RECQL4, mutated in the Rothmund–Thomson and RAPADILINO syndromes, interacts with ubiquitin ligases UBR1 and UBR2 of the N-end rule pathway

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The Rothmund–Thomson syndrome (growth retardation, skin and bone defects, predisposition to cancer) and the RAPADILINO syndrome are caused by mutations in the RECQL4 gene. The 133 kDa RECQL4 is a putative DNA helicase, a member of the family that includes the BLM and WRN helicases. The latter are mutated, respectively, in the Bloom and Werner syndromes, whose manifestations include predisposition to cancer. Using antibodies to human RECQL4, we found that the bulk of RECQL4 was present in a cytoplasmic extract of HeLa cells, in contrast to the largely nuclear BLM and WRN helicases. However, in untransformed WI-38 fibroblasts, RECQL4 was found to be largely nuclear, and was present at significantly lower total levels than in transformed HeLa cells. RECQL4 from HeLa cells was isolated as a stable complex with UBR1 and UBR2. These 200 kDa proteins are ubiquitin ligases of the N-end rule pathway, whose substrates include proteins with destabilizing N-terminal residues. The functions of this proteolytic pathway include the regulation of peptide import, chromosome stability, meiosis, apoptosis and cardiovascular development. Although the known role of UBR1 and UBR2 is to mediate polyubiquitylation (and subsequent degradation) of their substrates, the UBR1/2-bound RECQL4 was not ubiquitylated in vivo, and was a long-lived protein in HeLa cells. The isolated RECQL4–UBR1/2 complex had a DNA-stimulated ATPase activity, but was inactive in DNA-based assays for helicases and translocases, the assays in which the BLM helicase was active. We discuss ramifications of these results, possible functions of RECQL4, and the involvement of the N-end rule pathway.

INTRODUCTION

Helicases are ATP-dependent RNA- or DNA-unwinding enzymes (1,2). Humans and other mammals contain at least five distinct helicases of the RecQ family, named after the single RecQ gene of Escherichia coli (3,4). A malfunction or absence of specific RecQ-family helicases causes several human diseases: the Bloom syndrome (BS: mutations in the BLM helicase), the Werner syndrome (WS: mutations in the WRN helicase), and the Rothmund–Thomson syndrome (RTS: mutations in the RECQL4 helicase). One common property of these syndromes is predisposition to cancer (reviewed in 4–10). Clinical features of the RTS include postnatal growth retardation; skeletal abnormalities such as osteopenia (a decrease in bone density) and the radial ray defect (appendages on the thumb, missing thumbs, and other malformations); skin and nail abnormalities, including poikiloderma (skin rash); some aspects of premature aging; and predisposition to cancer, especially osteosarcoma (4,11–13). In the latter and several other respects, the phenotype of RTS differs from that of the BS, which predisposes to a large variety of cancers, and from the WS, in which the pattern of cancer predisposition is also broader than in RTS, with a prevalence of various sarcomas (4,8).

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Most (but apparently not all) cases of RTS are caused by autosomal recessive null or hypomorphic mutations in the RECQL4 gene (11,12,14–17). An RTS-related disease, termed the RAPADILINO syndrome (radial hypoplasia, patella hypoplasia and cleft or arched palate, diarrhea and dislocated joints, little size and limb malformation, nose slender and normal intelligence), with a lower predisposition to cancer, was found to be also caused by mutations in the RECQL4 gene (18). Most alterations of RECQL4 in RTS patients are compound heterozygotes of nonsense or frameshift mutations, resulting in truncated RECQL4 polypeptides that often lack a large part of the RECQL4 helicase domain (4,11,14,17). In contrast, most RECQL4 mutations in RAPADILINO patients examined so far are compound heterozygotes in which at least one of two defective RECQL4 alleles contains a deletion that causes, upon pre-mRNA splicing, the skipping of exon 7 without frameshift, and largely spares the putative helicase domain of RECQL4 (18). The (apparently) null phenotype of RECQL4 in mice is death in early embryogenesis (19). Mice bearing a homozygous hypomorphic mutation in RECQL4 survived until birth, with massive lethality afterwards (~5% survival by 2 weeks of age) (20). The surviving mice were growth-re retarded and exhibited a number of other defects as well, some of which were similar to abnormalities in RTS patients (20).

Though several lines of evidence suggest that cells from RTS patients are genetically unstable (4,21), the understanding of these phenotypes in RTS cells is far from advanced. One difference between RTS and, for example, BS is a near-normal frequency of sister chromatid exchanges (SCEs) in RTS cells (11), in contrast to high frequency of SCEs in BS cells.

