Summary

Cellular as well as viral RNAs are usually found complexed with proteins. In an attempt to identify proteins that interact with transcripts of hepatitis B virus (HBV), a DNA virus that replicates through reverse transcription, a partial cDNA was isolated from a human cDNA expression library whose gene product bound to an HBV-derived RNA. Using an overlapping clone from a molecular hybridization screen a full-length cDNA was assembled. It contained a large open reading frame for a 1208 amino-acid protein of 138 kDa identical to the hypothetical product of the KIAA0675 clone. Closely related sequences are present in mouse cDNA libraries but not in the genomes of lower organisms. The protein sequence contained no known RNA-binding domain and, apart from a probable coiled-coil domain, the only significant homology involved a complete RING-H2 motif. This suggested that the protein might be a novel RNA-binding RING-dependent ubiquitin-protein ligase or E3 enzyme. A motif critical for RNA binding was experimentally mapped to a central Lys-rich region. Binding specificity is either broad or the protein has as yet unknown physiological targets; hence, at present, a potential importance for HBV biology remains open. The RING-H2 domain was functional in and essential for self- and trans-ubiquitylation in vitro and for proteasome-mediated turnover of the protein in vivo. We therefore termed it hRUL138 for human RNA-binding ubiquitin ligase of 138 kDa. hRUL138 mRNAs are expressed at low levels in most tissues. GFP-tagged hRUL138 derivatives were found associated with cytoplasmic structures, possibly the ER, but excluded from the nucleus. The combined presence of RNA binding and E3 activity in hRUL138 raises the possibility that both are mechanistically linked.

Key words: E3 enzyme, ubiquitin ligase, ubiquitylation, RING finger

Introduction

RNA-protein interactions are central to cellular metabolism, and they are likewise essential for viruses, most obviously for those replicating through RNA intermediates. Hepatitis B virus (HBV), the causative agent of B-type hepatitis in humans (Blumberg, 1997), is a small enveloped virus whose 3.2 kb DNA genome is generated by reverse transcription. The template is a viral transcript called pregenomic RNA (pgRNA) that also serves as mRNA for the virus capsid protein and the reverse transcriptase. Binding of the enzyme to a 5'-proximal stem-loop structure, ε, on the RNA mediates its selective packaging into capsids and initiation of DNA synthesis (Nassal, 1999; Nassal, 2000). Previous evidence for cellular proteins that bind to HBV RNA near the ε signal (Perri and Ganem, 1996; Perri and Ganem, 1997) or to the similarly structured HIV-1 trans-activation response (TAR) element, for example, the TAR RNA-binding protein TRBP (Daher et al., 2001; Gatignol et al., 1991), prompted us to screen, using Northwestern assays, a human liver cDNA expression library whose gene product bound to an HBV-derived RNA probe. A 3’ terminally truncated 2.1 kb cDNA clone obtained by this procedure was completed by fusion to an overlapping clone identified by molecular hybridization. This assembled sequence contained a continuous large open reading frame (ORF) for a 1208 amino acid protein with a calculated molecular mass of 138 kDa. On the basis that it was isolated as an RNA-binding protein and has ubiquitin-protein ligase (E3) activity (see below), it was termed hRUL138 (human RNA-binding ubiquitin ligase of 138 kDa). The relevance of the assembled clone was independently confirmed by the publication, during the course of this study, of a continuous cDNA in the HUGE (human unidentified gene-encoded large proteins) collection (Kikuno et al., 2000), KIAA0675 (Ishikawa et al., 1998), that encodes an identical protein. The corresponding gene is located on chromosome 3 but the function of its predicted gene product is unknown.

Database searches using the full-length sequence confirmed that hRUL138 represents a hitherto unknown human protein with no homologs in the completely sequenced genomes of S. cerevisiae, C. elegans or A. thaliana; however, highly related mouse cDNAs were recently identified (S. G. Kreft, PhD thesis, University of Heidelberg, 2000). No known RNA-binding motif (Perez-Canadillas and Varani, 2001) was detectable. Motif searches indicated a central coiled-coil domain and the potential presence of one to three transmembrane regions; however, all had a weak score. The only really informative similarity to other known proteins involved a short sequence stretch close to the C terminus of hRUL138 that contains all of the essential Cys- and His-residues of an intact RING (‘really interesting new gene’)
domain (Borden and Freemont, 1996) of the RING-H2 type [consensus: CX₂CX₉₋₁₉CX₁₋₃HX₂₋₃H/CX₂CX₄₋₁₅CX₂C; C=Cys, H=His, X=any aa; H/C=His in RING-H2 and Cys in RING-HC fingers (Borden, 2000)]. The recognizable features within the hRUL138 protein are schematically shown in Fig. 1B (see Results for further details).

RING domains are Zn²⁺-binding structures that were first recognized for their ability to mediate protein-protein interactions (Saurin et al., 1996). Recently, they have been shown to be integral parts of a second major class, in addition to HECT-domain proteins, of ubiquitin ligases (or E3 enzymes) in which the RING is essential for function (Borden, 2000; Freemont, 2000; Lorick et al., 1999). Ubiquitylation is a key event in proteosome-dependent protein degradation (Pickart, 2000; Pickart and VanDemark, 2000); in addition it is important in many other processes that do not necessarily involve proteolysis (Pickart, 2001) such as signal transduction (Wang et al., 2001), endocytosis (Hicke, 2001) and virus budding (Vogt, 2000). The mechanism of ubiquitylation (Pickart, 2001) involves energy-dependent activation of the 76 amino acid ubiquitin protein with an ubiquitin-activating (or E1) enzyme and its transfer to a ubiquitin-conjugating (or E2) enzyme. Covalent ubiquitin attachment to the ε-amino group of lysines, or sometimes to the N terminus, of target proteins is mediated by E3 enzymes; further ubiquitins are usually, but not always, attached to the first one, leading to polyubiquitylation. E3s are the main determinant for target specificity; consistently, there is a greater variety of E3 than E2 enzymes, whereas only one E1 is known in most eukaryotes including humans.

