Differential expression, localization and activity of two alternatively spliced isoforms of human APC regulator CDH1

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The timely destruction of key regulators through ubiquitin-mediated proteolysis ensures the orderly progression of the cell cycle. The APC (anaphase-promoting complex) is a major component of this degradation machinery and its activation is required for the execution of critical events. Recent studies have just begun to reveal the complex control of the APC through a regulatory network involving WD40 repeat proteins CDC20 and CDH1. In the present paper, we report on the identification and characterization of human CDH1 β , a novel alternatively spliced isoform of CDH1. Both CDH1 α and CDH1 β can bind to the APC and stimulate the degradation of cyclin B1, but they are differentially expressed in human tissues and cells. CDH1 α contains a nuclear localization signal which is absent in CDH1 β . Intra-

cellularly, CDH1 α appears in the nucleus whereas CDH1 β is a predominantly cytoplasmic protein. The forced overexpression of CDH1 α in cultured cells correlates with the reduction of nuclear cyclin A, but the steady-state amount of cyclin A does not change noticeably in CDH1 β -overexpressed cells. In *Xenopus* embryos, ectopic overexpression of human CDH1 α , but not of CDH1 β , induces cell-cycle arrest during the first G₁ phase at the midblastula transition. Taken together, our findings document the differential expression, subcellular localization and cell-cycle-regulatory activity of human CDH1 isoforms.

Key words: anaphase-promoting complex (APC), CDH1, cell-cycle control.

INTRODUCTION

The APC (anaphase-promoting complex) is a major cellular ubiquitination system that controls the precise order and timing of the eukaryotic cell cycle by targeting mitotic cyclins, anaphase inhibitors and other regulators for degradation by the proteasome [1,2]. The composition of the APC is highly conserved in eukaryotes. The APC activity is tightly regulated during the cell cycle through phosphorylation and dephosphorylation of its subunits [3,4]. In addition, biochemical and genetic analyses in yeast [5,6], fruit flies [7–9] and frogs [10] have identified the homologous proteins CDC20/Fizzy and CDH1/Fizzy-related/HCT1 as activators and specificity factors of APC.

CDC20 and CDH1 are members of the WD40 repeat protein superfamily. They bind transiently to the APC and are thought to facilitate ubiquitination through direct interaction with different sets of targets [11,12]. Many of these targets share a recognition motif known as the D box [13,14]. Distinct motifs termed the KEN box [15,16] and the A box [17] have also been identified in substrate proteins recognized by the CDH1-activated APC.

The abundance of CDC20 and CDH1 peaks in G₂/M and drops abruptly at the exit from mitosis [4,14,18,19]. CDH1 is widely expressed in differentiated tissues including postmitotic neurons [20], but its expression is significantly decreased during malignant progression of a B-lymphoma cell line [21]. In keeping with this, genetic analysis in *Caenorhabditis elegans* indicates that CDH1 regulates cell proliferation [22]. Both CDC20 and CDH1 can be phosphorylated [12]. While it remains controversial as to whether phosphorylation of CDC20 may stimulate [3], inactivate [23] or have no influence on the APC [4], the inhibitory

phosphorylation of CDH1 has been demonstrated by several groups [3,24,25].

The APC has emerged as a downstream target of the mitotic checkpoint, which prevents the onset of anaphase until all chromosomes are properly aligned [26]. To date, more than six components (MAD1, MAD2, MAD3, BUB1, BUB2, and BUB3) of the mitotic checkpoint have been identified. Both MAD2 and BUBR1 associate with and inhibit CDC20, thereby transducing a stop signal to APC [27]. In addition, yeast BUB2 and human MAD2B/MAD2L2 can target CDH1 [28–30]. However, the mechanisms by which BUBR1, MAD2 and MAD2B inhibit CDC20/CDH1 remain to be elucidated.

We have previously characterized human MAD1 and MAD2 [31]. Given that CDC20 and CDH1 are effectors of MAD1 and MAD2, we set out to characterize CDC20 and CDH1 in human cells. In the present study, we identified and characterized a novel alternatively spliced isoform of human CDH1. We demonstrated the differential expression, subcellular localization and APC-activating activity of the two functional isoforms of CDH1, designated CDH1 α and CDH1 β . Our findings suggest an additional level of spatial regulation in the activation of the APC.

EXPERIMENTAL

Antibodies

Rabbit polyclonal anti-CDH1 antibody α -CDH1C was raised against a C-terminal peptide (amino acids 472–493 of human CDH1 α , underlined in Figure 1A) conjugated to keyhole limpet haemocyanin. Rabbit polyclonal anti-CDH1 antibody α -CDH1N

Abbreviations used: APC, anaphase-promoting complex; CDK, cyclin-dependent kinase; HA, haemagglutinin; GFP, green fluorescent protein; MBT, midblastula transition; NFIC, nuclear factor I/C; RT, reverse transcription.

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The human CDH1 α , human CDH1 β and mouse CDH1 nucleotide sequences reported have been deposited in the DDBJ, EMBL, GenBank® and GSDB nucleotide sequence databases under accession numbers AF083810, AF433157 and AF083809 respectively.