Human RECQL4 encodes a 1208-residue (133 kDa) protein that contains characteristic sequences of the RecQ-family’s helicase domain, but lacks other significant similarities to known proteins. RECQL4 has not been shown, thus far, to actually possess an RNA- or DNA-helicase activity, in contrast to the BLM and WRN DNA-helicases, whose enzymatic properties have been characterized extensively (reviewed in 5,6). No RECQL4-binding proteins have been identified so far, also in contrast to the BLM and WRN helicases, which are known to interact with each other (22) and to function as components of large multiprotein complexes (4,23–28).

Here we describe the initial characterization of the human RECQL4 protein, and in particular the finding that RECQL4 isolated from HeLa cells occurs as a stable complex with UB1R and UB2R. These highly similar 200 kDa proteins are the E3 components of ubiquitin (Ub) ligases of the N-end rule pathway, one pathway of the Ub–proteasome system. Ub-dependent proteolysis controls the concentrations of many regulatory proteins, and is also essential for selective elimination of damaged or otherwise abnormal proteins (29–35). A substrate of the Ub system is conjugated to Ub through the action of three enzymes, E1, E2 and E3. The selectivity of ubiquitylation is determined by E3, which recognizes a substrate’s degradation signal, or degron (29,30). The term ‘Ub ligase’ denotes either an E2–E3 complex or its E3 component. The rate of degradation of specific proteins is regulated by modulating the structure of degrons or their steric exposure, and also by controlling the activity of Ub ligases (35,36). The multiple proteolytic pathways of the Ub system have in common their dependence on Ub conjugation and the proteasome, and differ through their utilization of distinct E2–E3 complexes.

The substrates of the N-end rule pathway include proteins with destabilizing N-terminal residues (37–40). The corresponding degron, called the N-degron, consists of two determinants: a destabilizing N-terminal residue and an internal Lys residue of a substrate (41). The Lys residue is the site of formation of a substrate-linked poly-Ub chain. A ubiquitylated substrate is processively degraded by the 26S proteasome (42). The N-end rule pathway is present in all organisms examined, from mammals and plants to fungi and prokaryotes (32,38,40). The pathway’s currently known functions include the regulation of peptide import in yeast, through the conditional (peptides-modulated) degradation of the repressor CUP9 that controls the expression of a peptide transporter (36); the maintenance of chromosome stability, through the degradation of a separate-produced fragment of SCC1 (a subunit of cohesin) at the metaphase–anaphase transition (43); the regulation of meiosis in yeast and mammals (38,44); an essential role in the mammalian cardiovascular development (39); the regulation of apoptosis in Drosophila, through the degradation of DIAP1, an inhibitor of apoptosis (32,45), and a role in the leaf senescence in plants (46).

In the yeast Saccharomyces cerevisiae, two substrate-binding sites of the 225 kDa, RING-domain Ub ligase UB1R (sc-UBR1) recognize primary destabilizing N-terminal residues of two types, basic (type 1: Arg, Lys and His) and bulky hydrophobic (type 2: Phe, Leu, Tyr, Trp and Ile) (47). Several other N-terminal residues function as tertiary (Asn, Gln) and secondary (Asp, Glu) destabilizing residues, in that they are recognized by sc-UBR1 after their enzymatic conjugation to Arg, a primary destabilizing residue (40). In the case of N-terminal Asn and Gln (as well as Cys in metazoans), the conjugation of Arg is preceded by other enzymatic modifications (39,48). sc-UBR1 also contains a third substrate-binding site that targets proteins such as CUP9, a transcriptional repressor, through its internal (non-N-terminal) degron (47). In contrast to yeast, more than one Ub ligase mediates the N-end rule pathway in mammals. Two of these E3s, the 200 kDa UB1R and UB2R, have been characterized so far (37,38). As demonstrated below, both of these enzymes are associated with RECQL4 in HeLa cells. Although the known function of UB1R and UB2R is to mediate polyubiquitylation (and subsequent degradation) of their substrates, the UB1R/2-bound RECQL4 was not ubiquitylated in vivo, and was a long-lived protein. We discuss ramifications of these results, possible functions of RECQL4, and the involvement of the N-end rule pathway.