Together these considerations suggested that hRUL138 might be a novel RING-H2 E3 that can bind RNA. The functional analysis of hRUL138 reported here fully supports this notion.

**Materials and Methods**

**Identification of hRUL138 cDNAs**

The initial cDNA clone was obtained by Northwestern screening of a human liver cDNA expression library in lambda Uni-ZAP XR phages (Stratagene) using an in-vitro-transcribed digoxigenin (DIG)-labeled RNA probe comprising the first 184 nt of HBV (subtype ayw) pgRNA, including the 60 nt ε signal. Phages were plated on *E. coli* XL1-Blue MRf° cells and screened essentially as previously described (Gatignol et al., 1991), except that positive plaques were identified using an anti-DIG antibody (DIG Nucleic Acid detection kit, Roche). After plaque purification a Bluescript SK(–) plasmid form of the 2.1 kb cDNA was derived by excision of the cDNA insert from the lambda phage; the plasmid was termed pBlis-NIII. Five additional human cDNA libraries were screened using a 3’-labeled DNA probe encompassing nt 1497-1998 of the hRUL138 ORF (performed at RZPD screening service, German Cancer Research Center, Heidelberg, Germany; URL: http://www.rzpd.de). Four positive clones were obtained, as confirmed by sequencing: ICRFp512 H14173Q2 and B2260Q2 (both fetal liver); DKFZ586 C175Q3 (uterus); and IMAGp998 N0865Q6 (derived from the same Stratagene liver cDNA library as NIII). Clone ICRFp512 H14173Q2 comprised an apparently intact 3’ end and was used to reconstitute the full-length hRUL138 ORF. The general structures of the individual cDNAs are outlined in Fig. 1A.

**Plasmid constructs**

A full-length hRUL138 cDNA was assembled from the cDNA fragments contained in plasmids pBlisNIII and ICRFp512H14173Q2 using an AvrII restriction site located in the overlap region (nt position 1704 of the hRUL138 ORF). Both fragments were cloned into the vector pT7AMVpol16 (Weber et al., 1994). The resulting plasmid, pT7-hRUL138, comprises the complete hRUL138 ORF under control of the bacteriophage T7 promoter. It also served as starting material for various hRUL138 derivatives. For expression in *E. coli*, hRUL138 or wildtype and mutant fragments thereof were fused to the maltose-binding protein (MBP) using the pMal-c2 vector (New England Biolabs); His-tagged variants were obtained using the pET-30a(+) vector (Novagen). In some constructs a FLAG epitope sequence (DYKDDDDK) was inserted using oligonucleotide-mediated mutagenesis. Point mutations in the Lys-rich motif (amino acids 662 to 666 KKKTK changed to SGSTA) and in the RING-H2 domain (Cys1187 changed to Ser, i.e. C1187S) were generated by PCR-mediated mutagenesis. Eukaryotic hRUL138 expression vectors were based on plasmid pTR-UP5 (Zolotukhin et al., 1996), which encodes
a codon-optimized version of the enhanced green fluorescent protein (eGFP) under control of the CMV-IE enhancer/promoter and an SV40 intron plus poly-adenylation signal. Appropriate hRUL138-encoding fragments were inserted so as to generate C-terminal fusions with eGFP.

All plasmids were named according to the following scheme: the first letters indicate the parental plasmid (pT7, pMBP, pET, pTR-UF); the numbers following the term RUL give the amino-acid positions of the full-length hRUL138 present in the construct; amino acid substitutions are indicated in parentheses and the presence of tags by an acronym either in front of (N-terminal tags) or after (C-terminal tags) the RUL term. For instance, pMBP-RUL681-1128-FLAG stands for a plasmid encoding hRUL138 amino acids 681 to 1128 with a N-terminal MBP and a C-terminal FLAG tag; the same names without the plasmid-specific term denote the corresponding proteins. As a marker for the endoplasmatic reticulum (ER), the dsRed2 gene from plasmid pdsRed2 (Clontech) was used to replace the eYFP gene in pEYFP-ER (Clontech), thus maintaining the original calreticulin ER-targeting and KDEL ER retrieval signals. The corresponding plasmid was named pFP2-ER. Detailed outlines of all cloning procedures are available from the authors upon request.

In vitro translation
In vitro translations were performed in rabbit reticulocyte lysate using the TNT T7 Quick Coupled Transcription/Translation system (Promega) programmed with appropriately linearized pT7 plasmids. For 35S-labeling, [35S]methionine (specific activity 1,000 Ci/mmol; Amersham/Pharmacia) was used at 25 μCi per 50 μl reaction.

Expression of recombinant proteins
MBP fusion proteins were expressed in E. coli strain Top10 (Invitrogen), pET constructs in E. coli BL21(DE3) cells (Novagen). MBP-tagged proteins were enriched by using amylose resin (New England Biolabs) and His-tagged variants by using Ni2+ nitriiotriacetate (Ni-NTA) agarose (Qiagen). For co-expression, BL21(DE3) cells were double-transformed with ampicillin-resistance-mediating pMBP and kanamycin-resistance-mediating pET vectors and propagated in the presence of both antibiotics. Protein concentrations were estimated by comparing, on Coomassie blue stained SDS-PAGE gels, the corresponding band intensities with those of serial dilutions of a bovine serum albumin (BSA) standard of known concentration.

Cell culture and DNA transfection
Huh7 human hepatoma cells were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (Nassal, 1992). DNA transfections were carried out using FuGENE 6 transfection reagent (Roche) as suggested by the manufacturer.

Northern blot analysis
A human multiple tissue Northern blot (Clontech) was hybridized with a [32P]dATP random primed probe (High Prime DNA labeling kit, Roche) corresponding to nt 1497-1998 of the hRUL138 ORF or a full-length actin control probe (Clontech) using standard conditions; hybridizing bands were visualized on a BAS-1500 Phosphorimager (Fuji).