Figure 1 CDC20 and CDH1 represent two families of WD40 proteins

(A) Amino-acid sequence of human and mouse CDH1. The human sequence is in capitals. The mouse sequence, where different from the human sequence, is indicated above in lower case letters in parentheses. Sequences of the N-terminal and C-terminal synthetic peptides used to raise antisera in rabbits are underlined. Putative CDK phosphorylation sites are shaded. Residues absent from the alternatively spliced variant CDH1 β are boxed. The positively charged residues that act putatively as a nuclear localization signal are indicated by #. The seven WD40 repeats are indicated. (B) Consensus phylogenetic tree of all CDC20/CDH1-related proteins in the extant databases. Phylogenies were inferred from protein sequences aligned with the ClustalW program (<http://www.ch.embnet.org/software/ClustalW.html>). Kimura's distances were computed with the ProtDist program in the Phylip package (version 3.573; J. Felsenstein, University of Washington). Tree reconstruction was based on the neighbour-joining method (Neighbor). SeqBoot and Consensus programs in the same package were used to perform bootstrap replication and to produce the majority rule consensus tree from 100 replicates. Numbers on the nodes are bootstrap confidence probabilities (%). GenPept (gp), EMBL (emb), PIR (pir), or SWISS-PROT (sp) protein accession numbers of the sequences are: CDC20B-thale cress, gp/AF160760; CDC20C-thale cress, pir/T01768; CDC20-rape, emb/CAA11819.1; CDC20A-thale cress, gp/AAF14048.1; CDC20-carrot, pir/T14352; CDC20-rat, gp/AAC14741.1; CDC20-human, gp/AAD16405.1; CDC20-frog, gp/AAC41376.1; CDC20-clam, gp/AAC06232.1; CDC20A-fission yeast, pir/T41719; CDC20-budding yeast, gp/BAA03957.1; CDC20-nematode, pir/T27762; CDC20-trichomonad, gp/AAB51112.1; CDC20B-fission yeast, pir/T41034; CDC20C-fission yeast, pir/T41148; AMA1/CDC20B-budding yeast, gp/NP_011741.2; CDH1-mouse, gp/AAD52029.1; CDH1-human, gp/AAC62835.1; CDH1-frog, emb/CAA74576.1; CDH1-fly, gp/AAF45973.1; CDH1-nematode, pir/T27730; CDH2-fly, gp/AAF47111.1; CDH1-barrel medic, gp/AAD22612.1; CDH1-thale cress, pir/T09351; CDH1 β -fission yeast, pir/T40614; CDH1 α -fission yeast, pir/T37680; CDH1-budding yeast, gp/NP_011512.1.

was raised to a keyhole-limpet-haemocyanin-conjugated peptide corresponding to the N-terminus of human CDH1 (amino acids 2–21; underlined in Figure 1A). Mouse monoclonal anti-FLAG antibody M2 was from Sigma. Mouse monoclonal anti-HA (haemagglutinin) antibody F-7, rabbit polyclonal anti-cyclin A antibody H-432 and rabbit polyclonal anti-CDC27 antibody H-300 were from Santa Cruz Biotechnology.

RNA and protein analyses

Human multiple tissue Northern blots (Clontech) were probed with a 307 bp ³²P-labelled *NcoI*–*NaeI* fragment shared by both CDH1 α and CDH1 β cDNAs. A 2 kb human β -actin cDNA was used as a control for RNA loading. Northern blotting was carried out according to the manufacturer's protocol.

Polyadenylated RNAs from human whole brain, heart, liver and spleen were purchased from Clontech. Polyadenylated RNAs from cultured cells were isolated using the TRIzol[®] reagent

and oligo(dT)–cellulose (Invitrogen). RT (reverse transcription) PCR was performed in the presence of 5% (v/v) DMSO with a reagent kit (Advantage RT-for-PCR from Clontech) according to the manufacturer's protocol. Synthesis of the first-strand cDNA was primed with oligo(dT) and random hexamers. Images of ethidium-bromide-stained gels were obtained in a Fotodyne gel documentation system (Hartland, WI, U.S.A.).

HeLa and HepG2 cells were cultured as described in [32]. Protein samples from HeLa cells were solubilized directly in SDS gel loading buffer [60 mM Tris base, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (w/v) 2-mercaptoethanol and 0.1% (w/v) Bromophenol Blue], separated by SDS/PAGE (12% gels), and electroblotted on to Immobilon-P membrane (Millipore) using a semidry blotter (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). Blots were visualized by enhanced chemiluminescence (ECL[®], Amersham Biosciences). Co-immunoprecipitation was performed as previously described in [33]. Expression vectors for CDH1 α and CDH1 β (pHACDH1 α and pHACDH1 β) were derived from plasmid pHA [34].

Luciferase assay

Luciferase assay was performed as described in [35]. Human cyclin B1 cDNA has been described in [36]. pCYCB1-Luc was derived from reporter plasmid pGL3-Control (Promega) and it expresses a luciferase fusion protein with the first 119 amino-acid residues of cyclin B1 at the N-terminus. Proteasome inhibitor MG-132 (benzyloxycarbonyl-Leu-Leu-L-leuciny) was purchased from the Peptide Institute, based in Osaka, Japan.

Embryo manipulations

Eggs were collected from *Xenopus laevis* females (Xenopus Express, Cape, South Africa), which had been injected with 500–700 units of human chorionic gonadotrophin (Sigma) 12 h before egg collection. Eggs were fertilized *in vitro* with minced testis. Embryos were staged as described in [37]. RNAs were synthesized and capped with reagent kits from Ambion (Austin, TX, U.S.A.).