RESULTS AND DISCUSSION

**RECQL4 is present in the nucleus and cytoplasm**

Three polyclonal antibodies were raised against recombinant proteins containing, respectively, three regions of the human RECQL4: residues 60–111, 1013–1159 and 1090–1159. Each of these antibodies, affinity-purified against the corresponding RECQL4 fragments (see Materials and Methods), could detect, upon immunoblotting, a protein with the apparent Mr of ~150 kDa in a whole-cell extract from HeLa cells (Fig. 1A). (The antibody used in the experiments described
below was against the RECQL4 fragment 60–111.) That the detected protein was, in fact, RECQL4 was confirmed by mass spectrometry (see below), and was also indicated by immunoblotting with whole-cell extracts of AG05013 fibroblasts, from an RTS patient with mutations in RECQL4 gene (14) (Fig. 1C; see also Materials and Methods). Specifically, the anti-RECQL4 antibody, which detected a ~150 kDa protein in extracts from HeLa, MCF7 and Jurkat cells (Fig. 1A and C), did not detect this protein in the identically prepared extract from AG05013 fibroblasts (Fig. 1C).

Unlike the BAF57 protein (a component of the SWI–SNF complex) (49), as well as the WRN and BLM helicases, which were present largely in the nuclear extract, the bulk of isolated RECQL4 was in the cytoplasmic extract, with a significant but much smaller amount in the nuclear extract (Fig. 1B). Similar results were obtained with cytoplasmic versus nuclear extracts of two other transformed human cell lines, MCF7 and Jurkat (Fig. 1B). In contrast to these findings, RECQL4 was shown to reside largely (but not exclusively) in the nuclear extract of WI-38, a line of untransformed diploid human fibroblasts (Fig. 2J). Similar results were obtained with two other tested lines of untransformed human fibroblasts, Detroit-551 (skin-derived) and IMR-90 (lung-derived) cell lines (data not shown). In addition, the total amount of RECQL4 (both nuclear and cytoplasmic extracts, together) was consistently and significantly lower in untransformed cell lines, in comparison to transformed ones such as HeLa (Fig. 1B and data not shown).

Indirect immunofluorescence with anti-RECQL4 antibody and HeLa cells indicated the presence of RECQL4 in both...
In the cytoplasm and the nucleus, with a punctate (rather than uniform) distribution of RECQL4 in both compartments (Fig. 1D and E). A test with antibody to human BRG1, a chromatin remodeling nuclear protein (50), confirmed the nuclear location of BRG1 (Fig. 1F). The cytoplasmic and nuclear distribution of RECQL4 varied somewhat from cell to cell, with some patterns (data not shown) suggesting higher levels of RECQL4 in perinuclear regions of the cytoplasm.

Isolated RECQL4 has DNA-stimulated ATPase activity but no helicase or translocase activity

Several mammalian helicases of the RecQ family, including WRN and BLM, have been shown to possess an ATP-dependent helicase activity with a variety of DNA substrates (4,5,8,28). However, there has been no evidence, so far, about helicase activity of RECQL4, either as a recombinant protein or as a part of an endogenous protein complex. As shown in Figure 3A and B, either cytoplasmic or nuclear RECQL4 isolated from HeLa cells through immunoprecipitation with peak gel-filtration fractions of RECQL4 from nuclear extract in (B). An asterisk in (D) indicates the band of unidentified protein that may or may not be a fragment of either RECQL4 or UBR1/UBR2. The sequencing of proteins by mass spectrometry in the anti-RECQL4 precipitate from the fractionated nuclear extract would have required a major scale-up, in contrast to cytoplasmic extract (see Results.) (E) Immunoblotting with either anti-RECQL4 or anti-UBR1 antibodies of anti-RECQL4 immunoprecipitate that had been fractionated by SDS-PAGE. The left, middle and right lanes are the initial (input) sample of cytoplasmic HeLa extract, the unbound (flow-through, FT) fraction, and the immunoprecipitate (IP). (F) Same as in (E) but with anti-UBR1 immunoprecipitate of cytoplasmic HeLa extract. (G) Same as in (E) but with nuclear HeLa extract. (H) Same as in (E) but with nuclear HeLa extract. (I) Same as in (E) but with cytoplasmic extract from AG05013 RTS fibroblasts, with immunoblotting for UBR1 only. (J) Immunoblotting of cytoplasmic and nuclear extracts from WI-38 cells with either anti-UBR1-antibody (upper panel, left two lanes) or anti-RECQL4 antibody (no. 0103) (upper panel, right two lanes), and also with anti-BAF57 antibody (lower panel).
stranded or double-stranded DNA (Fig. 3C). Specifically, in the absence of added DNA the ATPase activity of RECQL4 was indistinguishable from the background level of ATPase activity in the sample produced with preimmune serum (Fig. 3C).

