Cloning, Mapping, Expression, Function, and Mutation Analyses of the Human Ortholog of the Hamster Putative Tumor Suppressor Gene doc-1*

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Takanori Tsuji‡‡‡, Fuh-Mei Duh‡‡‡, Farida Latif, Nicolas C. Popescu**, Drazen B. Zimonjic**, Jim McBride†, Kou Matsuo†, Hiroe Ohyama†, Randy Todd†, Emi Nagata†, Nagaaki Terakado†††, Akira Sasaki‡‡‡, Tomohiro Matsumura‡‡‡, Michael I. Lerman‡, and David T. W. Wong§§§

From the ‡‡‡Laboratory of Molecular Pathology, Division of Oral Pathology, Harvard School of Dental Medicine, Boston, Massachusetts 02115, †††Intramural Research Support Program, SAIC Frederick and ‡‡‡Laboratory of Immunobiology, Division of Basic Sciences, NCI-Frederick Cancer Research and Development Center, Frederick, Maryland 21702, **Laboratory of Experimental Carcinogenesis, Division of Basic Sciences, NCI, National Institutes of Health, Bethesda, Maryland 20892, and the †††Department of Oral and Maxillofacial Surgery II, Okayama University Dental School, Okayama 700, Japan

doc-1 is a putative tumor suppressor gene isolated and identified from the hamster oral cancer model. Here, we report the molecular cloning and the functional characterization of the human ortholog of the hamster doc-1 gene. Human doc-1 cDNA is 1.6 kilobase pairs in length and encodes for a 115-amino acid polypeptide (12.4 kDa, pI 9.53). Sequence analysis showed 98% identity between human and hamster doc-1 protein sequences. DOC-1 is expressed in all normal human tissues examined. In oral keratinocytes, expression of DOC-1 is restricted to normal oral keratinocytes. By immunostaining of normal human mucosa, DOC-1 is detected in both the cytoplasm and nuclei of basal oral keratinocytes; while in suprabasilar cells, it is primarily found in the nuclei. Human oral cancers in vivo did not exhibit immunostaining for DOC-1. Like murine DOC-1, human DOC-1 associates with DNA polymerase α/primase and mediates the phosphorylation of the large p180 catalytic subunit, suggesting it may be a potential regulator of DNA replication in the S phase of the cell cycle. Using a human doc-1 cDNA as a probe, human doc-1 is mapped to chromosome 12q24. We identified four exons in the entire human doc-1 gene and determined the intron-exon boundaries. By polymerase chain reaction and direct sequencing, we examined premalignant oral lesion and oral cancer cell lines and found no intragenic mutations.

Squamous cell carcinoma (SCC)† of the oral cavity is newly diagnosed in 38,000 Americans each year and in 350,000 people worldwide (1, 2). Approximately half of the patients afflicted die within 5 years of diagnosis, while surviving patients may be left with severe cosmetic and/or functional compromise (1–3). Survival curves of oral cancer patients have plateaued over the past 2 decades and remained among the worst of all cancer sites.

The hamster oral cancer model is an excellent model to study the molecular event during oral carcinogenesis (4–7). doc-1 is a putative tumor suppressor gene identified and isolated from the carcinogen-induced hamster oral cancer model (8). DOC-1 is predicted to be a 114-amino acid peptide with a molecular mass of 12.4 kDa. Transfection of doc-1 into malignant oral keratinocytes led to the reversion of transformation phenotypes including anchorage independence, doubling time, and morphology. The genetic sequence of doc-1 matched to a tumor necrosis factor-α-induced early-response murine transcript, TU-166 (9), suggesting that doc-1 may be a downstream event in the tumor necrosis factor-α signaling pathway. We have recently cloned the full-length mouse doc-1 cDNA (GenBank™ number AF011644); its DNA sequence in the open reading frame is 94% identical to that of the hamster. The predicted amino acid peptides encoded by the mouse and hamster doc-1 open reading frames are identical. Each open reading frame encodes for a 114-amino acid peptide that has a predicted molecular mass of 12.4 kDa and a pI of 9.53.

The highly conserved nature of the rodent doc-1 genes prompted us to clone and examine the role of doc-1 in human oral and other cancers. This paper describes the cDNA and genomic DNA cloning of the human doc-1 gene, its chromosome localization, and its expression in normal and transformed human tissues. In addition, we examined for intragenic mutation of the human doc-1 gene. To obtain an insight into the biology of DOC-1, we made use of our recent finding that murine DOC-1 associates with DNA polymerase α/primase (DNA-PP) and regulates DNA replication in the S phase of the cell cycle. The potential interaction of human DOC-1 with DNA-PP was therefore examined.