Confocal microscopy

Laser scanning confocal immunofluorescence microscopy was performed on a Zeiss Axiophot microscope as previously described in [31,32]. Dual labelling was achieved with primary antibodies from different species and pre-adsorbed species-specific secondary antibodies: Cy5-conjugated goat anti-mouse immunoglobulin G (Zymed Laboratories, South San Francisco, CA, U.S.A.) and fluorescein-conjugated goat anti-rabbit immunoglobulin G (Zymax). To express a GFP (green fluorescent protein) fusion protein with peptide sequences encoded by exons 6 and 7 of human CDH1 α at the C-terminus, exons 6 and 7 were amplified by PCR using primers 5'-CCCAAGCTTATCCCTTAGACACCAAG-3' (forward) and 5'-CGGGATCCCTGGCTGTACAGGCACT-3' (reverse). The 267 bp fragment was inserted into pEGFP-C1 (Clontech) via restriction sites *Hind*III and *Bam*HI. GFP experiments were conducted as described in [38].

RESULTS

CDC20 and CDH1 represent two multigene families

The identification of *Drosophila* and *Xenopus* CDH1 [8,10] facilitates the cloning of human and mouse orthologues. The isolation and characterization of human CDH1 has been reported previously [14,19,39]. In an early and independent attempt to assemble full-length CDH1 cDNAs, we also obtained several human and mouse EST clones that share striking sequence homologies with frog CDH1. The human and mouse CDH1 sequences with a complete coding region were determined and deposited in GenBank® under accession numbers AF083810 and AF083809 (submitted on 12 August 1998).

Human and mouse CDH1 proteins (Figure 1A) share 98.4% identity in an overlap of 493 amino-acid residues. The seven WD40 repeats are identical in the two proteins except for a substitution of aspartic acid for glutamic acid at residue 193. The nine putative CDK (cyclin-dependent kinase) phosphorylation sites (shaded in Figure 1A) are also conserved. Since a similarity search generated more CDC20/CDH1-related sequences than was expected, we constructed a phylogenetic tree of all CDC20/CDH1 homologues from various species (Figure 1B) in order to better understand the genetic relationship.

Notably, there is one additional CDC20 paralogue in budding yeast. This protein, named AMA1/CDC20B (Figure 1B), is required for sporulation and has been implicated as a meiosis-specific activator of APC [40]. In fission yeast, five CDC20/CDH1

Figure 2 Genomic characterization of human CDH1

(A) Schematic delineation of human CDH1 at 19p13.3. Arrows underlying the genes indicate the orientation of transcription. FZR1/HCDH1, human fizzy-related 1 or CDH1; INSR, insulin receptor; NF1C, nuclear factor I/C; HMG20B, high mobility group 20B or Sox-like transcription factor; Tel, telomeric; Cen, centromeric. (B) Genomic structure of the human CDH1 gene. Non-coding (open boxes) and coding exons (filled boxes) are shown.

homologues can be identified. In addition to the documented CDC20A (SLP1) and CDH1A (SRW1/STE9), two CDC20 paralogues (CDC20B and CDC20C) and another CDH1 paralogue (CDH1B or MFR1 or FZR1) were found. In the phylogenetic tree, the fission yeast CDC20B and CDC20C cluster with budding yeast AMA1/CDC20B, while the fission yeast CDH1A and CDH1B group with budding yeast CDH1. Interestingly, CDH1B/MFR1/FZR1 has been shown to be another meiosis-specific activator of APC required for sporulation [41,42]. The bootstrap supports for these clusters are statistically very significant (100% or 95%). We postulate that different CDC20/CDH1 homologues in fission yeast might serve redundant and non-redundant functions. It would be of interest to see how they co-operate to ensure the precise spatiotemporal order of APC activation.

Multiple CDC20 and CDH1 homologues were also noted in higher eukaryotes (Figure 1B). Thus *Arabidopsis thaliana* (thale cress) has at least one CDH1 and three closely related CDC20 homologues (CDC20A, CDC20B and CDC20C). Likewise, *Drosophila* (fruit fly) possesses CDC20, CDH1/FZR and CDH2/FZR2 [9]. We predict that more vertebrate CDC20/CDH1 homologues will be identified as the genome projects progress. CDC20 and CDH1 represent two multigene families of WD40 repeat proteins critically involved in the regulation of APC. It is important to compare and contrast the functions of different CDC20/CDH1-like proteins in one species.

Chromosomal mapping and genomic organization of human CDH1 locus

We determined the chromosomal map location of human CDH1 gene by sequence alignment. Using cDNA information, we identified two genomic clones that contain the coding and non-coding regions of human CDH1. These were cosmids R33374 and R31109 from a genomic library constructed at the Lawrence Livermore National Laboratory, Livermore, CA, U.S.A. (GenBank® accession numbers AC005787 and AC005786). Both clones map to chromosome 19p13.3. The human CDH1 locus (FZR1/HCDH1) was noted to be closely linked to NFIC (nuclear factor I/C) and Sox-like transcription factor (HMG20B or SOXL). This map location of CDH1 was confirmed by PCR analysis and by restriction mapping. We verified that the distance between NFIC and FZR1 is within 30 kb, whereas the FZR1 and HMG20B genes are separated by no more than 37 kb (Figure 2A).