We also tested the possibility that RECQL4 might have a DNA translocase activity, using the triple-helix displacement assay. In this assay (52,53), a triple helical (H-DNA) region is prepared, in which each nucleotide of the third strand forms Hoogsteen base pairs with Watson–Crick base pairs of the duplex DNA. A translocase displaces the third strand of H-DNA. We employed this assay in an earlier work to show that a protein involved in the ATRX syndrome has an ATP-dependent translocase activity (54). The isolated RECQL4 showed no activity in the triple-helix displacement assay, in contrast to the BLM helicase (Fig. 3D).

Isolated RECQL4 is in a stable complex with ubiquitin ligases UBR1 and UBR2

Gel filtration of the cytoplasmic extract from HeLa cells on Superose-6 showed that RECQL4 (detected by immunoblotting) migrated as a species with the apparent molecular mass (for a globular particle) of ~500 kDa, indicating either a homo-oligomer of RECQL4 or its complex with other proteins (Fig. 2A). The peak RECQL4 fraction was precipitated with anti-RECQL4 antibody, followed by SDS–PAGE and silver staining. In addition to the ~150 kDa band of RECQL4 (confirmed by both immunoblotting and tryptic digestion/mass spectrometry), the immunoprecipitated sample also contained a doublet of bands corresponding to ~200 kDa proteins (Fig. 2C). The amounts of these co-immunoprecipitated ~200 kDa proteins were comparable to the amount of RECQL4 in the anti-RECQL4 precipitate, as estimated either by silver staining (Fig. 2C) or by staining with Coomassie (data not shown). The bands of the doublet were cut out together, and sequenced through tryptic digestion and mass spectrometry, yielding the sequences TVVQSCGHSLETK, NLPENENENETGLENVINK, YFEAVQLSSQQR, HELNTSEIEEEDPLVHLASEDIAR, YAVEILTWEK, TLHQYTGSAK and VLHLIGMALQEEK. Database searches with these sequences identified the two ~200 kDa proteins (Fig. 2C) as human sequelogs of mouse UBR1 and UBR2. The term ‘sequelog’ denotes a nucleotide or amino-acid sequence that is similar, to a specified extent, to another sequence (55). The recently introduced, evolutionarily neutral terms ‘spalog’ and ‘sequelog’ make it possible to state the facts of spatial or sequence similarity without having to

**Figure 3.** ATPase, helicase and translocase assays with RECQL4 and BLM. (A and B) DNA duplex-displacement assays with RECQL4 and BLM, with the structures of templates and reaction products depicted on the left of electrophoretograms. The 4-sided star denotes ^32P at the 5‘end of DNA strand. ‘S, heated’, a substrate was heated before electrophoresis, in the absence of added proteins (see Materials and Methods). (C) Assays for ATPase activity with immunoprecipitated RECQL4 and BLM, in the presence or absence of single-stranded and duplex DNA. (D) Triple-helix displacement assay with immunoprecipitated RECQL4 and BLM. While both RECQL4 and BLM were active as DNA-stimulated ATPases, only BLM was active in the helicase and translocase assays.
conjoin such facts, from the outset, to the often unproven infer-ences that the terms ‘homolog’, ‘ortholog’ and ‘paralog’ inher-ently imply (55).]

The sequences of the 1757-residue (200 kDa) mouse UBR1 (37,56) and the 1755-residue mouse UBR2 (38) are 47% identical. These RING-H2 domain-containing proteins are functionally overlapping Ub ligases of the N-end rule pathway, a universally present Ub–dependent proteolytic pathway that is a part of the Ub-proteasome system (32,38,40) (see Introduction). In the case of UBR1, its presence in the RECQL4-containing complex was independently confirmed by SDS–PAGE of anti-RECQL4 immunoprecipitate from the cytoplasmic HeLa extract that was followed by immunoblotting with anti-UBR1 and anti-RECQL4 antibodies (Fig. 2E). Moreover, no detectable UBR1 was immunoprecipitated by anti-RECQL4 antibody from the extract of RECQL4-lacking AG05013 fibroblasts, derived from an RTS patient (Fig. 2H). The gel-filtration profile of UBR1 in cytoplasmic HeLa extract (monitored by immunoblotting) was similar to that of RECQL4 (Fig. 2A), in agreement with the above results. A reciprocal immunoprecipitation of HeLa cytoplasmic extract with anti-UBR1 antibody, followed by SDS–PAGE and immunoblotting with either anti-RECQL4 or anti-UBR1 antibody has yielded, as expected, both RECQL4 and UBR1 (Fig. 2F).