Western blot analyses
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)-resolved proteins were transferred onto PVDF membranes (Amersham/Pharmacia) and incubated with the appropriate primary antibody followed by horseradish-peroxidase-conjugated secondary antibodies (Dianova) as described previously (König et al., 1998). Immunoreactive bands were visualized using the ECL-plus system (Amersham/Pharmacia). The following antibodies were used: anti-FLAG monoclonal antibody M2 (Sigma); anti-His monoclonal antibody anti-Tetra-His (Qiagen) and a polyclonal rabbit antisera recognizing the His-tag-containing linker sequence encoded by the pET-30 vector.

RNA-binding assays
DIG-labeled RNA probes were obtained by in vitro transcription (DIG RNA labeling kit; Roche) of an appropriately linearized T7 promoter containing plasmids as suggested by the manufacturer. The parental plasmid for the HBV-RT and AMV RNA probes was pT7HAMV-RT (provided by J. Beck) which contains HBV nucleotides 938-1130, that is, part of the reverse transcriptase ORF, behind the 49 nt AMV leader. The HBV-RT probe consisted of both segments, the AMV probe of only the AMV part. Labeled RNA was purified by gel filtration (G-25 Sephadex Quick Spin columns, Roche) and quantified via dot blotting (DIG Nucleic Acid Detection Kit, Roche). 35P-labeled RNAs were obtained by in vitro transcription in the presence of 32P-CTP (specific activity: 800 Ci/mmole; Amersham/Pharmacia) and quantified by Cerenkov scintillation counting.

Western blot analyses
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)-resolved proteins were transferred onto PVDF membranes (Amersham/Pharmacia) and incubated with the appropriate primary antibody followed by horseradish-peroxidase-conjugated secondary antibodies (Dianova) as described previously (König et al., 1998). Immunoreactive bands were visualized using the ECL-plus system (Amersham/Pharmacia). The following antibodies were used: anti-FLAG monoclonal antibody M2 (Sigma); anti-His monoclonal antibody anti-Tetra-His (Qiagen) and a polyclonal rabbit antisera recognizing the His-tag-containing linker sequence encoded by the pET-30 vector.
insect cells was used, lysates from E. coli cells overexpressing either UbcH5 or UbcH7 served as a source for E2 (Nuber et al., 1996). For trans-ubiquitylation assays E. coli lysates containing co-expressed MBP-RUL681-1208-FLAG and His-RUL681-1128-His were passed through amylase resin and the proteins bound to 6 μl of amylase gel were incubated in ubiquitylation buffer as described above, except that 10 μg of methylated ubiquitin were used. His-RUL681-1128-His-only ubiquitylation reactions were performed with crude bacterial lysates. Reactions were allowed to proceed for 90 minutes at 30°C and were terminated by boiling for 5 minutes in SDS sample buffer. Samples were analyzed by SDS-PAGE and autoradiography (when 35S labeled) or by western blotting.

Proteasome inhibition
Huh7 cells were transfected with expression constructs for GFP- or FLAG-tagged hRUL138 derivatives. 62 hours post-transfection, the cells were incubated with a final concentration of 50 μM MG132 (Calbiochem) in DMSO for 6 hours. Thereafter, they were washed twice with PBS, scraped off the plate and lysed in SDS sample buffer. Equal aliquots from the lysates were analyzed by western blotting using GFP- or FLAG-specific antibodies. Lysates from nontransfected untreated cells and from cells incubated with the same concentration of DMSO only served as controls.

Immunofluorescence
Transfected Huh7 cells were grown on glass coverslips. 48 hours post-transfection GFP and/or RFP derivatives were examined directly with a confocal laser scanning microscope (Zeiss LSM410). For nuclear counter-staining with TOTO-3 iodide (Molecular Probes) the cells were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes and incubated with 1 μg/ml of RNase A for 30 minutes, washed with PBS containing 0.05% saponin for 10 minutes and incubated with 1 μM TOTO-3 iodide in PBS. After rinsing with PBS, the coverslips were mounted with Vectashield H-1000 (Vector Laboratories). Image files were processed using Adobe Photoshop 5.5 software.

Results
Identification of human RUL138 cDNAs
As HBV is a liver-specific virus we initially screened a human liver cDNA expression library for HBV RNA-binding proteins. Out of about 100,000 recombinant λ phage plaques (of which one third would be expected to yield in-frame translation products) one gave a clearly positive signal when incubated with the HBV-β-containing RNA probe in the presence of an about 100-fold excess of yeast tRNA and herring sperm DNA. Its cDNA insert comprised 2166 nt and contained a 168 nt 5'-untranslated region followed by one large ORF extending to the very 3' end, without a stop codon or a poly-adenylation signal or sequence. Screening of additional human cDNA libraries, some derived from other tissues, by molecular hybridization with a DNA probe corresponding to nt positions 1497 to 1998 of the hRUL138 ORF yielded four independent related clones (see schematic representation in Fig. 1A). All of these shared extensive sequence homologies with the original cDNA; on the basis of the recently published draft sequence of the human genome, the gaps and/or insertions in some of the cDNAs can all be explained by alternative or incomplete splicing events. Two clones, for instance, had a 14 nt deletion after position 905, causing a frameshift and generating a translational stop after 303 amino acids. Since both were isolated from independent libraries they are probably derived from authentic mRNAs, implying the existence of a short form of hRUL138. One cDNA (ICRFp512 H14173Q2) that overlapped with the original clone by 332 nt had all the features of a complete 3' end. It was used to assemble a continuous sequence of 4590 bp containing an ORF of 3624 nt encoding a 138 kDa protein identical to the hypothetical product of the KIAA0675 clone [GenBank Accession Number AB014575 (Ishikawa et al., 1998)].