Figure 3 Expression of CDH1 mRNA in human tissues and cells

(A) Northern blot analysis of CDH1 mRNA in human tissues and cells. CDH1-specific probe hybridizes to a predominant mRNA migrating with a size of approx. 3 kb (arrow). This size corresponds to CDH1 α (3.0 kb). A band possibly derived from CDH1 β (2.7 kb) is highlighted by an asterisk (*). The hybridization signal of CDH1 β is much weaker (approx. 10-fold) than that of CDH1 α , but it is reproducibly seen in some tissues and cells. β -Actin-specific hybridization was performed on the same blot after stripping the CDH1 α signal in order to verify equivalent loading. Blots were from Clontech and all lanes contain approx. 2 μ g of polyadenylated RNA. Similar results were obtained with three different probes corresponding to the N-terminal, C-terminal and central parts of CDH1 α . (B) Isoform-specific PCR analysis of CDH1 α and CDH1 β . The PCR reactions contain the indicated primer sets (set A for lanes 1 and 4, and set B for lanes 2 and 3) and templates (plasmid pCDH1 α for lane 1, pCDH1 β for lane 2, and pCDH1 α + pCDH1 β for lanes 3 and 4). Results are representative of triplicate experiments. Sequences for the PCR primers are 5'-GTG CAG GAC CCG CAG ACT GAG-3' plus 5'-CTC CGT GTA CTG CTG CAC GGG-3' (primer set A; size of CDH1 α amplification product, 740 bp), and 5'-GAG GAC CGC AGG CTG CAG CCC-3' plus 5'-CTG CTG CAC GGG GCT CAG GCT CGA-3' (primer set B; size of CDH1 β amplification product, 443 bp). M: molecular mass marker. (C) RT-PCR analysis of CDH1 α mRNA in human tissues and cell lines. The PCR reactions contain primer set A and total RNAs from the indicated sources (HepG2, hepatoma cells; U2 OS, osteosarcoma cells; H1299, lung cancer cells; AGS, gastric adenocarcinoma cells; MKN, stomach adenocarcinoma cells; Chang, Chang cells possibly established via HeLa cell contaminant). Results are representative of duplicate amplifications of two independent preparations of RNA samples. (D) RT-PCR analysis of CDH1 β mRNA in human tissues and cell lines. The PCR reactions contain primer set B and total RNA from the indicated sources.

Approx. 43 kb of assembled sequence from cosmid R33374 was obtained. Human CDH1 locus spans 29.8 kb. The sizes of all exons and introns were verified by PCR analysis. The gene contains at least 14 exons varying in size from 63 bp to 1.4 kb (Figure 2B). The sequences of all splice junctions and of the 5' upstream region were confirmed by direct sequencing of PCR products amplified from human genomic DNA.

Identification of an alternatively spliced isoform of human CDH1

We isolated an alternative form of CDH1 from a human heart cDNA library (GenBank[®] accession number AF433157). We noted that this cDNA represents an alternatively spliced isoform. This novel isoform, designated CDH1 β , can be produced by splicing exon 8 to exon 5, skipping exons 6 and 7 (267 nucleotides; Figure 2B). The CDH1 β protein lost 89 residues containing one WD40 repeat, four CDK phosphorylation sites and a cluster of positively charged residues (Figure 1A). This raises the possibility that CDH1 β might function or be regulated in a manner different from the original isoform of human CDH1, renamed CDH1 α . Of note, CDH1 β has been independently isolated by S. Kotani, T. Oyamatsu and K. Todokoro (GenBank[®] ac-

cession number AB013463; unpublished work) and by the Mammalian Gene Collection at the National Institutes of Health (<http://mgc.nci.nih.gov>), suggesting that it is unlikely to be a cloning artifact or an erroneously spliced RNA.

Differential expression of CDH1 α and CDH1 β in human tissues and cancer cells

To reassess the expression pattern of CDH1 α and CDH1 β mRNA and in human tissues and cancer cells, we performed Northern blotting (Figure 3A) and RT-PCR (Figures 3B–3D). CDH1 α is the major isoform detected in Northern blot analysis (Figure 3A, indicated by arrows). We did not observe the 2.7 kb CDH1 β mRNA in most human tissues and cells by Northern blotting with a probe that was expected to hybridize equally well to both CDH1 α (3.0 kb) and CDH1 β (2.7 kb) transcripts (Figure 3A). However, a weak band of > 2.4 kb in size (indicated by asterisks) was reproducibly seen in brain (Figure 3A, lane 1), HeLa cells (Figure 3A, lane 14), and A549 cells (Figure 3A, lane 19). This transcript that is possibly derived from CDH1 β is much less abundant than the CDH1 α mRNA. Using antibodies raised against the N-terminal and C-terminal sequences shared by CDH1 α (55 kDa)