Although RECQL4 was present in HeLa nuclei as well, as evidenced by both immunofluorescence assays with anti-RECQL4 antibody (Fig. 1D and E) and biochemical fractionation, the relative amount of RECQL4 in the nuclear HeLa extract was significantly lower than in the cytoplasmic extract, in contrast to the WRN and BLM helicases, which were present largely in the nuclear extract (Fig. 1B). Both the relative content of RECQL4 in cytoplasmic extracts (Fig. 1B) and the immunofluorescence data (Fig. 1D and E) suggested the cytoplasmic location of most RECQL4 in HeLa cells. Nevertheless, we cannot preclude, at present, the unlikely possibility that the observed complex of RECQL4 with UBR1 and UBR2 is, to a significant extent, nuclear in vivo but can be readily extracted from nuclei by the low-salt buffer used to prepare the cytoplasmic extract.

Gel filtration of the nuclear extract from HeLa cells on Superose-6 yielded the results similar to those with cytoplasmic HeLa extract (Fig. 2B, compare with Fig. 2A), except for the amount of recovered RECQL4, which was consistently and significantly lower than in cytoplasmic extract. Specifically, RECQL4 (detected by immunoblotting) migrated as a species with the apparent molecular mass (for a globular particle) of ~500 kDa (Fig. 2B), as did the cytoplasmic extract-derived RECQL4 (Fig. 2A). In addition, the gel filtration profile of UBR1 was similar to that of RECQL4 in both extracts (Fig. 2A and B). Precipitation of nuclear extract-derived peak RECQL4 gel-filtration fractions with anti-RECQL4 antibody yielded a doublet of the ~200 kDa bands electrophoretically indistinguishable from the bands of UBR1 and UBR2 in the anti-RECQL4 precipitate from cytoplasmic extract (Fig. 2D; compare with Fig. 2C), but the amounts of these bands (and of the RECQL4 band) in the nuclear extract were too low for sequencing without a major scale-up. Independent evidence for the presence of UBR1 in the anti-RECQL4 precipitate from nuclear HeLa extract was produced through an SDS–PAGE of anti-RECQL4 immunoprecipitate that was followed by separate immunoblottings with anti-UBR1 and anti-RECQL4 antibodies (Fig. 2G).

Interestingly, RECQL4 from untransformed WI-38 fibroblasts, which was present largely in the nuclear extract (Fig. 2J), in contrast to RECQL4 from transformed cell lines (Fig. 1B), was found to migrate, upon gel filtration, similarly to RECQL4 in cytoplasmic extracts from transformed cells (Fig. 2I; compare with Fig. 2A and B). [Low levels of RECQL4 in WI-38 cells precluded a technically conclusive immunoprecipitation test, thus far (data not shown).]

These data, taken together, suggest that RECQL4 interacts with UBR1 and UBR2 in both the nucleus and cytoplasm of HeLa cells. Although it is clear that the RECQL4 complex with UBR1 and UBR2 is a major RECQL4-containing complex at least in the HeLa cytoplasm, the resolution and sensitivity of our analysis did not preclude the existence of other, less abundant RECQL4-containing complexes, particularly in the nucleus. The compositional simplicity of the RECQL4-containing complex(es) described in the present work (Fig. 2C) is in contrast to a large number of proteins that have been shown to interact, primarily in the nucleus, with BLM and WRN, the other mammalian RECQ-family helicases (23–28) (reviewed in 4,5,10).

**RECQL4 is not ubiquitylated in vivo, and is a long-lived protein in HeLa cells**

The known mechanistic function of UBR1 and UBR2 is tomediate polyubiquitylation and proteasome-dependent degrada-tion of substrates of the N-end rule pathway (37,38). To assess the extent of metabolic instability of RECQL4 in HeLa cells, we carried out pulse–chase analysis, with a 15-min pulse and the chase times of up to 2 h. The results (Fig. 4A) indicated that the bulk of newly formed RECQL4 was a relatively stable protein in vivo, with the half-life considerably longer than 2 h. In a different assay for the in vivo degradation of RECQL4, the HeLa cells were incubated for 16 h with either the calpain inhibitor ALLM or two different proteasome inhibitors, ALLN and lactacystin, followed by the detection of RECQL4 through immunoblotting of whole-cell extracts. Neither of these treatments caused a significant increase in the steady-state levels of RECQL4 (Fig. 4B), in agreement with the pulse–chase data (Fig. 4A). In contrast, β-catenin, a short-lived protein targeted by the Ub–proteasome dependent pathways distinct from the N-end rule pathway (57,58), was significantly upregulated in the presence of proteasome inhibitors, in addition to the appearance of bands of apparently polyubiquitylated β-catenin (Fig. 4B). No ubiquitylation of RECQL4 could be detected under the same conditions, by either immunoprecipitation or immunoblotting (Fig. 4A and B). Thus, the binding of the Ub ligases UBR1 and UBR2 to RECQL4 does not result in either ubiquitylation or metabolic destabilization of RECQL4, at least in (relatively) unstressed HeLa cells.