A tBLASTn search (NCBI; URL: http://www.ncbi.nlm.nih.gov:80/BLAST/) with the full-length hRUL138 protein sequence in the H. sapiens translated RNA database showed, besides the KIAA0675 product, only two hypothetical ORFs with extended similarity, corresponding to amino acids 128-339 and the region from amino acid 740 to near the C-terminus. One is the predicted tetraticopeptide repeat protein 3 (or TPR protein D. TPRD; GenBank Accession Number D83077) on chromosome 21 (Ohira et al., 1996), the other a similar, hypothetical gene on the X chromosome (GenBank Accession Number XM_032867). The function of neither of the two proteins is known, and the region of similarity between TPRD and hRUL138 does not involve the four TPR repeats (Small and Peeters, 2000) that gave TPRD its name. The central region of hRUL138, approximately from amino acids 600 to 950, showed some similarity to a variety of proteins containing myosin heavy chain tail-like potential coiled-coil sequences (Lupas, 1996); the coiled-coil prediction is strongest for the sequence between position 794 to 852, extending with weaker scores in both directions from there (T. Doerks and P. Bork, personal communication). Different search programs for transmembrane regions (PHDhtm, SOSU1, TMAP, TMHMM, TMed) predicted one, two or three transmembrane helices around amino acids 120 to 140, 390 to 410, and 1001 to 1019; however, all had a low score that varied from program to program.

Although these sequence analyses did not offer any distinct clues to the potential function of hRUL138, the C-terminal RING-H2 domain contained all of the absolutely conserved Cys- and His-residues that are typical for this motif, which occurs as a module in various proteins of otherwise unrelated primary structure (Borden, 2000); functionally, however, there is accumulating evidence that most RING finger proteins may be protein-ubiquitin ligases (Freemont, 2000). We therefore went on to experimentally address the RNA-binding potential, ubiquitin ligase activity, expression profile and intracellular localization of hRUL138.

A lysine-rich region in the center of hRUL138 is critical for RNA binding
The hRUL138 sequence did not contain a known RNA-binding motif; however, because the truncated protein encoded by the initial cDNA had been identified via its RNA-binding ability, the region responsible had to be present within the first 666 amino acids. Deletion mapping combined with Northwestern blotting was used to further define this region. A candidate was a stretch of basic residues (amino acid
RNA-binding ubiquitin-protein ligase hRUL138

positions 656 to 674, with 11 Lys plus 1 Arg residue within a total of 19 amino acids; see Fig. 2) that remotely resembled the Arg-rich RNA-binding part within the HIV-1 Tat protein (Cheng et al., 2001; Smith et al., 2000). In a first series of experiments hRUL138 fragments corresponding to residues 1 to 205, 1 to 499 and 1 to 666 were expressed in E. coli as maltose-binding protein (MBP) fusions. Crude bacterial lysates were subjected to Northwestern blotting with the HBV-e-containing RNA probe in the presence of 10 μg/ml of yeast RNA and herring sperm DNA as nonspecific competitors. Specific signals were obtained only with longest protein (data not shown). As this included, at its C-terminus, part of the suspected basic region, three variants lacking 3, 6 or 9 amino acids from the C-terminus were analysed in parallel to the 1 to 666 derivative (Fig. 2A). Again, only the latter generated signals in the Northwestern blot, one at the 120 kDa position expected for the intact fusion protein and a second band at about 50 kDa, most probably a cleavage product lacking the MBP part. Total protein loading on all lanes was similar, as shown by Coomassie blue staining of the SDSPAGE gel (right panel); this also excluded the possibility that the signals arose from RNA binding to E. coli proteins. Hence amino acids 664 to 666 (KTK) are important for RNA binding.

RNA binding was independent of the C-terminal disposition of the basic motif and of the fusion to MBP, as shown by the variant K4+. In this pET30-vector-derived His-tagged construct, the four central Lys residues between positions 662 to 666 were replaced by neutral amino acids (KKTK>SGSTA) in the context of hRUL138 amino acids 510 to 878; hence it still contained the rest of the Lys-rich motif including four further lysines, that is, the total number of basic residues was maintained. The corresponding wild-type construct, and an additional one coding for a C-proximal hRUL138 fragment of similar size (amino acids 832 to 1176) served as controls. The E. coli-expressed proteins were enriched by Ni-NTA agarose chromatography and analyzed by Northwestern blotting (Fig. 2B, left panel). No signals were observed for the C-proximal fragment and for the mutated 510 to 878 protein;т by contrast, a series of bands was generated with the wild-type fragment 510-878; the largest was present at the 50 kDa position expected for the intact fusion protein. Reprobing the same blot with an antibody against the N-terminal His-tag (Fig. 2B, right) revealed similar amounts of the two 510 to 878 proteins plus several smaller, probably proteolytically derived products of similar mobility to the faster migrating bands on the Northwestern blot. The nonbinding C-proximal fragment was present in much larger amounts, indicating that the Northwestern signals did not originate from unspecific binding of the probe. In some experiments, and upon long exposure of the autoradiograms, weak signals were also observed with the K4– mutant 510 to 878 protein. Hence mutating the four central Lys-residues in the Lys-rich motif led to a drastic reduction, though possibly not complete abolishment, of RNA binding.

This was further corroborated by in-solution RNA-binding assays. The wild-type and the mutated 510 to 878 fragments...
RNA: HBV-RT  AMV
RUL: wt K4 wt K4 wt K4 wt K4 wt K4 wt K4
NW α-His NW α-His

Fig. 3. RNA-binding specificity of hRUL138. The same wild-type and K4− variant hRUL138 fragments as in Fig. 2 were expressed as pET30 linker fusions and processed as before for Northern blotting, except that RNA probes derived from a different part of the HBV genome (panel HBV-RT) or an unrelated RNA derived from the alfalfa mosaic virus leader RNA (panel AMV) were used as probes. Loading was controlled by reprobing the blots with an anti-His antibody. Note that weak but detectable signals were also observed in the Northern blot lanes containing the K4− mutant proteins.

were expressed as fusions with the pET30 linker; an N-proximal hRUL138 fragment (amino acids 1 to 205) fused in the same way served as a control. The proteins were incubated with 32P-labeled HBV RNA in the presence of 10 μg/ml of yeast tRNA and 350 mM NaCl, bound to Ni-NTA agarose, and after several washing steps the radioactivity remaining on the beads was determined by liquid scintillation counting; the presence of similar amounts of immobilized protein was confirmed by SDS-PAGE (data not shown). Compared to the wild-type protein, about 10-fold less RNA was retained by the K4− variant and about 20-fold less by the 1-205 fragment (Fig. 2C). These data confirmed that, also in solution, the Lys-rich motif is crucially involved in RNA binding.