and CDH1 β (45 kDa), we can't detect the 45-kDa CDH1 β protein in HeLa and HepG2 cells by immunoblotting (results not shown; see Figure 5A for an example). These results implicate CDH1 α as a predominant isoform in many tissues and cells. The possibly low abundance of CDH1 β transcript and protein in cells prompted us to use more sensitive and accurate technology for RNA detection. To this end, we tested several sets of primers for amplification of human CDH1 α and CDH1 β in model experiments using CDH1 α and CDH1 β plasmids as template. In our experiments, primer sets A and B were able to differentially amplify CDH1 α and CDH1 β . Figure 3(B) shows that primer set A can specifically amplify CDH1 α from templates containing either CDH1 α alone (Figure 3B, lane 1) or CDH1 α plus CDH1 β (Figure 3B, lane 4). Likewise, primer set B is specific for CDH1 β from templates containing CDH1 β alone (Figure 3B, lane 2) or CDH1 α plus CDH1 β (Figure 3B, lane 3). Next, we tested RNAs from various human tissues and cells using primer sets A (Figure 3C) and B (Figure 3D). We noted that both CDH1 α and CDH1 β were detected in multiple sources. The CDH1 α transcript is relatively more abundant in heart, liver, spleen, HeLa cells, HepG2 cells and H1299 cells (Figure 3C). In contrast, the CDH1 β mRNA is expressed in heart, spleen, and H1299 lung cancer cells (Figure 3D). The identity of the CDH1 α and CDH1 β amplification products was verified by DNA sequencing. Thus CDH1 β can be found in some tissues and cells, albeit in low abundance. The distinct expression patterns of CDH1 α and CDH1 β suggest that they might serve different and complementary functions.

APC-binding and APC-activating activity of CDH1 β in HeLa cells

During the progress of the present study, four CDH1 homologues have been identified in chicken [43]. All of them bind and activate the APC both *in vitro* and in cultured cells. To our surprise, all four chicken CDH1 homologues contain sequences corresponding to exons 6 and 7 of human CDH1 gene. They represent four different gene loci and were not produced by alternative splicing. Thus none of them are structurally equivalent to human CDH1 β . However, vertebrate CDH1 variants generated through distinct mechanisms could still be functionally related. Based on this reasoning, human CDH1 β isoform and chicken CDH1 homologues could serve similar or related functions intracellularly. To test this hypothesis, we asked whether human CDH1 β binds to and activates the APC in HeLa cells.

First, we expressed HA-tagged CDH1 α and CDH1 β proteins in HeLa cells. As expected, cells transfected with CDH1 α and CDH1 β expression plasmids produced 55 kDa and 45 kDa proteins reactive to anti-HA antibody respectively (Figure 4A, compare lane 2 with lane 1). This provides the direct evidence that a CDH1 β protein distinct to CDH1 α can be translated from the CDH1 β transcript we have identified. We next performed co-immunoprecipitation experiments with extracts of CDH1 α - and CDH1 β -expressing cells. Figure 4(B) shows that the precipitates prepared with an irrelevant antibody against the FLAG tag did not contain CDC27, a subunit of the APC complex. In contrast, CDC27 was found in the protein complex pulled down by the anti-HA antibody (Figure 4C). These data suggest that CDH1 β associates with CDC27 in HeLa cells as tightly as CDH1 α (Figure 4C, compare lane 2 with lane 1).

To determine whether or not the association of CDH1 β with APC leads to activation of proteolysis, we constructed a reporter plasmid expressing the firefly luciferase fused to the D box of cyclin B1. This plasmid (pCYCB1-Luc), when transfected into cells, can reflect the *in vivo* activity of the APC-dependent ubiquitination and degradation system in real time [24,44]. We observed that the overexpression of either CDH1 α or CDH1 β

Figure 4 Both CDH1 α and CDH1 β associate with APC and activate APC-dependent degradation of cyclin B1

(A) Expression of CDH1 α and CDH1 β in HeLa cells. Expression plasmids for HA-tagged CDH1 α and CDH1 β were transiently transfected into cells. Extracts of transfected cells were probed with a monoclonal anti-HA antibody. (B) and (C) Co-immunoprecipitation experiments. HeLa cells expressing HA-tagged CDH1 α (lane 1) and CDH1 β (lane 2) were immunoprecipitated with mouse anti-FLAG (panel B) or anti-HA (panel C). The precipitates were resolved by SDS/PAGE and the Western blots were probed with rabbit anti-CDC27. (D) CDH1 α and CDH1 β stimulate cyclin B1 degradation. Cells were co-transfected with pCYCB1-Luc reporter plus empty vector, or plus CDH1 α expression plasmid, or plus CDH1 β expression plasmid. Transfected cells were untreated (□) or treated (■) with proteasome inhibitor MG-132 (25 μ M) for 12 h before harvest.

protein significantly increased the degradation of the cyclin B1-luciferase fusion protein (Figure 4D). APC-dependent proteolysis is through the proteasome. If CDH1 α and CDH1 β specifically target the APC, their stimulatory effect on cyclin B1 degradation should be responsive to proteasome inhibitors. Indeed, when we incubated the CDH1 α - and CDH1 β -expressing cells with the proteasome inhibitor MG-132, the CDH1 α /CDH1 β -induced degradation of cyclin B1-luciferase was prevented (Figure 4D, compare filled with unfilled columns). Thus both CDH1 α and CDH1 β are specific activators of APC-dependent and proteasome-mediated destruction of cyclin B1 in HeLa cells.