**RECQL4, genetic stability and the N-end rule pathway**

Until the results of this study, Ub ligases (E3s) of the N-end rule pathway have been known to target their substrates for...
polyubiquitylation and proteasome-mediated degradation (32,36–38,43). The discovery, in the present work, that these Ub ligases, UBR1 and UBR2, interact with the putative helicase RECQL4 (Fig. 2), and that RECQL4 is a long-lived, non-ubiquitylated protein in HeLa cells (Fig. 4), suggests an additional role for these E3 proteins, and reveals a link between the N-end rule pathway and RECQL4. Both UBR1 and UBR2 are N-recognins, that is, Ub ligases that recognize (bind to) proteins bearing primary destabilizing N-terminal residues (38,40). It is unlikely, however, that the full-length RECQL4 bears a destabilizing N-terminal residue, because the known Met-aminopeptidases (MetAPs) would not remove N-terminal Met from Met–Glu, the N-terminal sequence of nascent RECQL4 (15,16). Specifically, a MetAP would cleave the peptide bond of an N-terminal Met-X sequence in a polypeptide if, and only if, the residue X is stabilizing in the yeast-type N-end rule (32,38,40). (Met and Glu are, respectively, a stabilizing and a destabilizing residue in the N-end rule.) By analogy with S. cerevisiae UBR1, which is known to contain a distinct (third) substrate-binding site that recognizes proteins through their internal sequences (36,47), we presume that mammalian UBR1 and UBR2 also contain such a site, and that it binds to RECQL4.

The isolated RECQL4, assayed as a complex with UBR1 and UBR2, exhibited DNA-stimulated ATPase activity but was inactive as either DNA helicase or DNA translocase, in contrast to the (also tested) BLM helicase (Fig. 3). The cancer-prone phenotype of patients with the RTS (see Introduction) suggests that RECQL4 will be found to mediate processes that contribute to genetic stability of mammalian cells. If so, a possibly direct (but mechanistically still unclear) involvement of the N-end rule pathway in the function of RECQL4 is unsurprising in hindsight, as this pathway has already been demonstrated to be essential for chromosome stability in the yeast S. cerevisiae, through the degradation of a separase-produced fragment of SCC1, a subunit of cohesin that mediates the conditional association of sister chromatids in mitosis and meiosis (43). The N-end rule pathway is required for degradation of the analogous cohesin fragment in mouse cells as well (J. Zhou, R. Hu, J. Sheng, Y.T. Kwon and A. Varshavsky, unpublished data).

Previous work has shown that PARC, a PARKIN-like Ub ligase, functions as a cytoplasmic anchor for the tumor suppressor protein p53 in unstressed mammalian cells (59). It is unknown whether the functionally overlapping Ub ligases UBR1 and UBR2 might play an analogous role in preventing the entry of RECQL4 to the nucleus, until a change in the state of a cell, brought about, for example, by DNA damage. Too many aspects of the revealed RECQL4–UBR1/2 interaction are insufficiently defined, at present, for a detailed discussion of specific models. One example, which remains to be understood both functionally and mechanistically, is the finding that RECQL4 is present largely in nuclear extracts from untransformed diploid fibroblast lines such as WI-38, Detroit-551 and IMR-90, but largely in cytoplasmic extracts from transformed cell lines such as HeLa, MCF7 and Jurkat (Figs 1B and 2J and data not shown). A transiently overexpressed, epitope-tagged human RECQL4 has previously been shown, through anti-epitope immunofluorescence, to be present predominantly in the nucleus of HeLa cells (15), in apparent contrast to our observation that the endogenous (untagged) RECQL4 is present in both the nucleus and cytoplasm (Fig. 1). One possibility is that a transient overexpression of epitope-tagged RECQL4 titrates the pool of available UBR1 and UBR2, neither of which is a highly expressed protein. If one function of UBR1/UBR2 is to sequester RECQL4 in the cytoplasm (discussed earlier), this model would predict that the bulk of overexpressed RECQL4 would enter the nucleus, as observed in (15). A circuit that mediates the (presumably) conditional partitioning of RECQL4 between the nucleus and cytoplasm remains to be analyzed. It is also possible that UBR1 and UBR2 mediate the destruction of RECQL4 under certain conditions, for example after a molecule of RECQL4 had carried out its (unknown) function in chromosome mechanics. Although it appears quite unlikely that RECQL4 may be a component of the N-end rule pathway that is required for some of pathway’s activity, even this possibility cannot be formally precluded by the data at hand. An attempt to verify the above conjecture, through pulse–chase analyses of the N-end rule pathway in RECQL4-lacking AG05013 cells, with transiently transfected cDNAs encoding reporter substrates (38), did not succeed.
so far, owing to poor transfectability of these cells (data not shown).