In order to test whether hRUL138 binds only to HBV-e-containing RNA, similar Northern experiments were performed using other RNA probes. These included sequences from another part of the HBV genome (HBV-RT, without the ε stem-loop) and an A-U-rich unstructured RNA derived from the alfalfa mosaic virus (AMV) leader (Weber et al., 1994). On Northern blot loading with the same wild-type and K4− mutant 510-878 proteins as in Fig. 2, all RNAs gave signals, with the wild-type protein, of similar intensity to the original probe (Fig. 3). These data did therefore not reveal a significant preference of hRUL138 for certain RNA sequences or structures. Additional in-solution experiments (data not shown), in which RNA homopolymers were used as competitors, showed that poly-A and poly-U, at 10 μg/ml, reduced binding of the labeled HBV ε probe to the immobilized wild-type 510 to 878 fragment by about 80%, whereas 100 μg/ml of poly-G and poly-C were required to achieve a similar reduction; double-stranded poly-IC was ineffective even at this concentration. These data showed that hRUL does not indiscriminately bind to all RNAs but, because the in vitro assays may not faithfully mimic physiological conditions, it remains an open question whether RNA binding specificity is naturally broad or whether specific natural targets exist that we currently do not know.

A  Ub  =  +  +  +  +  =
UbcH5 =  =  =  =  +  +
UbcH7 =  =  =  =  =  =
Incub. [min] 0  90  90  90  90

B  RUL  RUL138  681-1208  MeUb
  =  +  =  +  =  +  +
  =  +  =  +  =  =  =
  0  90  90  90  90

C  RUL  681-1208  RUL681-1208
  681-1208  681-1208
  =  +  =  +  =  =
  =  +  =  =  =  =  =

Fig. 4. Self-ubiquitylation of hRUL138. (A) Polyubiquitylation of full-length hRUL138. hRUL138 was in vitro translated in the presence of 35S-Met and incubated with ATP and E1 plus ubiquitin (Ub) and UbcH5 or UbcH7 as indicated, and the reaction products were analyzed by SDS-PAGE and autoradiography. The borders of the stacking gel are indicated; material accumulating at the upper edge of the stacking gel is marked poly-Ub RUL. (B) Effect of methyl ubiquitin. hRUL138, and the RING containing fragment 681-1208 were in vitro translated and processed as in A, except that methyl ubiquitin (MeUb) was used in two reactions. Both proteins were efficiently modified except that the reaction products were smaller (smear extending to the top of the separating gel; lanes 9 and 12). (C) Requirement for an intact RING-H2 domain. RUL681-1208 and its C1187S mutant were in vitro translated and subjected to ubiquitylation assays as in A.
The hRUL138 RING-H2 domain is functionally active in self- and trans-ubiquitylation.

Many, if not all, RING proteins may be ubiquitin ligases, with an intact RING being essential for ubiquitylation activity. E3 activity can be reconstituted in vitro by providing to an E3 enzyme ubiquitin, an ubiquitin-activating E1 and a conjugating E2 enzyme plus ATP and the target protein; successful reaction results in an increased molecular weight caused by polyubiquitylation. In the absence of a trans-target, various E3s have been shown to self-ubiquitylate (Bays et al., 2001; Fang et al., 2000; Lorick et al., 1999; Nuber et al., 1998). As a first test for E3 activity we investigated whether hRUL138 is capable of self-ubiquitylation. The protein was in vitro translated in rabbit reticulocyte lysate (RRL) in the presence of 35S-Met and incubated with a reaction mix containing ATP, ubiquitin and recombinant E1 plus either UbcH5 or UbcH7 as the E2. In control reactions, individual components were omitted. Incubation with the complete mix containing UbcH5 dramatically decreased the mobility of hRUL138, preventing most of it from entering the stacking gel (Fig. 4A, lane 4); only a small proportion remained at the original position. UbcH7, by contrast, had only minor, if any, effects. In the UbcH5 reaction without added ubiquitin, some decrease in the intensity of the unmodified hRUL138 band and a concomitant reaction without added ubiquitin, some decrease in the efficiency of modification and the differences between ubiquitin and MeUb were even more obvious when a C-terminal RING-H2 motif in close proximity to a self-ubiquitylation-competent hRUL138 molecule, potentially leading to trans-ubiquitylation. Because full-length hRUL138 was poorly expressed in bacteria, RUL681-1208, known to be active after in vitro translation (Fig. 4B), was fused with an N-terminal MBP and a C-terminal FLAG tag (MBP-RUL681-1208-FLAG) and co-expressed, in E. coli, with a His-tagged fragment lacking the RING-H2 motif (His-RUL681-1128-His). Binding of both proteins to the amylose resin was confirmed by western blotting (data not shown). The immobilized complexes were then subjected to ubiquitylation assays with MeUb. The reaction products were separated by SDS-PAGE and visualized by western blots using anti-His (for the trans-target) or anti-FLAG antibodies. The anti-His blot at 0 minutes incubation time showed the expected 58 kDa product plus a second anti-His reactive band slightly above the 97 kDa marker, possibly a dimer. Irrespective of the exact identity of this additional product (which was observed only in the co-expression experiments), it was evident that upon incubation with the ubiquitylation-proficient derivative (Fig. 5, α-His, lane 2), but not in its absence (lane 6), a series of new products with lower mobility was generated at the cost of the unmodified proteins. Interestingly, only a weak reaction was observed when both proteins were separately expressed and then mixed (lane 8); we assume this difference is mainly caused by a more efficient hetero-oligomerization when the two proteins are co-expressed, whereas subunit exchange between separately expressed homo-oligomers is slow. Anti-FLAG blots (Fig. 5, α-FLAG) confirmed that MBP-RUL681-1208-FLAG expressed in E. coli was competent for auto-ubiquitylation. These data demonstrated that hRUL138 is able to mediate trans-ubiquitylation and therefore has genuine E3 ligase activity. We note that these experiments were performed with RUL fragments lacking the Lys-rich region; RNA binding was
therefore not required for the auto-ubiquitylation and the special setting of the trans-ubiquitylation assays.