EXPERIMENTAL PROCEDURES

Cell Culture and Culture Media—Normal, diploid human oral keratinocyte cell strains, having a limited replicative lifespan, have been cultured from floor of mouth (OKF4 and OKF6) and cryopreserved within their first two serial passages in culture. Oral squamous cell carcinoma-derived, immortal cell lines (SCC-9, SCC-15, and SCC-25) were cryopreserved within their first five serial passages in culture (10, 11). POE-1 and POE-9 are dysplastic “premalignant” oral lesions from oral carcinomas (9). POE-1 and POE-9 are dysplastic “premalignant” oral lesions from oral carcinomas (9). POE-1 and POE-9 are dysplastic “premalignant” oral lesions from oral carcinomas (9). POE-1 and POE-9 are dysplastic “premalignant” oral lesions from oral carcinomas (9).

The human oral keratinocyte cultures were grown in Life Technologies, Inc. keratinocyte serum-free medium supplemented with hydrocortisone, insulin, transferrin, 0.1 μg/ml epidermal growth factor, and 50 μg/ml bovine pituitary extract. We added additional CaCl₂ to bring the total [Ca²⁺] to 0.4 mM (12), a concentration that is sufficient to permit cadherin- and desmosome-mediated cell-cell junction formation.
This results in closer association of sister cells in growing colonies and permits stratification of terminally differentiated cells in the central regions of larger colonies, thereby aiding the identification of individual colonies and the evaluation for growth rate and relative proportions of proliferative and differentiated cells. This calcium concentration does not support proliferation of human keratinocytes. Media were supplemented with penicillin and streptomycin. Cells were cryopreserved in medium containing 10% MeSO and 10% serum and stored in liquid nitrogen freezers.

Cloning of doc-1 cDNA and Genomic DNA—Using the plaque hybridization method (13), cDNA clones were obtained by screening a human testis cDNA library (Stratagene, La Jolla, CA) with the hamster doc-1 cDNA (8). Genomic clones were isolated from a total human genomic placent al cosmid library (Stratagene, La Jolla, CA) by colony hybridization using a human doc-1 cDNA clone as a probe (14).

DNA Sequencing and Sequence Analyses—Plasmid cDNA clones and genomic cosmid clones were sequenced by automated sequencing as was described by Duh et al. (15). Sequence editing, sequence analyses, and homology search were performed following previously published methods (15).

Fluorescent in Situ Hybridization—Chromosomes obtained from human peripheral lymphocyte cultures after methotrexate/thymidine release treatments and normal mouse spleen cultures, were used for fluorescence in situ hybridization (FISH). The probes were labeled with biotin-digoxigenin using a random-prime DNA labeling kit (Boehringer Mannheim). The FISH protocol as described in detail elsewhere was followed (16, 17). The slides were pretreated with RNase, denatured in 2× SSC, 70% (v/v) formamide for 2 min at 70 °C. Human cosmid DNA probes (200 ng) together with human Cot-1 DNA (Life Technologies) in 2× SSC, 50% (v/v) formamide, 10% (w/v) dextran sulfate, 2× Denhardt’s solution, 1% Tween 20 (v/v) were denatured for 5 min at 70 °C, reannealed for 2 h at 37 °C, and hybridized in a humid environment for 18 h at 37 °C. Posthybridization final wash was in 0.1× SSC at 60 °C. Biotin and digoxigenin-labeled DNA was detected by fluorescein isothiocyanate-conjugated avidin DCS (Vector Laboratories) and rhodamine-conjugated antidigoxigenin (Boehringer Mannheim), respectively.

