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, a novel alternatively spliced isoform of CDH1. Both CDH1α and CDH1β can bind to the APC and stimulate the degradation of cyclin B1, but they are differentially expressed in human tissues and cells. CDH1α contains a nuclear localization signal which is absent in CDH1β. Intracellularly, CDH1α appears in the nucleus whereas CDH1β is a predominantly cytoplasmic protein. The forced overexpression of CDH1α in cultured cells correlates with the reduction of nuclear cyclin A, but the steady-state amount of cyclin A does not change noticeably in CDH1β-overexpressed cells.

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 CDH1α and CDH1β. 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 CDH1α, 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 CDH1α, human CDH1β 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. 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 CDH1β 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/A14048.1; CDC20-carrot, gp/T14352; CDC20-rat, gp/AA14741.1; CDC20-human, gp/AAD6405.1; CDC20-frog, gp/AAC41776.1; CDC20-clam, gp/AA06232.1; CDC20A-fission yeast, gp/AA035957.1; CDC20-nematode, gp/T17762; CDC20-trichomonad, gp/AA061712; CDC20A-fission yeast, gp/T14034; CDC20C-fission yeast, gp/T141148; AM1/CD20B-budding yeast, gp/10117412; CDH1-mouse, gp/AAD52029; CDH1-human, gp/AAC282835; CDH1A-frog, emb/CAA74561; CDH1A-frog, gp/A45973; CDH1A-nematode, gp/T17730; CDH2-frog, gp/A47711.1; CDH1-barrel medic, gp/AAD22612; CDH1-thale cress, gp/T09351; CDH1A-fission yeast, gp/T10614; CDH1A-fission yeast, gp/T37680; CDH1A-fission yeast, gp/1011512.1.

was raised to a keyhole-limpet-haemocyanin-conjugated peptide corresponding to the keyhole-limpet-haemocyanin-conjugated peptide

RNA and protein analyses

Human multiple tissue Northern blots (Clontech) were probed with a 307 bp 32P-labelled NcoI–NaeI fragment shared by both CDH1α and CDH1β 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 (Advan 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 SDSPAGE (12% gels), and electroblotted on to Immobilon-P membrane (Millipore) using a semidy 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 CDH1α and CDH1β (pHACDH1α and pHACDH1β) were derived from plasmid pHA [34].

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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 (benzlyoxycarbonyl-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 700 units of human chorionic gonadotrophin (Sigma) 12 h before

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 CDH1α at the C-terminus, exons 6 and 7 were amplified by PCR using primers 5′-CCCAAGCTTATTCCC-TTAGCACAAG-3′ (forward) and 5′-CAGGATCCCTGGCTG-CCCAAGCTTATTCCC-TTAGCACAAG-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 parologue 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 homologues can be identified. In addition to the documented CDC20A (SLP1) and CDH1A (SRW1/STE9), two CDC20 paralogues (CDC20B and CDC20C) and another CDH1 parologue (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 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/HDCH1) was noted to be closely linked to NFIC (nuclear factor I/C) and 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. (A) Schematic delineation of human CDH1 at 19p13.3. Arrows underlying the genes indicate the orientation of transcription. FZR1/HCDH1, human fuzzy-related 1 or CDH1; INSR, insulin receptor; NFIC, nuclear factor I/C; HMG20B, high mobility group 20B or Sox-like transcription factor; Tel, telomeric; Cen, centromeric.)

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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, denoted CDH1β, can be produced by splicing exon 8 to exon 5, skipping exons 6 and 7 (267 nucleotides; Figure 2B). The CDH1β 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 CDH1β might function or be regulated in a manner different from the original isoform of human CDH1, renamed CDH1α. Of note, CDH1β has been independently isolated by S. Kotani, T. Oyamatsu and K. Todokoro (GenBank® accession 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 CDH1α and CDH1β in human tissues and cancer cells