Fig. 6. Upon proteasome inhibition, hRUL138 steady-state levels in intact cells increase in a RING-dependent fashion. Transiently transfected Huh7 cells expressing GFP fusions of hRUL138 or the RING-deleted variant RUL1-1128 were treated for 6 hours with the proteasome inhibitor MG132, or DMSO alone, and equal aliquots from total cell lysates were analyzed by western blotting using a monoclonal anti-GFP antibody (left panel). The stabilizing effect of MG132 was also observed when the FLAG epitope instead of GFP was fused hRUL138; no signal at the corresponding position was detectable in nontransfected MG132-treated Huh7 cells (right panel).

The RING-H2 domain mediates proteasome-dependent degradation of hRUL138 in vivo

To address the potential physiological relevance of the in vitro auto-ubiquitylation reaction, we examined the influence on steady-state levels of hRUL138 in transfected Huh7 cells of the established inhibitor of proteasome-dependent protein degradation, MG132 (Palombella et al., 1994; Rock et al., 1994). For detection by western blotting (and for fluorescence microscopy; see below) the full-length hRUL138 protein and the RING-deleted hRUL1-1128 variant were fused to GFP, against which highly specific antibodies are available. Because the inhibitor is used dissolved in DMSO, control cells were treated with solvent alone. Aliquots from SDS lysates of MG132 and DMSO-only treated cells containing equal amounts of total protein were then analyzed by using a monoclonal anti-GFP antibody (Fig. 6). For the full-length fusion protein, the signals at the expected position were markedly enhanced by the inhibitor; the RING-deleted fusion, by contrast, produced much stronger signals without the inhibitor, and the signals remained unchanged in its presence. To exclude the possibility that GFP mediated the effects, hRUL138 was, in addition, fused to the FLAG peptide instead of GFP and analyzed correspondingly using an anti-FLAG antibody (Fig. 6, right panel). Again, the presence of MG132 led to a strong signal increase; specificity of detection was confirmed by the absence of signals at the relevant position in non-transfected MG132-treated Huh7 cells. Therefore, the RING-H2 domain in hRUL138-mediated in vitro auto-ubiquitylation is also involved in regulating, in a proteasome-dependent fashion, the steady-state levels of hRUL138 in intact cells. The stabilizing effect of deleting the RING-H2 domain was also observed when the GFP fusions were analyzed by fluorescence microscopy (see below).

The rul138 gene is expressed at low levels in most tissues and slightly higher in skeletal muscle, heart and kidney

To analyze the tissue specificity of hRUL138 RNA expression, premade Northern blots containing polyA+ RNA from eight different tissues were hybridized with the above-described 502 bp random-primed hRUL138 DNA (nt positions 1497-1998 of the hRUL ORF) and analyzed by phosphorimaging. For control, the same blot was reprobed with an actin probe (lower panel). Numbers on the left show the positions of RNA size markers (in kb); the major hRUL transcripts are indicated by the arrowheads on the right.
hRUL138 is excluded from nucleus and associated with a structured cytoplasmic compartment.

To investigate the intracellular distribution of hRUL138, plasmids encoding the above-described GFP fusions plus several additional derivatives were transiently transfected into Huh7 human hepatoma cells. Confocal laser scanning fluorescence microscopy showed, for the full-length fusion protein, a weak fluorescence concentrated in some cytoplasmic structures but excluded from the nucleus (Fig. 8A). This was seen in various experiments but always only very few cells per dish exhibited clearly visible signals, suggesting that the full-length protein was poorly expressed, or unstable, or its expression led to a preferential loss of transfected cells. On the basis of the increased steady-state levels of full-length hRUL upon proteasome inhibition demonstrated by western blotting (see above) we incubated the transfected cells for 6 hours with MG132. This increased the fraction of detectably positive cells, as monitored by visual inspection; however fluorescence intensities were still very low. Substantially more and stronger fluorescent cells were reproducibly observed with a C-terminally truncated construct lacking the RING domain (RUL1-1128-GFP), consistent with the anti-GFP western blot data shown above (Fig. 6). Its intracellular distribution appeared very similar to that of the full-length fusion protein (Fig. 8B), indicating that the RING domain is not responsible for the distinct localization. Exclusion from the nucleus was confirmed by nuclear counterstaining using TOTO-3 iodide (Fig. 8B, right panel); hence the K-rich motif, despite some sequence similarity, is not active as a nuclear localization signal. Suspecting that the compartment in question might be the ER, we generated a variant of an improved version of the red fluorescent protein with ER targeting and retention signals (RFP2-ER) on the basis of the commercially available yellow fluorescent protein (YFP)-ER derivative (Clontech). Huh7 cells transfected with this construct showed cytoplasmic structures, sometimes accumulating around the nuclei, that were indistinguishable from those stained with the YFP-ER variant (data not shown). Overall the fluorescence distribution appeared very similar to that observed with the hRUL138 derivatives. For confirmation we sought to demonstrate colocalization of the proteins by co-transfection. The patterns looked very similar upon separate inspection of the green and red channels and regions of apparent co-staining were revealed (Fig. 8C). However, there were always regions with preferential red or green staining; hence the localization of the two proteins was not completely overlapping. Such a partial but incomplete overlap was observed in numerous experiments. We also noted that in some cells, and increasing with time in culture, the hRUL138-derived fluorescence appeared to accumulate in the nuclear periphery, whereas the RFP2-ER signals remained more dispersed in the cytoplasm, suggesting a segregation of the two proteins. Preliminary experiments aimed at defining the localization signal(s) within the RUL138 sequence using variously truncated derivatives showed similar patterns even for a variant consisting of only amino acids 510 to 878 (Fig. 8D). This suggests that none of the putative transmembrane regions is crucial for association with the compartment; possibly, association is mediated by the coiled-coil region in hRUL138 but the exact nature of that compartment as well as the localization mechanism remains to be determined.

