% of UbcH10-17 cells and 28% of V-15 cells entered into S phase as indicated by incorporation of BrdUrd, respectively. These results strongly suggest that overexpression of UbcH10 stimulated an ability of cells to proceed through the S phase.

Next, we examined the growth of parental NIH3T3, V-15, UbcH10-17, and UbcH10-20 cells in different serum culture conditions (10% and 2% serum). The growth rate of each cell line was determined by counting the number of cells daily. As shown in Fig. 5A, UbcH10-overexpressing transfectants grew at a much faster rate than NIH3T3 or the control transfectants. Similar results were obtained under a low serum culture condition (Fig. 5B). In addition, cells overexpressing UbcH10 overgrew a monolayer by doubling the saturation density of control cells (Table 2). The accelerated growth rate of UbcH10-overexpressing transfectants raised the possibility that they might have become transformed. To this end, we examined their ability to grow in soft agar medium. As shown in Fig. 5C, UbcH10-17 and UbcH10-20 cells formed colonies in soft agar medium (13 ± 1 and 14 ± 2 colonies/dish, respectively) more efficiently than the parental and the empty plasmid-transfected V-15 cells (4 ± 1 and 2 ± 1 colonies/dish, respectively). Under our experimental conditions, NIH3T3 transfectants overexpressing UbcH10 created distinct colonies in soft agar medium after 3 weeks; however, we could detect only small colonies after 2 weeks of culture. Taken together, our present results provide the first evidence that the E2 ubiquitin-conjugating enzyme gene, UbcH10, is highly expressed in various human primary tumors compared with their corresponding normal tissues and that UbcH10 has an ability to promote cell growth and transformation.
**DISCUSSION**

In the present study, we evaluated the expression level of the E2 gene family in a wide variety of human normal tissues and 24 cell lines derived from multiple tumor origins by quantitative real-time RT-PCR analysis. We found that, among the 17 E2 genes examined, **UbcH10** was expressed at very low to undetectable levels in normal tissues, whereas numerous cancerous cell lines expressed it at high levels. Of note, **UbcH10** was highly expressed in primary tumors of the lung, stomach, uterus, and bladder compared with the matched normal tissues. Consistent with the above-mentioned observations, overexpression of **UbcH10** caused an efficient incorporation of BrdUrd, an accelerated growth rate, an increase in saturation density, and a promotion of anchorage-independent growth. These data provide evidence that the deregulated overexpression of **UbcH10** may lead to growth promotion as well as malignant transformation and that **UbcH10** may be an essential oncogenic factor in a variety of tumors.

In our experiments, the expression of **UbcH10** in tumor tissues as well as cancerous cell lines was examined by RNA levels. As described by Shekhar et al. (21), the expression levels of HR6B protein, which is the mammalian homologue of yeast ubiquitin-conjugating enzyme Rad6, in mouse mammary tumor cells were in good agreement with those detected by RT-PCR. Additionally, Arvand et al. (18) reported that endogenous **UbcH10** was up-regulated at both the mRNA and protein level in transformed NIH3T3 cells, suggesting that the expression level of **UbcH10** mRNA might reflect in part the intracellular level of **UbcH10** protein. To confirm our present results, it is necessary to analyze **UbcH10** protein levels in tumor tissues as well as cancerous cell lines.

**UbcH10** has been identified as a human homologue of the cyclin-selective E2 (E2-C) that is required for the destruction of mitotic cyclins (14–16), Townsley et al. (16) described that enforced expression of the dominant-negative type of **UbcH10** inhibited the ubiquitination and the subsequent degradation of mitotic cyclins. In accordance with the above-mentioned observations, **UbcH10** is functionally associated with the APC, which acts as the E3 ubiquitin ligase to catalyze the transfer of ubiquitin to mitotic cyclins (9, 14, 15). APC is activated during mitosis, remains active throughout the G2 phase, and is degraded at the G1-S boundary (22, 23). Intriguingly, Arvand et al. (18) reported that **UbcH10** protein was highly expressed in G2-M phase, but its expression level was extremely low in G0-G1 phase. Yamanaka et al. (24) found that **UbcH10** that contains the destruction box (D box) underwent APC-dependent degradation at early G1 phase, suggesting that the cell cycle-dependent expression of **UbcH10** might be a unique autoregulatory feedback loop for the regulation of APC activity. Consistently, the dominant-negative **UbcH10** arrests mammalian cells in M phase and inhibits the onset of anaphase, it is possible that the function of **UbcH10** is closely linked to cell cycle progression (16). Thus, the enforced overproduction of **UbcH10** might disrupt the autoregulatory feedback loop and thereby lead to deregulated cell growth; however, the precise molecular mechanisms by which **UbcH10** promotes cell growth are unclear.

Recently, Shekhar et al. (21) found that overexpression of **Ubc2/Rad6** induced anchorage-independent growth of recipient cells, indicating that deregulated expression of **Ubc2/Rad6** is involved in malignant transformation. In addition, McDoniels-Silvers et al. (19) reported that the expression levels of **Ubc9** were increased in human lung adenocarcinomas compared with those of their corresponding normal tissues. These findings imply that certain E2 proteins might be closely linked to tumorogenesis. As described previously, overexpression of EWS/FLI1 in NIH3T3 cells induced anchorage-independent growth in soft agar medium and generated tumors in nude mice (25–28). Of note, Arvand et al. (18) reported that endogenous **UbcH10** was up-regulated in NIH3T3 cells transformed with EWS/FLI1 but not in nontransformed NIH3T3 cells, indicating that **UbcH10** could play an important regulatory role in the transformation. According to their results, however, stable NIH3T3 transfectants overexpressing mouse **UbcH10** did not form colonies in soft agar medium under their experimental conditions. They suggested that other gene products in addition to **UbcH10** might be required for cellular transformation. Their findings differ from our present results, which showed **UbcH10**-induced colony formation in soft agar medium. Intriguingly, they also demonstrated that YAL-7 cells expressing EWS/FLI1 did not grow in soft agar medium, and there existed a significant difference in the EWS/FLI1-mediated induction level of **UbcH10** between NIH3T3 and YAL-7 cells, raising the possibility that a certain threshold level of **UbcH10** protein might be required to render cells for being transformed. Although the underlying cause of this discrepancy is not clear, it could be explained in part by the differences in intracellular expression levels of **UbcH10** between our stable transfectants and their clones.

Pagano et al. (17) reported that **Ubc2/Rad6** and **Ubc3/CDC34** were specifically involved in the ubiquitination-dependent degradation of cyclin-dependent kinase inhibitor p27. **UbcH7/E2-F1** was reported to support E6-AP-dependent ubiquitination and function in the conjugation and subsequent degradation of tumor suppressor p53 (29–31). Thus, it is possible that **UbcH10**, together with a particular E3 protein(s), might recognize and break down substrate proteins with growth-regulatory function. In this connection, identification of target proteins of **UbcH10** should help promote understanding of its role in malignant transformation and tumor cell growth.

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**REFERENCES**

ROLE OF UbcH10 IN CANCERS


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