It remains to be determined whether RECQL4 translocates to the nucleus in association with UBR1 and UBR2, or whether the nuclear import requires at least a transient dissociation of the RECQL4–UBR1/2 complex. Yet another possibility is that cytoplasmic RECQL4 gains a functionally relevant access to chromosomes only during mitosis, after breakdown of the nuclear envelope. At the present time, when mechanistic aspects of RECQL4 are largely a mystery, it is also possible that some RECQL4 functions are in the cytosol, where it might act, for example, as an RNA helicase. Given the nearly identical sizes of UBR1 and UBR2, we do not know, as yet, whether a single molecule of the 133 kDa RECQL4 forms a complex with two molecules each of the 200 kDa UBR1 and UBR2, or whether UBR1 and UBR2 form separate complexes with RECQL4 that co-migrate upon gel filtration. Specific regions of the three proteins that mediate their interactions in this complex(es) remain to be mapped as well.

The discovery that RECQL4 is associated with UBR1 and UBR2, and is neither ubiquitylated nor degraded as a result of this interaction opens up new vistas in studies of both RECQL4 and the N-end rule pathway.

MATERIALS AND METHODS

Chemicals, antibodies and cell cultures

Calpain inhibitor N-acetyl-leucyl-leucyl-methionial (ALLM) and calpain/proteasome inhibitor N-acetyl-leucyl-leucyl-norleucinal (ALLN) (Bachem, King of Prussia, PA, USA) were stored as 25 mM solutions in anhydrous dimethylsulfoxide (DMSO), and used at 1:1000 dilution. Proteasome inhibitor lactacystin (Biomol, Plymouth Meeting, PA, USA) was stored as a 2.5 mM solution in anhydrous DMSO, and used at 1:100 dilution. Rabbit anti-RECQL4 antisera 0103, 3034 and 3234 were raised, respectively, against

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\text{rabbit anti-RECQL4 (1-250);} \\
\text{rabbit anti-RECQL4 (1-200);} \\
\text{rabbit anti-RECQL4 (1-750);}
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and analyzed by SDS–PAGE, in 8% or 8–16% (gradient) polyacrylamide gels, and by immunoblotting with anti-RECQL4 and anti-UBR1 antibodies (60).

Cytoplasmic and nuclear extracts were prepared as described in (50). Briefly, a HeLa cell pellet was resuspended in 5 volumes of buffer A (10 mM KCl, 1.5 mM MgCl2, 10 mM HEPES, pH 7.9), and cells were disrupted in a homogenizer with a rotating teflon pestle. The supernatant (cytoplasmic fraction) was clarified by centrifugation at 20 000 g. The nuclear pellet was collected by centrifugation, washed once with buffer A, resuspended in 2 volumes of buffer C (25% glycerol, 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 20 mM HEPES, pH 7.9), using the above homogenizer, and incubated for 30 min at 4°C. The nuclear extract was clarified by centrifugation at 20 000 g. Either cytoplasmic or nuclear extracts were applied to a Superose-6 column (HR16/50; Amersham-Pharmacia, Piscataway, NJ, USA) equilibrated with buffer D [5% glycerol, 0.1% NP40, 0.2 M NaCl, 1 mM dithiothreitol (DTT), 20 mM HEPES, pH 7.9], containing also 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml leupeptin, and 2 μg/ml aprotinin. Fractions were collected and analyzed by SDS–PAGE, in 8% or 8–16% (gradient) polyacrylamide gels, and by immunoblotting with anti-RECQL4 and anti-UBR1 antibodies (60). The RECQL4-containing fractions from Superose-6 were pooled and immunoprecipitated as described in (28). Briefly, the sample was incubated with affinity-purified anti-RECQL4 antibody and protein A-beads (Amersham-Pharmacia) for ~12 h at 4°C. The immunoprecipitate on protein-A beads was washed 4 times with IP buffer (10% glycerol, 0.1% NP-40, 0.2 M NaCl, 1 mM DTT, 20 mM HEPES (pH 7.9), 0.2 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin). The RECQL4 sample on protein-A beads was used for ATPase and helicase assays directly, or the proteins were eluted with 0.1 M glycine–HCl, pH 2.5, followed by SDS–PAGE, silver staining and immunoblotting. Specific (Coomassie-stained) bands were excised, and the proteins were sequenced through tryptic digestion/mass spectrometry, as described in (28). The BLM helicase, employed as a control in helicase assays, was isolated similarly, using anti-BLM antibody.