**Discussion**

In this study we have identified a large human RNA-binding protein, hRUL138, that is associated with intracytoplasmic structures and contains a RING-H2 domain mediating self- and trans-ubiquitylation. These data indicate that hRUL138 represents a novel type of RNA-binding E3 enzyme. Below we discuss this possibility in the light of the data obtained so far.
RNA binding by hRUL138
In the absence of a known RNA recognition motif within the hRUL138 sequence we used deletion mapping and Northwestern blotting to identify a central, basic region (amino acids 656 to 674) as important for RNA binding. The first seven lysines, present in the product of the initial cDNA clone, but not the first five, were sufficient for RNA binding. Binding was also detected when the basic motif was disposed internally rather than at the C-terminus and was drastically reduced, though not completely abolished, when the central KKKTKK sequence was mutated to SGSTA; this was also observed in solution assays. Because of the poly-cationic nature of the K-rich motif it might be argued that the RNA binding detected in these assays is merely electrostatic. However, several lines of evidence argue strongly against this view. First, all assays were performed in the presence of more than a hundred-fold excess of tRNA and herring sperm DNA. This included the initial expression library screen, which was based on a procedure that allowed successful isolation of another RNA-binding protein, TRBP (Gatignol et al., 1991). Further, during the solution assays NaCl was present at the rather high concentration of 350 mM. Second, the total number of basic residues (7 Lys and 1 Arg) in the K-rich region of the K+ mutants with drastically reduced RNA binding is the same as in the strongly binding C-terminally truncated hRUL1-666 variant; this was further corroborated by the absence of a signal on the Northwestern blots when a fragment from an unrelated protein containing the sequence K2NK3EKSK (i.e. 8 Lys in 11 amino acids in total) was run in parallel. Finally, the Northwestern signal from the hRUL510-878 was at least partially resistant to a washing step with a solution of 1 mg/ml of heparin (Konarska, 1989; Pinol-Roma et al., 1988), and the extent of resistance was similar to that observed for the core protein of HBV, which is an established nucleic-acid-binding protein (Hatton et al., 1992; Nassal, 1992). Hence the central KKKTKK element of the K-rich motif appears to be a critical part of a larger RNA-binding domain.

Regarding binding specificity, our Northwestern assays using three different RNA probes did not reveal a preference for HBV-ε-containing RNA, at least not under the conditions used. Although database searches revealed stretches of multiple lysines in various predicted gene products, including putative RNA helicases, no protein containing the identical motif, let alone one with characterized RNA-binding capacity, was found that would have allowed for inferences from the hRUL138-RNA interaction. Remotely, however, the preponderance of Lys-residues resembles that of the arginines in the arginine-rich RNA-binding motif present, for instance, in the HIV-1 Tat protein. Peptides derived from this sequence bind specifically to TAR RNA, mainly by a single Arg-residue surrounded by other basic residues that recognize a bulge; however, with about 10-fold lower affinity they also interact with other RNAs (Calnan et al., 1991). Possibly, our assays measure a similar basal RNA-binding activity of hRUL138. More quantitative binding studies using the same probe RNAs might reveal more explicit differences. To this end, we have also tested, using the in-solution binding assay, the ability of homopolymeric RNAs to compete with binding of the labeled probe RNA as described in Fig. 2C. Poly-A and poly-U at 10 µg/ml reduced binding by about 80% whereas ten-fold higher concentrations of poly-G and poly-C were required for a similar reduction; double-stranded poly-IC was ineffective even at this concentration (data not shown). Hence there is some discrimination between different kinds of RNA, which again argues against mere electrostatic binding; at present, however, these data do not allow for clear-cut conclusions on the actual RNA targets of hRUL138. Given its large size, the fragments used in the assays may only be imperfect mimics of the entire protein; furthermore, other factors associating with hRUL138 could affect its binding specificity in vivo. Altogether we envisage two alternatives for physiological RNA binding by hRUL138: either it has a truely broad selectivity or, more likely, there are specific RNA targets that remain to be identified, possibly by similar approaches to those recently used for finding target RNAs of the FMRP protein whose absence causes fragile X syndrome (Brown et al., 2001; Darnell et al., 2001).

Ubiquitin ligase activity of hRUL138
When complemented with ubiquitin (or methyl ubiquitin), E1, E2 and ATP, both in vitro translated hRUL138 and bacterially expressed fragments containing the RING-H2 domain were capable of self-ubiquitylation. Efficient reaction required all of the aforementioned components and an intact RING domain, as demonstrated by the inactivity of variants that lacked the RING domain or carried a Cys to Ser mutation in the RING motif (Bays et al., 2001). hRUL138 is also capable of trans-ubiquitylation, as shown by the efficient ubiquitylation of a RING-deleted variant upon co-expression in E. coli. Plausibly, the spatial proximity between the E2-E3 complex and the target protein required for trans-ubiquitylation was brought about by the ability of hRUL138 to undergo homomeric interactions, and these might be mediated by the coiled-coil domain in the center of hRUL138. Whether homomeric interactions between hRUL138 molecules exist in vivo and, if so, whether trans-ubiquitylation from one to another molecule within the complex occurs cannot be answered by these in vitro experiments. However, preliminary data show that a non-related RNA-binding protein, selenocysteine-insertion-sequence (SECIS)-RNA-binding protein 2 [SBP2 (Copeland et al., 2001)] can also be ubiquitylated by hRUL138. Together these data demonstrated that hRUL138 meets the criteria of a genuine E3 enzyme. The physiological relevance of the auto-ubiquitylation activity of hRUL138 is strongly supported by its marked accumulation in transfected cells upon inhibition of proteasome-dependent turnover, which, by contrast, had no effect on a RING-deficient variant. Since the latter was expressed much better even without proteasome inhibition, it is likely that the authentic protein is subject to RING-mediated autoregulation as, for example, reported for the RING E3 Mdm2 (Fang et al., 2000).