Differential localization of CDH1 α and CDH1 β

Next we investigated the subcellular localization of CDH1 α and CDH1 β . We generated two specific antibodies against CDH1

Figure 5 Differential localization of CDH1 α and CDH1 β

(A) Western blotting. Extracts of HepG2 cells (approx. 20 μ g) were resolved by SDS/PAGE. Immunoblotting was performed using purified α -CDH1C serum. The position of CDH1 α (55 kDa) is highlighted. The α -CDH1C antibody was purified through a HiTrap NHS (N-hydroxysuccinimide)-activated affinity column (Amersham Biosciences) coupled to the immunizing peptide. A duplicate blot was separately probed with α -CDH1C pre-incubated with 3 μ g of immunizing peptide (α -CDH1C w/pep.; lane 2). Similar results were obtained using another antibody, α -CDH1N, raised against the N-terminal sequences shared by CDH1 α and CDH1 β (results not shown). (B) Subcellular localization of exogenously expressed CDH1 α and CDH1 β in HeLa cells. Cells were transfected respectively with expression vectors pHACDH1 α (panels 1 and 2) and pHACDH1 β (panel 3), in which the expression of HA-tagged CDH1 α /CDH1 β is driven by the SV40 (simian virus 40) promoter. Cells were fixed 36 h after transfection and stained with α -CDH1N (panel 1) or α -HA (panels 2 and 3) antibodies. The same fields of the cells were also monitored by phase contrast microscopy (panels 1', 2' and 3'). vec., expression vector; ab., antibody. Bar, 20 μ m. The patterns shown in panels 2 and 3 are representative of 85% and 79% respectively of 200 CDH1 α /CDH1 β -expressing cells.

(α -CDH1N and α -CDH1C; Figure 1A) and stained cultured cells using the purified antibodies. As a first step, the specificity of the anti-CDH1 antibodies was verified by Western blotting. As an example, HepG2 cell lysates were probed with either α -CDH1C or α -CDH1C pre-incubated with an excessive amount of immunizing peptide (Figure 5A). A single protein species corresponding to the 55 kDa CDH1 α was detected from HepG2 cells (Figure 5A, lane 1) and the band was not seen with a pre-absorbed serum (Figure 5A, lane 2). HepG2 cells were then stained with either α -CDH1C or α -CDH1C pre-absorbed with immunizing peptide. The endogenous CDH1 α in HepG2 cells localized to the nucleus and the specificity of this nuclear staining was corroborated by a peptide-blocking experiment (results not shown). These results generally agree with recent findings on the nuclear localization of CDH1 [45,46].

We were concerned that CDH1 β was not detected in Western blotting and immunostaining due to the low abundance (Figure 5A). To remedy this and to distinguish the CDH1 β staining from that of CDH1 α , we stained for epitope-tagged CDH1 α and CDH1 β expressed from exogenously introduced plasmids (Figure 5B). HeLa cells transiently transfected with expression plasmids for HA-tagged CDH1 α (Figure 5B, panels 1 and 2) and CDH1 β (Figure 5B, panel 3) were stained with α -CDH1N (Figure 5B, panel 1) or anti-HA (α -HA; Figure 5B, panels 2 and 3) antibodies. These antibodies reacted with HA-CDH1 α /HA-CDH1 β in a highly specific manner. Notably, a nuclear localization pattern of CDH1 α was consistently seen with both α -CDH1N and α -HA antibodies. By sharp contrast, CDH1 β localized homogeneously to the cytoplasm (Figure 5B, panel 3). The identity of the HA-CDH1 β protein was further verified by immunostaining with α -CDH1N and α -CDH1C antibodies. Both antibodies reacted with the overexpressed HA-CDH1 β as well as the endogenous CDH1 α (results not shown).

Both CDH1 α and CDH1 β bind to and activate the APC (Figure 4), but they localize to different subcellular compartments (Figure 5B). In this setting, CDH1 α and CDH1 β could encounter distinct sets of APC substrates at different locales. For example, they are able to activate cyclin B1 destruction (Figure 4B) and might co-operate with each other to mediate the timely and orderly degradation of nuclear and cytoplasmic cyclin B1. In another case, they might act differentially on the nuclear cyclins such as cyclin A and cyclin E. To explore how expression of CDH1 α and CDH1 β might influence the proteolysis of cyclin A, HA-CDH1 α and HA-CDH1 β were overexpressed in HeLa cells and were co-stained for CDH1 and cyclins (Figure 5C). In agreement with previous studies [47], ambient cyclin A is predominantly nuclear (Figure 5C, panels 2 and 5). We observed that CDH1 α co-localized with cyclin A to the nucleus (Figure 5C, panel 3). In addition, the forced overexpression of CDH1 α correlated with the reduction of nuclear cyclin A (Figure 5C, panel 2, compare cell with an arrow with cells without an arrow). This observation is generally consistent with reported findings [44]. On the other hand, CDH1 β and cyclin A were found in different subcellular locations (Figure 5C, panel 6) and CDH1 β overexpression did not lead to noticeable degradation of cyclin A (Figure 5C, panel 5, compare cell with an arrow with cells without an arrow). These results support the notion that human CDH1 α

(C) Differential co-localization of CDH1 α and CDH1 β with cyclin A. HeLa cells were transfected with pHACDH1 α (panels 1–3) or pHACDH1 β (panels 4–6). Cells were co-stained with mouse α -HA (panels 1 and 4) and rabbit α -cyclin A (panels 2 and 5) antibodies. In panels 3 and 6, the CDH1 (green; probed with α -HA) and cyclin A (red) fluorescent signals were overlaid by computer assistance. Co-localization is shown in yellow. The same fields are shown in panels 1–3 and 4–6. Bar, 20 μ m. The patterns shown in panels 3 and 6 are representative of 68% and 63% respectively of 200 CDH1 α /CDH1 β -expressing cells.