Chromosomes were counterstained with propidium iodide (PI) or 4,6-diamine-2-phenylindole (DAPI) and examined with a Zeiss Axios phot epifluorescent microscope with a 100-wat mercury lamp. Digital images of selected metaphase spreads were obtained using a cold charge-coupled device camera CH250 (Photometrics, Tuscon, AZ) and a filter system consisting of a triple band pass beam splitter and emission filters. Excitation of each of fluorochromes used was accomplished by single band pass excitation filters in a computer-controlled motorized filter wheel. This made it possible to acquire sequential, registration shift-free gray scale images of two or three fluorochromes (DAPI, fluorescein isothiocyanate, and/or rhodamine). Images were processed and analyzed on an Apple Power Macintosh 8100/100 computer using Oncor recording and analytic program Image, and well as NIH Image and Yale University’s Gene Join. To identify individual chromosomes and to assign a designation to specific chromosome regions, the method for direct visualization of fluorescent spots on LUT-inverted digital images of DAPI-banded chromosomes was used (18). To confirm the identity of chromosomes, preparations were rehybridized with human chromosome 12-specific painting probes (Oncor, Gaithersburg, MD), and previously observed labeled metaphases were retained.

Northern Blot Analysis—Total RNA was isolated from human oral keratinocytes using the guanidine isothiocyanate method described by Davis et al. (19). Details of Northern blot analysis using the Zetabind membrane were described previously (20, 21). Random priming was used to label the cDNA inserts.

Immunohistochemical Staining for DOC-1 in Normal and Tumor Oral Epithelia—Three normal oral mucosal tissues (two tongues and one gingival tissue sample) and five primary oral squamous cell carcinomas were subjected to DOC-1 immunohistochemical study. These tissues were obtained from biopsy and surgical operations at the Department of Oral and Maxillofacial Surgery II, Okayama University Dental School Hospital (Okayama, Japan). All samples were embedded in OCT compound without fixation, snap-frozen, and stored at −80 °C until used. Frozen sections (4–6 μm) were air-dried after sectioning and then rehydrated in phosphate-buffered saline (pH 7.4). Immunohistochemical staining was performed using a rabbit polyclonal anti-hamster DOC-1 antibody at a dilution of 1:200 to 1:500 for overnight at 4 °C. DOC-1 immunoreactivity was detected by the indirect immunoperoxidase method with an Envision kit (DAKO, Tokyo, Japan).

Interaction of DOC-1 with DNA Polymerase α/Primase—The human doc-1 cDNA was cloned into the GST fusion protein vector pGEX-4T-1

Human $doc-1$ Gene

Table I

| Exon | Length | Primer | Sequence |
|------|--------|--------|----------|
| 1    | 289    | E1F    | 5′-ACC CGG FFC CTG GGT GAG ACTG-3′ |
| 2    | 238    | E2F    | 5′-CTG TTG CGG CAG CGG ACTG-3′ |
| 3    | 266    | E3F    | 5′-TCA GCC TCC TGA ACT GGT-3′ |
| 4    | 181    | E4F    | 5′-TGC TGT GCA CCT GGT GAC-3′ |

FIG. 1. Detection of doc-1 in the genomes in various species.

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|------|---|---|---|---|---|---|---|---|---|
| kbp  | 60 | 23.1 | 23.1 | 6.6 | 6.6 | 3.3 | 3.3 | 2.3 | 2.3 |

TABLE I

From: Human $doc-1$ Gene. Published in The Journal of Biological Chemistry. 2001, 276(19), 17021-17028. doi:10.1074/jbc.276.19.17021.
we identified three splice junctions in the gene. The positions of the splice sites were positioned at the cDNA sequence (GenBank AF006484) nucleotide numbers 577 and 578, 675 and 676, and 802 and 803, respectively. The small coding exons ranged from 55 to 127 bp (Fig. 3).

Chromosome Mapping of the Human doc-1 Gene—FISH was used to map the chromosome location of the human doc-1 gene. In duplicate experiments, a high efficiency of FISH was observed when biotin- and digoxigenin-labeled genomic doc-1 probe was hybridized to normal human chromosomes. The majority of 200 metaphases had fluorescent label on the chromosomes. The majority of 200 metaphases had fluorescent label on the distal region of the short arm of both chromosomes 12 regardless of detection protocol (fluorescein isothiocyanate or rhodamine), and symmetrical fluorescent spots were detected on the long arm of chromosome 12. The signal was localized at region 12q24 in 50 nonoverlapped and variably contracted chromosomes 12 showing a G-like banding pattern generated by contrast enhancement and LUT inversion of digital images of DAPI-counterstained chromosomes (Fig. 4). Within the region 12q24, where we assign the locus of the doc-1 gene, is a common fragile site where several genes are located (22).

doc-1 Expression in Normal Human Tissues and Oral Keratinocytes—Expression of doc-1 in normal human tissues was examined using a multiple tissue Northern blot (CLONTECH) containing mRNA isolated from various normal human tissues. All tissues examined (heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas) demonstrated high cellular levels of the 1.5-kb doc-1 transcript (Fig. 5A). The bottom panel A and B shows the rehybridization of the same blot with the chicken β-actin gene to demonstrate RNA loading.