To reassess the expression pattern of CDH1α and CDH1β mRNA and in human tissues and cancer cells, we performed Northern blotting (Figure 3A) and RT-PCR (Figures 3B–3D). CDH1α is the major isoform detected in Northern blot analysis (Figure 3A, indicated by arrows). We did not observe the 2.7 kb CDH1β mRNA in most human tissues and cells by Northern blotting with a probe that was expected to hybridize equally well to both CDH1α (3.0 kb) and CDH1β (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 CDH1β is much less abundant than the CDH1α mRNA. Using antibodies raised against the N-terminal and C-terminal sequences shared by CDH1α (55 kDa)
and CDH1β (45 kDa), we can’t detect the 45-kDa CDH1β protein in HeLa and HepG2 cells by immunoblotting (results not shown; see Figure 5A for an example). These results implicate CDH1α as a predominant isoform in many tissues and cells. The possibly low abundance of CDH1β 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 CDH1α and CDH1β in model experiments using CDH1α and CDH1β plasmids as template. In our experiments, primer sets A and B were able to differentially amplify CDH1α and CDH1β. Figure 3(B) shows that primer set A can specifically amplify CDH1α from templates containing either CDH1α alone (Figure 3B, lane 1) or CDH1α plus CDH1β (Figure 3B, lane 4). Likewise, primer set B is specific for CDH1β from templates containing CDH1β alone (Figure 3B, lane 2) or CDH1α plus CDH1β (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 CDH1α and CDH1β were detected in multiple sources. The CDH1α transcript is relatively more abundant in heart, liver, spleen, HeLa cells, HepG2 cells and H1299 cells (Figure 3C). In contrast, the CDH1β mRNA is expressed in heart, spleen, and H1299 lung cancer cells (Figure 3D). The identity of the CDH1α and CDH1β amplification products was verified by DNA sequencing. Thus CDH1β can be found in some tissues and cells, albeit in low abundance. The distinct expression patterns of CDH1α and CDH1β suggest that they might serve different and complementary functions.

**APC-binding and APC-activating activity of CDH1β 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 CDH1β. However, vertebrate CDH1 variants generated through distinct mechanisms could still be functionally related. Based on this reasoning, human CDH1β isoform and chicken CDH1 homologues could serve similar or related functions intracellularly. To test this hypothesis, we asked whether human CDH1β binds to and activates the APC in HeLa cells.

First, we expressed HA-tagged CDH1α and CDH1β proteins in HeLa cells. As expected, cells transfected with CDH1α and CDH1β 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 CDH1β protein distinct to CDH1α can be translated from the CDH1β transcript we have identified. We next performed co-immunoprecipitation experiments with extracts of CDH1α- and CDH1β-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 CDH1β associates with CDC27 in HeLa cells as tightly as CDH1α (Figure 4C, compare lane 2 with lane 1).

To determine whether or not the association of CDH1β 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 CDH1α or CDH1β protein significantly increased the degradation of the cyclin B1-luciferase fusion protein (Figure 4D). APC-dependent proteolysis is through the proteasome. If CDH1α and CDH1β specifically target the APC, their stimulatory effect on cyclin B1 degradation should be responsive to proteasome inhibitors. Indeed, when we incubated the CDH1α- and CDH1β-expressing cells with the proteasome inhibitor MG-132, the CDH1α/CDH1β-induced degradation of cyclin B1-luciferase was prevented (Figure 4D, compare filled with unfilled columns). Thus both CDH1α and CDH1β are specific activators of APC-dependent and proteasome-mediated destruction of cyclin B1 in HeLa cells.

**Differential localization of CDH1α and CDH1β**

Next we investigated the subcellular localization of CDH1α and CDH1β. We generated two specific antibodies against CDH1
µ with 3 hydroxysuccinimide)-activated affinity column (Amersham Biosciences) coupled to the CDH1 and CDH1α are representative of 85% and 79% respectively of 200 CDH1α.

Immunoblotting was performed using purified (Figure 5). Western blotting. Extracts of HepG2 cells (approx. 20 µg) were resolved by SDS/PAGE. Immunoblotting was performed using purified α-CDH1C serum. The position of CDH1α (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 µg of immunizing peptide (α-CDH1C w/pop., lane 2). Similar results were obtained using another antibody, α-CDH1N, raised against the N-terminal sequences shared by CDH1α and CDH1β (results not shown). (B) Subcellular localization of exogenously expressed CDH1α and CDH1β in HeLa cells. Cells were transfected respectively with expression vectors pHACDH1α (panels 1 and 2) and pHACDH1β (panel 3), in which the expression of HA-tagged CDH1α/CDH1β 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 µm. The patterns shown in panels 2 and 3 are representative of 85% and 79% respectively of 200 CDH1α/CDH1β-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 CDH1α 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 CDH1α 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 CDH1β was not detected in Western blotting and immunostaining due to the low abundance (Figure 5A). To remedy this and to distinguish the CDH1β staining from that of CDH1α, we stained for epitope-tagged CDH1α and CDH1β expressed from exogenously introduced plasmids (Figure 5B). HeLa cells transiently transfected with expression plasmids for HA-tagged CDH1α (Figure 5B, panels 1 and 2) and CDH1β (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–CDH1α/HA–CDH1β in a highly specific manner. Notably, a nuclear localization pattern of CDH1α was consistently seen with both α-CDH1N and α-HA antibodies. By sharp contrast, CDH1β localized homogenously to the cytoplasm (Figure 5B, panel 3). The identity of the HA–CDH1β protein was further verified by immunostaining with α-CDH1N and α-CDH1C antibodies. Both antibodies reacted with the overexpressed HA–CDH1β as well as the endogenous CDH1α (results not shown).