Figure 6 Peptide sequences encoded by exons 6 and 7 of human CDH1 α target heterologous GFP to the nucleus

HeLa cells were transfected with pEGFP-C1 (panel 1) or pEGFPE67 (panel 2). Unfixed cells were observed directly under the fluorescence microscope 24 h after transfection. The same fields of the cells were also monitored by phase-contrast microscopy (panels 1' and 2'). Arrows indicate transfected cells. The pattern shown in panel 2 is representative of 62 % of 200 GFPE67-expressing cells. Bar, 20 μ m.

and CDH1 β fulfil different functions at different locales of the cell.

CDH1 α contains a nuclear localization signal that is absent in CDH1 β

The differential localization of CDH1 α and CDH1 β (Figure 5) suggests that the peptide fragment that is present in CDH1 α , but is absent in CDH1 β , might dictate nuclear import. Indeed, a cluster of nine positively charged residues can be found in that fragment (Figure 1, residues indicated by #). This cluster might act as a nuclear localization signal in CDH1 α . To test this hypothesis, we fused the peptide sequence encoded by exons 6 and 7 of CDH1 α to GFP. When overexpressed in HeLa cells, GFP alone was diffusely found in both the cytoplasm and the nucleus (Figure 6, panel 1). In contrast, when we overexpressed the chimaeric protein GFPE67 in cells, the heterologous GFP protein was targeted into the nucleus by the nuclear localization signal apparently resided in the sequences encoded by exons 6 and 7 of CDH1 α (Figure 6, panel 2). Thus CDH1 α harbours a nuclear localization signal that is absent in CDH1 β . Identification and further characterization of this nuclear localization signal are reported elsewhere [46].

Differential activity of human CDH1 α and CDH1 β in *Xenopus* embryos

The co-localization studies shown above suggest that human CDH1 β might be a poor activator of the APC-mediated degradation of nuclear cyclin A (Figure 5C). To compare the global influence of human CDH1 α and CDH1 β on cell-cycle progression, we used the *Xenopus* embryo microinjection system. We and others have shown that the injection of *Xenopus* CDH1 RNA into one blastomere of two-cell-stage *Xenopus* embryos sufficiently induced a cell-cycle arrest at the MBT (midblastula transition) owing to continued activation of the APC [30,48]. As such, the over-

expression of CDH1 was phenotypically silent before the MBT (stage 8.5) and the cell-division cycles seemed normal. However, immediately after the MBT, the cells on the CDH1-injected side were significantly larger than those on the uninjected side. Indeed, when we overexpressed human CDH1 α in early *Xenopus* embryos by microinjecting CDH1 α RNA into one of the two blastomeres in stage 2, we observed a post-MBT arrest (Figure 7A, panel 3) exactly as described for *Xenopus* CDH1 (Figure 7B). As shown in a CDH1 α -injected embryo at late stage 9 (Figure 7A, panel 3), the cells overexpressing CDH1 α were > 5 times bigger, implicating that the inhibition lasted for at least several cycles of cell division. In control groups, the uninjected, mock-injected, and antisense CDH1 α /CDH1 β -injected embryos did not arrest cell division during the MBT (Figure 7A, panels 1 and 2; Figure 7B). Interestingly, the injection of human CDH1 β RNA is phenotypically silent (Figure 7A, panel 4; Figure 7B), and the injected embryos were indistinguishable from the controls (Figure 7A, compare panel 4 with panels 1 and 2). Thus human CDH1 α and CDH1 β have differential APC-activating activity in *Xenopus* embryos. CDH1 β is incapable of inducing the post-MBT cell-cycle arrest.

DISCUSSION

In the present paper, we documented the differential expression, subcellular localization and activity of CDH1 α and CDH1 β , two functional isoforms of human CDH1. CDC20 and CDH1 represent two multigene families of WD40 repeat proteins that are key regulators of APC (Figure 1). A novel alternatively spliced isoform of human CDH1, named CDH1 β , was identified from a heart cDNA library (Figures 1 and 2). The two CDH1 isoforms were expressed to different levels in various human tissues and cells (Figure 3). Both isoforms associate with the APC subunit CDC27 and stimulate the APC-dependent proteolysis of cyclin B1 in cultured cells (Figure 4). CDH1 α , which harbours a nuclear localization signal that is absent in CDH1 β (Figure 6), localizes to the nucleus (Figure 5). In contrast, CDH1 β is cytosolic (Figure 5). Finally, CDH1 α , but not CDH1 β , co-localizes with nuclear cyclin A, activates cyclin A degradation and induces a post-MBT arrest in cell-cycle progression in *Xenopus* embryos (Figures 5 and 7). The present study suggests a complex spatial and temporal control of APC activation during the cell cycle.

One salient finding of the present study is the differential expression, localization and activity of human CDH1 α and CDH1 β generated through alternative splicing. Alternative splicing is very common in human cells and approx. 60% of human genes are estimated to have two or more transcripts [49]. Among the various types of alternative splicing that give rise to multiple isoforms of a gene, exon skipping can be caused by exonic and intronic mutations [50]. In this context, it would be of interest to identify and characterize the signals and mechanisms through which the production of CDH1 β is induced.