To determine whether doc-1 is differentially expressed in normal and malignant human oral keratinocytes, a Northern blot containing mRNA from cultures of two normal human oral keratinocyte lines (OKF4 and OKF6) and three malignant human oral keratinocyte lines (SCC9, SCC15, and SCC25) was hybridized with 32P-labeled human doc-1 cDNA. doc-1 transcripts were detectable only in the two normal oral keratinocyte cultures (Fig. 5B); none of the three malignant SCC lines contained detectable doc-1 mRNA. The lower panel is the rehybridization of the same blot with the chicken β-actin gene to demonstrate RNA loading.

Immunohistochemistry was used to examine DOC-1 expression in five resected oral cancers and three normal mucosal tissues. DOC-1 protein is localized to normal human oral mucosa in all three cases (Fig. 6A). DOC-1 is not detectable in four of the five oral cancer specimens (Fig. 6B). In normal oral mucosa, localization of DOC-1 protein is intense and perinuclear in the basal cells but predominantly nuclear in the spinous cell layer (Fig. 6A).

Human DOC-1 Associates with the p180 Catalytic Subunit of DNA-PP—Our laboratory has recently demonstrated that murine DOC-1 associates and mediates the phosphorylation of the p180 catalytic subunit of DNA-PP. A interaction of DOC-1 with DNA-PP is associated with suppression of DNA replica-

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3 Available on the World Wide Web at http://ch.nus.sg/bio/proscan/proscan.html.
tion, shown by the SV40 in vitro DNA replication assay. These findings provide an insight into the mechanism whereby DOC-1 can mediate its growth and/or tumor suppressor function, by regulating DNA replication in the S phase of the cell cycle.

To determine whether human DOC-1 similarly associates with DNA-PP and mediates phosphorylation of the p180 subunit, the human doc-1 cDNA was cloned in frame into the pGEX plasmid vector (pGEX-4T-1) at the SalI and NotI sites (see “Experimental Procedures”). Human DOC-1 was expressed as a GST-DOC-1 fusion protein. SDS-polyacrylamide gel electrophoresis analysis showed that the human DOC-1 peptide was correctly expressed (data not shown). Digestion with thrombin released the DOC-1 peptide at the expected size. Immunoblotting with a DOC-1-specific rabbit polyclonal antibody detected the GST-DOC-1 and DOC-1 peptides (data not shown).

500 μg of lysate prepared from the normal human oral keratinocytes OKF4 was mixed with 5 μg of GST-DOC-1 fusion protein. The DOC-1-OKF4 lysate binding was carried out at 4 °C for 18 h, followed by centrifugation to separate from unbound proteins. A standard phosphorylation assay was set up using the DOC-1-OKF4 pull-down lysate complex in the presence of [γ-32P]ATP. Fig. 7A is the autoradiograph of the phosphorylation assay showing that an ~180-kDa phosphorylated protein band can be seen in the GST-DOC-1 lysate pull-down complex (lane 1) but not in the GST lysate pull-down sample (lane 2). Fig. 7B is a Western blot of the GST pull-down assay for DNA-PP. The p180 and p70 subunits of DNA-PP are associated with the GST-DOC-1 pull-down complex (lane 1), while GST alone did not associate with any of the DNA-PP subunits (lane 2). To confirm that human DOC-1 can associate with DNA-PP and mediates the phosphorylation of the p180 subunit, GST-DOC-1 was used in an in vitro pull-down/phosphorylation reaction with baculovirus-expressed recombinant human DNA-PP (panel C). Fig. 7C showed that GST-DOC-1 associates with recombinant DNA-PP and mediated the phosphorylation of the p180 subunit (lane 1), while GST alone did not mediate the phosphorylation of the p180 subunit.

Intragenic Mutation Analysis of the Coding Exons of Human doc-1 Gene in Normal and Malignant Oral Keratinocyte Cell Lines—The availability of the intron-exon boundaries and the flanking sequences allow the design of primers to amplify the exon sequences to compare the coding regions of
doc-1 gene between normal and tumor oral keratinocytes. We performed mutation analysis on two premalignant (POE-1 and POE-9) and three oral cancer cell lines (SCC-9, SCC-15, and SCC-25) using the four primer sets in Table I and did not detect any intragenic mutation.