Whole-cell extracts for immunoblotting were produced by suspending cell pellet that had been washed with phosphate-buffered saline (PBS) in the SDS–PAGE sample buffer (2% SDS, 10% glycerol, 50 mM DTT, 0.1% bromophenol, 50 mM Tris–HCl, pH 6.8), passing the sample through a 21-gauge needle to reduce viscosity, and heating at 100°C for 3 min, followed by SDS–PAGE.
Immunofluorescence

Indirect immunofluorescence was carried out as described in (61), using methanol fixation (formaldehyde fixation yielded similar results). After fixation and blocking, the cells were incubated with either anti-BRG1 or anti-RECQL4 antibody in the blocking buffer (5% milk in PBS containing 0.2% Triton-X 100) at 4°C overnight, then stained with anti-rabbit secondary antibody conjugated to Alexa fluor 568 (Molecular Probes, Eugene, OR, USA). DNA was stained with TOTO-3 (Molecular Probes). The images were produced using either the laser scanning microscope Zeiss LSM 410 (Carl Zeiss, Germany) (Fig. 1D, F and G) or the Olympus IX70 microscope and Delta Vision Image Restoration System (Applied Precision, Issaquah, WA, USA) (Fig. 1E). Staining of AG05013 fibroblasts that lacked RECQL4 (Fig. 1C) with anti-RECQL4 antibody [it yielded negligible fluorescence (data not shown)] and staining with secondary antibody alone (Fig. 1G) were used as negative controls.

Pulse–chase assay

For the pulse–chase assay, HeLa S3 cells, grown to confluence in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, were incubated for 1 h at 37°C in methionine/cysteine-free DMEM containing 5% dialyzed FBS. The cells were labeled with 35S-Promix (Amer-sham-Pharmacia), at 200 μCi per plate, for 15 min. The labeling medium was removed, and the cells were washed with DMEM, followed by a chase in DMEM containing 5% FBS. At 20, 60 and 120 min of chase, the cells were harvested, washed with ice-cold PBS and extracted for 30 min on ice with RIPA buffer (1% NP40, 0.5% Na-deoxycholate, 0.1% SDS, PBS, pH 7.4) containing 0.2 mM PMSF and 1 μM leupeptin. The lysate was passed through a 21-gauge needle, incubated for another 30 min, and clarified by centrifugation at 20 000 g, followed by immunoprecipitation of the supernatant with anti-RECQL4 antibody, SDS-8% PAGE and either autoradiography or immunoblotting with anti-RECQL4 antibody.

ATPase and helicase assays

Reaction mixtures (20 μl) for ATPase activity contained 4 mM MgCl2, 40 mM KCl, 1 mM DTT, 20 mM phosphate buffer (pH 7.0), 0.1 mg/ml bovine serum albumin, 0.2 mM ATP, [γ-32P]ATP and either single- or double-stranded DNA. Reactions were initiated by the addition of immunoprecipitated complexes (either RECQL4-specific or BLM-specific), and the radioactivity was quantitated using PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA). The DNA substrates and procedures for the triple-helix and the double-stranded substrates and procedures for the triple-helix and the double-stranded DNA helicases assays were described in (54,62). Partial duplex/fork substrates were constructed by annealing the unlabeled oligonucleotide 5'-GGCGGGAGCTTGGCAGATATTGTGCAGGGAATCTGGCGC-3' with one of the 5' end [32P]-labeled oligonucleotides, 5'-TTATATCG TTATAAGAGCGCCAAGCTCCGGCG-3' or 5'-CCGG CGATTTCGGCTACGGTTAAAGACGTC-3'.

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