Both CDH1α and CDH1β bind to and activate the APC (Figure 4), but they localize to different subcellular compartments (Figure 5B). In this setting, CDH1α and CDH1β 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 CDH1α and CDH1β might influence the proteolysis of cyclin A, HA–CDH1α and HA–CDH1β 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 CDH1α co-localized with cyclin A to the nucleus (Figure 5C, panel 3). In addition, the forced overexpression of CDH1α 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, CDH1β and cyclin A were found in different subcellular locations (Figure 5C, panel 6) and CDH1β 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 CDH1α...
Others have shown that the injection of CDH1 RNA into early Xenopus embryos by microinjecting CDH1 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 CDH1α-injected embryo at late stage 9 (Figure 7A, panel 3), the cells overexpressing CDH1α 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 CDH1α/CDH1β-injected embryos did not arrest cell division during the MBT (Figure 7A, panels 1 and 2; Figure 7B). Interestingly, the injection of human CDH1β 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 CDH1α and CDH1β have differential APC-activating activity in Xenopus embryos. CDH1β 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 CDH1α and CDH1β, 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 CDH1β, 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). CDH1α, which harbours a nuclear localization signal that is absent in CDH1β (Figure 6), localizes to the nucleus (Figure 5). In contrast, CDH1β is cytosolic (Figure 5). Finally, CDH1α, but not CDH1β, 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 CDH1α and CDH1β 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 CDH1β is induced.

Both CDH1α and CDH1β can bind to the APC complex and activates the APC-dependent degradation of cyclin B1 in cultured cells (Figure 4). However, the presence of CDH1α and CDH1β 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 CDH1α and CDH1β may be active on different sets of substrates. We note that some of our data are in support of this.
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Figure 7 Differential activity of human CDH1\(\alpha\) and CDH1\(\beta\) in Xenopus embryos

(A) Representative images of embryos. (B) Graphic quantification of post-MBT cell-cycle arrest phenotype induced by expression of CDH1\(\alpha\). 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 \(\mu\)g of the indicated RNAs: Xenopus CDH1 (XCDH1), human CDH1\(\alpha\) (HCDH1\(\alpha\), panel 3), human CDH1\(\beta\) (HCDH1\(\beta\), panel 4), antisense human CDH1\(\alpha\) (as-HCDH1\(\alpha\)), or antisense human CDH1\(\beta\) (as-HCDH1\(\beta\)). 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\(\alpha\) and CDH1\(\beta\) 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\(\alpha\) and CDH1\(\beta\). 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\(\alpha\) and CDH1\(\beta\). On the other hand, CDH1\(\beta\) is apparently a poor activator of the APC-mediated proteolysis of nuclear cyclin A (Figure 5). Plausibly, CDH1\(\alpha\) and CDH1\(\beta\) 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\(\alpha\) and CDH1\(\beta\) 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\(\alpha\), 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\(\beta\) 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\(\alpha\) 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\(\beta\) 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\(\beta\), probably represent two major mechanisms by which CDH1 isoforms could be generated. According to our model, the differential activity of CDH1\(\alpha\) and CDH1\(\beta\) 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\(\alpha\) as a nuclear activator of the APC. Consistent with this, the forced overexpression of CDH1\(\alpha\) in the nucleus correlates with the degradation of nuclear cyclin A (Figure 5C). The nuclear localization of human CDH1\(\alpha\) 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\(\alpha\) and cyclin A (Figure 4C) provides the opportunity for the suggested regulation of CDH1\(\alpha\)-APC by cyclin A [24,55]. In contrast with the nuclear pattern of CDH1\(\alpha\), CDC20 resides ambiently in the cytoplasm [18]. Thus CDC20 and CDH1\(\alpha\) 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 phosphorylation and regulatory factors. J. Cell Biol. 146, 791–800


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