**DISCUSSION**

This paper presents the cDNA and genomic cloning as well as partial characterizations of the human doc-1 gene. doc-1 is a highly conserved gene present in the genomes of all mammalian species examined. doc-1 mapped to human chromosome 12q24. Expression of DOC-1 can be detected in all normal human tissues examined. Interestingly, expression of doc-1 is not detectable in malignant oral keratinocytes, in cell lines, and in primary tumor tissues. Intragenic mutation analysis of the doc-1 in premalignant and malignant oral keratinocytes did not reveal any mutations. Like the hamster DOC-1, human DOC-1 associates with DNA-PP and mediates the phosphorylation of the p180 subunit.

The existence of a human homolog and the highly conserved nature of the doc-1 gene prompted us to examine the role of the doc-1 gene in human oral and other forms of cancers. The doc-1 gene was initially identified and cloned from the hamster oral cancer model. In this chemically induced oral cancer model, expression of doc-1 is consistently reduced and/or lost. Loss of heterozygosity was observed in two out of three malignant oral keratinocyte cell lines (8). Transfection of doc-1 into malignant hamster oral keratinocytes altered a number of phenotypes in culture including anchorage dependent growth, doubling time, and morphology. 83% of the doc-1 transfectants lost the ability to grow in soft agar ($p < 0.05$).

The human doc-1 gene was mapped to chromosome 12q24. While this site has not been noted to be altered in oral/head and neck cancers, 12q24 is a recurrent break point in high grade B cell non-Hodgkin lymphoma (23). The cytogenetic abnormalities included both translocations and interstitial deletions (22).

Expression of doc-1 is consistently reduced/lost in transformed oral keratinocytes. Neither doc-1 mRNA nor protein could be detected in the malignant human keratinocyte cell lines or in freshly resected oral cancers. While no intragenic mutations could be detected in the three tumor cell lines examined, a much larger sample size of human oral and nonoral tumor cell lines needs to be examined to evaluate the potential involvement of the doc-1 gene in carcinogenesis. In addition, cis-regulatory modifications of the doc-1 promoter such as CpG methylation can alter expression of the gene, which has been demonstrated to be of importance in the inactivation of p16$^{ink4A/CDKN2A}$ (24).

The association of human DOC-1 with DNA-PP is of importance and sheds some light on the biochemical function of the doc-1 gene. DNA-PP is the only eukaryotic enzyme that can initiate DNA replication de novo. Based on data from our murine DOC-1 studies, we propose that DOC-1 is a negative regulator of DNA replication through the phosphorylation of the p180 subunit of DNA-PP. In tumor cells, where DOC-1 expression is absent or reduced, these DOC-1-mediated DNA-PP regulatory mechanisms might be compromised.

We are beginning to understand the biochemical function of doc-1. DOC-1 is likely to be a regulator of DNA replication in the S phase of the cell cycle, of importance in normal cells as well as in carcinogenesis. Gordon et al. (9) have shown that doc-1 is a tumor necrosis factor-$\alpha$-inducible gene, suggesting that doc-1 is a downstream event in the tumor necrosis factor-$\alpha$ signaling pathway. Recently, Hatakeyama et al. (25) have shown that DOC-1 is constitutively ubiquitinated in vivo and is a substrate of the mammalian ubiquitin ligase Nedd-4 (neural precursor cells expressed developmentally down-regulated) (25), thus providing a mechanism whereby intracellular DOC-1 activity is tightly regulated by the ubiquitin-mediated proteosomal degradation pathway (26). The interconnections of DOC-1 and these various signaling pathways are of importance in carcinogenesis and are now being addressed in our ongoing investigations.

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**FIG. 7.** Human DOC-1 associates with and mediates phosphorylation of the p180 subunit of DNA-PP in human OKF4 oral keratinocytes. Panel A, GST-DOC-1 associates with a phosphorylated cellular protein of about ~200 kDa. Panel B, Western blot of the GST pull-down assay for DNA-PP. Panel C, GST-DOC-1 associates with recombinant DNA-PP and mediates phosphorylation of the p180 subunit. Molecular weight markers are indicated on the left. The position of p180/DNA-PP is indicated (arrow).
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