Both CDH1 α and CDH1 β can bind to the APC complex and activates the APC-dependent degradation of cyclin B1 in cultured cells (Figure 4). However, the presence of CDH1 α and CDH1 β in different abundance (Figure 3) and at different locales (Figure 5) suggests that they might have different roles in cell-cycle regulation. In line with this, we observed that they showed differential activity in the activation of the APC in both cultured human cells (Figure 5C) and *Xenopus* embryos (Figure 7). As yet the mechanism of the differential APC-activating activity of the two isoforms is not fully understood. One possibility is that CDH1 α and CDH1 β may be active on different sets of substrates. We note that some of our data are in support of this

Figure 7 Differential activity of human CDH1 α and CDH1 β in *Xenopus* embryos

(A) Representative images of embryos. (B) Graphic quantification of post-MBT cell-cycle arrest phenotype induced by expression of CDH1 α . One blastomere of the two-cell stage embryos was uninjected (uninj.; panel 1), mock-injected with PBS (mock; panel 2) or injected separately with 4 μ g of the indicated RNAs: *Xenopus* CDH1 (XCDH1), human CDH1 α (HCDH1 α ; panel 3), human CDH1 β (HCDH1 β ; panel 4), antisense human CDH1 α (as-HCDH1 α), or antisense human CDH1 β (as-HCDH1 β). For each determination, 30 embryos were counted. Each bar represents the mean value from three experiments. Images in A were photographed at stage 8.5–9.5. The arrow indicates the arrested large cells on the injected side.

model. Thus both CDH1 α and CDH1 β bind to APC and stimulate the degradation of cyclin B1 (Figure 4). Cyclin B1 shuttles between the nucleus and the cytoplasm [47], whereas the cyclin B1–luciferase fusion protein used in the present study is both cytoplasmic and nuclear (results not shown). This localization pattern is compatible with the observed degradation of cyclin B1 by both CDH1 α and CDH1 β . It is also noteworthy that cyclin B1 destruction also takes place at metaphase when the nuclear envelope has been broken down [51]. In this scenario, cyclin B1 and possibly other cyclins are accessible to both CDH1 α and CDH1 β . On the other hand, CDH1 β is apparently a poor activator of the APC-mediated proteolysis of nuclear cyclin A (Figure 5). Plausibly, CDH1 α and CDH1 β may have differential activity on cyclin A and other cell-cycle regulators. In this regard, further biochemical analyses are required to compare in parallel the ubiquitination-stimulating activity of CDH1 α and CDH1 β on different targets.

Four CDH1 homologues in chicken derived from four different loci have recently been documented [43]. These chicken CDH1 homologues share 62–95% identical amino-acid residues with human CDH1 α , but are differentially expressed and localized. Importantly, they exhibit quantitatively different APC-stimulating activity on different substrates [43]. These CDH1 homologues are plausibly derived from gene duplications. None of the four chicken CDH1 homologues were generated through alternative splicing. Neither were they structurally equivalent to human CDH1 β identified in this work. Up-to-date human orthologues of the four chicken CDH1 proteins have not been described, except for the original human CDH1 α isoform and another EST clone possibly orthologous to the most divergent ChkCDH1-D. With

the working draft of the human genome sequence available, we are still uncertain whether or not there are four human CDH1 loci corresponding to those in chicken. It is not impossible that at least some of the gene-duplication events leading to the production of four CDH1 homologues in chicken might have taken place after the separation of birds from other vertebrates. On the other hand, it remains to be seen whether or not CDH1 isoforms orthologous to human CDH1 β exist in other species, including chicken. Nevertheless, our working model is that the various CDH1 isoforms in vertebrates have differential activity on different sets of targets. In this regard, gene duplication, which produced four CDH1 homologues in chicken, and alternative splicing, which led to the formation of human CDH1 β , probably represent two major mechanisms by which CDH1 isoforms could be generated. According to our model, the differential activity of CDH1 α and CDH1 β in *Xenopus* embryos is attributed to their differential ability to activate the degradation of different targets such as cyclin A.

The subcellular localization pattern (Figure 5B) implicates human CDH1 α as a nuclear activator of the APC. Consistent with this, the forced overexpression of CDH1 α in the nucleus correlates with the degradation of nuclear cyclin A (Figure 5C). The nuclear localization of human CDH1 α ensures its accessibility to nuclear regulators and targets such as CDC14 [52], Emi1 [53], APC subunits [54] and cyclins [47]. For example, the co-localization of CDH1 α and cyclin A (Figure 4C) provides the opportunity for the suggested regulation of CDH1 α -APC by cyclin A [24,55]. In contrast with the nuclear pattern of CDH1 α , CDC20 resides ambiently in the cytoplasm [18]. Thus CDC20 and CDH1 α are activated in different compartments of a cell. This adds another

level of complexity to the regulation of the APC. In another perspective, the steady-state localizations of many cell-cycle regulatory proteins are determined by the relative rates of nuclear import and export, because they shuttle continuously between the nucleus and the cytoplasm [47,56,57]. In this regard, it will be of great interest to investigate the dynamic nucleocytoplasmic shuttling of CDH1 α and CDH1 β .

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