Expression and intracellular localization of hRUL138
Northern blotting revealed a weak hRUL138-specific band of about 4.8 kb in all tissues tested, with the highest intensity in skeletal muscle followed by heart, kidney and brain, and finally pancreas, lung, liver and placenta. These data agree well with RT-PCR-derived semiquantifications for KIAA0675 RNA (available at: http://www.kazusa.or.jp/huge/). In that study, the highest levels (10-50 fg/ng specific plasmid DNA equivalents...
per ng poly-A+ RNA) were found in the heart, skeletal muscle, kidney and testis, lower amounts (1-10 fg/ng poly-A+ RNA) were found in the brain and very low levels (<1 fg/ng poly-A+ RNA) in the lung, liver, pancreas, spleen and ovary. Hence hRUL138 mRNAs are expressed at very low levels; the same is probably true for the protein. Whether the higher levels of hRUL138 mRNA in skeletal muscle and heart are related to the low proliferative activity of these tissues is unclear; notably, two other ubiquitin ligases, MuRF1 and MAFbx, are also predominantly expressed in these tissues and have been shown to be critically involved in muscle atrophy (Bodine et al., 2001). However, hRUL138 mRNA expression may also be inducible in these and in other tissues and, in addition, be regulated on the protein level (see above). Two larger cross-reactive bands of about 5.5 kb and 7.5 kb were detected in some tissues. Given that several of the cDNA clones obtained by molecular hybridization contained intron sequences, these larger mRNAs are probably derived from alternative splicing events. In two of the clones, for instance, a 805 bp intron is not spliced out between positions 1962 and 1963 of the hRUL138 ORF (see Fig. 1A); the larger mRNAs might correspond to these cDNAs. Although the full range of potential splicing variants remains to be explored it is highly likely from the appearance of more than one hRUL mRNA species that alternative splicing does occur physiologically, implying the existence of various isoforms.

Regarding the intracellular distribution of hRUL138, the weakly expressed full-length GFP fusion protein was clearly located outside the nucleus in what appeared to be a structured cytoplasmic compartment. The RING-deleted variant hRUL138-1128 gave much stronger signals with a very similar distribution. On the basis of the similar appearance of cells transfected with an ER-targeted RFP2 variant these structures might represent the ER, or a subcompartment thereof, because the two proteins did not fully colocalize and appeared to segregate with time. This localization was apparently independent of the RING-H2 domain, which was not too surprising given that examples for both RING-dependent and RING-independent localization of RING proteins are known (Hu and Fearon, 1999; Shin et al., 2001). We note that this conclusion rests on the similar appearance of the truncated hRUL138-GFP proteins and that of the very few detectably GFP-positive cells transfected with full-length hRUL138-GFP. We cannot rule out the possibility that these few cells might be defective in ubiquitylation or proteasome-mediated turnover. Hence it remains formally possible that, in a normal cellular environment, an active hRUL138 RING domain does affect the protein's localization. However, even a GFP fusion with hRUL510-878 had a very similar intracellular distribution; hence, neither of the three weakly predicted transmembrane helices is essential for localization. This suggests that compartment association occurs, different from that of the transmembrane-domain-containing ubiquitin ligases Hrd1p (Bays et al., 2001) or Tu1 (Reggiori and Pelham, 2002), in an indirect fashion, possibly via interactions mediated by the predicted coiled-coil region (amino acids 600 to 950), which is part of the localizing variant 510 to 878.

What is the physiological role of hRUL138?

As shown above, hRUL138 is a non-abundant RNA-binding RING-H2 ubiquitin-protein ligase that localizes to an intracytoplasmic compartment, possibly the ER. At present we can only speculate about its physiological function. The absence of homologs in lower eukaryotes not only prevents straightforward genetic experiments but also indicates a function restricted to higher organisms; together with its low abundance this makes a specialized function more likely than a role in general cellular metabolism. In this light, a relationship with ER-associated degradation (ERAD), a mechanism involved in cellular quality control and regulation of normal ER-resident proteins (Buys et al., 2001; Fang et al., 2001), does not seem likely. That hRUL138 cooperates, in vitro, productively with UbcH5 but not UbcH7 indicates the specificity of the E2-E3 interaction. However, each E2 enzyme can interact with more than one E3 and, in this respect, UbcH5 appears to be relatively promiscuous, not permitting direct conclusions on the physiological function of hRUL138. Eventually the natural RNA and protein ubiquitylation targets will have to be identified; both are probably major endeavours, especially when considering that the 138 kDa protein is probably only one out of several splicing variants. Perhaps the most intriguing and at the same time tractable question is whether the RNA-binding and the ubiquitylation activity of hRUL138 are functionally related. If so, hRUL138 may be an E3 enzyme that achieves target specificity using RNA as a mediator. In the auto-ubiquitylation and homo-oligomer-based trans-ubiquitylation assays described above we did not find an influence of RNA on ubiquitylation activity; however, in preliminary studies hRUL138-mediated ubiquitylation of SBP2 was markedly enhanced by the presence of RNA. Although the underlying mechanism remains to be elucidated this suggests that RNA-binding proteins or ribonucleoprotein particles (RNPs) might be physiological targets of hRUL138; whether this includes RNPs derived from HBV, or other viruses, remains to be determined.

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References

Bays, N. W., Gardner, R. G., Seelig, L. P., Joazeiro, C. A. and Hampton, R. Y. (2001). Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER-associated degradation. Nat. Cell Biol. 3, 24-29.

Blumberg, B. S. (1997). Hepatitis B virus, the vaccine, and the control of primary cancer of the liver. Proc. Natl. Acad. Sci. USA 94, 7121-7125.

Bodine, S. C., Latres, E., Baumhueter, S., Lai, V. K., Nunez, L., Clarke, B. A., Poueymirou, W. T., Panaro, F. J., Na, E., Dharmarajan, K. et al. (2001). Identification of ubiquitin ligases required for skeletal muscle atrophy. Science 294, 1704-1708.

Borden, K. L. (2000). RING domains: master builders of molecular scaffolds? J. Mol. Biol. 295, 1103-1112.

Borden, K. L. and Freemont, P. S. (1996). The RING finger domain: a recent example of a sequence-structure family. Curr. Opin. Struct. Biol. 6, 395-401.
